Identification of *Urop11*, a novel leptin-modulated gene that is upregulated in the hypothalamus of mice with virus-induced obesity

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Abstract

Obesity results from disturbances of tightly regulated interactions between the nervous, endocrine, and metabolic systems that can be caused by external factors, such as viral infections. A mouse model of obesity induced by brain infection with a morbillivirus, canine distemper virus, allowed us to identify obesity-related genes. Using a subtractive library for the hypothalamus, the main brain structure regulating energy homeostasis, we identified a new gene on mouse chromosome 19 which we named upregulated obese product (Urop) 11 and, which has no homology with any known mRNA. A step-by-step molecular approach allowed us to isolate the full-length mRNA, predict the protein sequence, and identify consensus sites. Urop11 was mainly detected in the hypothalamus and adipocytes, and was dramatically upregulated in these central and peripheral structures in obese mice. Urop11 was also expressed in human neural and lymphoid samples and its expression seemed to be regulated by the state of lymphocyte activation. Interestingly, Urop11 expression was strongly upregulated both *in vivo* in mouse hypothalamus and *in vitro* in mouse neural cell lines, after leptin treatment. Taken together, our data show that Urop11 is a target of leptin, the satiety factor produced by adipocytes, in physiological and pathological conditions, including obesity. This new gene can be considered a key molecule in the hypothalamic integration pathway and demonstrates the importance of Urop11 as a target of leptin action.

Journal of Molecular Endocrinology (2007) **38**, 3–17

Introduction

The maintenance of body weight around a set point requires a long-term balance between energy intake and expenditure, and depends on tightly regulated interactions between the nervous and the endocrine systems. Obesity, a pathological result of energy homeostasis deregulation, is thought to be a complex disorder caused by multiple environmental and genetic factors or following a hypothalamic lesion. However, one overlooked possibility is that obesity may have an infectious origin. Several viruses have been reported to cause obesity in animal models following central nervous system infection (Bernard *et al.* 1983, Herden *et al.* 2000, Dhurandhar 2001, Lyons & Nagashima 2002). Animal models have emphasized the role of viral infection in the etiology of human obesity (Dhurandhar *et al.* 1997, 2000, 2002) and provided a better knowledge of the molecular changes associated with obesity. In order to investigate brain damage leading to obesity, we used our well-

characterized mouse model of obesity induced by canine distemper virus (CDV; Bernard *et al.* 1983, 1999), a virus closely related to human measles virus. Interestingly, in infected mice, the neuroadapted strain of CDV predominantly targets the hypothalamus, the brain structure regulating energy homeostasis (Harrold 2004) and known to be a key site for the integration of central and peripheral signals, including leptin, a satiety factor produced by adipocytes, that act via specific hypothalamic receptors (Elmqvist *et al.* 2005). Deregulation of the neural pathways coordinating these molecular signals could undoubtedly lead to profound neuronal changes, which could result in obesity. We have previously shown that CDV infection leads to altered expression of hypothalamic neuropeptides implicated in obesity (Verlaeten *et al.* 2001, Griffond *et al.* 2004), decreases the expression of the functional leptin receptor Ob-Rb in the hypothalamus of obese mice (Bernard *et al.* 1999), and markedly alters the cytokine and protease content of the hypothalamus (Khuth *et al.* 2001). Hypothalamic

infection may therefore cause neuronal loss or transcriptional changes of genes involved in food intake and energetic metabolism. To obtain a better understanding of changes in the hypothalamus, we used a subtractive library to identify molecules involved in virus-induced obesity. Hypothalamic mRNA profiles in CDV-infected lean and obese mice were compared, and one gene, named upregulated obese product (Urop) 11, was selected because of the magnitude of the expression increase and the novelty of the sequence. Urop11 full-length mRNA was obtained and sequenced, the predicted amino acid sequence and central and peripheral localization were determined, and its physiological relevance to obesity analyzed in non-virus-induced obesity model (*ob/ob*, *db/db*, high fat diet (HFD)). Furthermore, leptin regulation of Urop11 expression was examined *in vivo* and *in vitro*. Our data show that Urop11, a novel gene, is regulated both *in vivo* and *in vitro* by leptin, and is mainly expressed in hypothalamic neurons involved in energy homeostasis, which are implicated in the development or maintenance of obesity. Its presence in human lymphoid samples emphasizes its relevance in human immune pathway.

Materials and methods

Experimental schedule

Virus

The neuroadapted CDV strain (10^5 PFU/ml) obtained from the Onderstepoort strain serially passaged in suckling mouse brain was used.

Animals

(i) Four-week-old female Swiss or SV129 mice (Harlan, France) were injected intracerebrally with brain homogenates from either CDV-infected neonatal mice or non-infected neonatal mice and were used for RT-PCR, western blot, immunocytochemistry (ICC), and *in situ* hybridization (ISH) studies. The mice were housed according to EEC (86/609/EEC) and French (Decree 87-848) animal care regulations with food and water and available *ad libitum*; the HFD mice received special food (Moraes *et al.* 2003).

(ii) Four-week-old male C57Bl/6J mice (IFFA-CREDO, L'Arbresle, France), control and obese mice (HFD; Moraes *et al.* 2003) were used for quantitative reverse transcription (Q-RT)-PCR experiments. Briefly, mice were acclimated for a week with standard chow A04 (UAR, Villemoisson-sur Orge, France) at 24 °C and allowed to feed *ad libitum*, lights for 12 h per day from 0630 h; they were then divided into two groups: one with standard chow = control (C) and another with HFD (36% lipids ref TD99249) from Harlan-Tecklad (Madison, WI,

USA) = HFD mice. After 7 weeks of each diet, each group was divided into two subgroups: one subgroup of each group received an Alzet osmotic pump (Alza Corp, Palo Alto, CA, USA) that delivered 10 mg/day recombinant leptin and the other subgroup received pumps with the diluent PBS. Animals were killed after 1-week leptin or vehicle infusion. Hypothalamus was collected and immediately frozen in liquid nitrogen and kept at –80 °C until use. Mice were clearly obese about 5 weeks after HFD (up to 20% more than C mice). After 8 weeks, the weight gain of HFD mice was twofold higher when compared with C mice (11 vs 6 g), the percentage of body fat was 27.9% (HFD) when compared with 10.9% (C) using dual-energy X-ray absorptiometry, and both the plasma leptin and insulin levels increased (six- and twofold respectively).

Experimental design

As described (Bernard *et al.* 1999), infected mice develop acute encephalitis during the active hypothalamic viral replication stage at 7–17 days post-inoculation (dpi) and a substantial proportion of the surviving mice become obese at 4–13 months post-inoculation (mpi), with a body weight ≥ 50 g and leptin levels up to 35 $\mu\text{g/ml}$ when compared with about 30 g and 7 $\mu\text{g/ml}$ respectively in controls. Non-obese surviving mice are referred to as infected lean mice.

Cell culture

The mouse N1E115 (ATCC) and hypothalamic neuronal GT1-7 (Mellon *et al.* 1990, Wetsel *et al.* 1991, Sandberg & Low 2005) cell lines were plated onto poly-L-lysine-coated (Sigma) plastic flasks as described previously (Bencsik *et al.* 1997). At 50–70% confluence, some of the cells were treated with 100–500 ng/ml recombinant 16 kDa mouse leptin (Tebu) for 1–4 days and were then processed for western blotting, ICC, or RT-PCR. GT1-7 cell line was obtained from immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis in transgenic mice using the promoter of the *GnRH* gene. The N1E115 cell line was established from a murine neuroblastoma (Amano *et al.* 1972).

Tissue preparation

At various times after inoculation (7–17 dpi and 4–13 mpi), mice were deeply anesthetized and perfused with ice-cold 0.1 M PBS (pH 7.4; intra-aortic route). For ISH and ICC, the brains, either untreated or fixed by intra-aortic perfusion with 1–4% paraformaldehyde (PFA), were removed, frozen, and stored at –80 °C until used to prepare serial 16 μm coronal sections (SuperFrost/Plus, Menzel-Glaser, VWR International France, Fortenay-Sous-Bois, France). For western blots,

cell lysates from infected and non-infected mice (7–14 dpi) and from neural cell lines were used. For RNA extraction, brain structures dissected according to precise anatomical landmarks and adipocytes were collected. RNAs were prepared from the hypothalamus of HFD (HFD) obese mice and age-matched controls (same genetic background) with or without leptin infusion (Moraes *et al.* 2003), and from untreated and leptin-treated N1E115 and GT1-7 mouse neuronal cells as well. For human samples, RNA was extracted from neural (DEV) or immune (C8166, C91PL, CEM, MT4) cell lines (10^6 cells) as already described (Szymocha *et al.* 2000). For RT-PCR, sham-inoculated and CDV-infected mice were killed during the early stage of infection (7–17 dpi) and sham-inoculated, infected asymptomatic, infected paralyzed, infected pre-obese, and infected obese mice were killed during the late stage of infection (4–13 mpi).

RNA preparation

RNA from homogenized brain structures, peripheral tissues, and neuronal cell lines was extracted using RNAzol (Bioprobe, Montreuil, France), or alternatively using Trizol (Invitrogen, Life Technologies) according to the manufacturer's protocols. The residual genomic DNA was removed (DNase-free, Ambion, Cambridge-shire, UK) and the RNA was quantified (Lab-on-Chip; Agilent Technologies, Santa Clara, CA, USA). The total RNA used for RACE-PCR was carefully examined for genomic contamination by attempting to amplify intronic sequences by PCR and Southern blotting.

Suppression subtraction hybridization (SSH) and reverse northern blot screening (RNB)

SSH (Diatchenko *et al.* 1996) was used to characterize the pattern of cellular gene responses to CDV infection. Hypothalamic mRNAs (50 ng total RNA) from an obese CDV-infected mouse at 5 mpi ('tester', 73 g) and an age-matched infected lean mouse ('driver', 31 g) from the same littermate were compared using SMART PCR cDNA synthesis kits and PCR-Select cDNA subtraction kits (both from Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France) according to the manufacturer's protocol. Differentially expressed transcripts were selected by RNB using ^{32}P cDNA probes from the tester and driver amplified RNA (aRNA; Verlaeten *et al.* 2001) and sequenced (GenomExpress, Meylan, France). Identification was attempted by a homology search of the GenBank, Ensembl, RIKEN, and TIGR DNA databases.

mRNA quantification

RT was performed using 500 ng DNA-free reverse-transcribed total RNA (1.5 h/42 °C), oligo(dT)_{12–18}, and MuLV (Invitrogen). Q-RT-PCR (LightCycler, Roche) was performed using FastStart DNA Master SYBR Green I (Roche) and U11-Es and U11-Er primers and cyclophilin (CyP) primers (Table 1) and reaction conditions of 95 °C/10 min, followed by 45 cycles of 95 °C/10 s, 60 °C/5 s, and 72 °C/10 s. Urop11 mRNA levels were expressed as relative units normalized to CyP mRNA levels and the data analyzed by the Student's

Table 1 Name, accession numbers, and sequences of primers (except primers from suppression subtraction hybridization provided by the manufacturer)

	Accession number	Name	Sequences
Target			
18S RNA	X00686	18S-s 18S-r	TGCAAAGCTGAACTTAAAGGA TCGTTTCGTTATCGGAATTAACC
Cyclophilin	50620	CyP-s CyP-r	ATAATGGCACTGGCGGCAGG CTTCAGTGAGAGCAGAGATTACAAGG
Urop11	AJ558021	Es Er P3r Zr Lr Ks	CAAAGCTACAGGAGGGCTACA TTATGGGGTGGGTTTA TGCATTGCTTTCCAGACAAGGTGTGA CACTGCTCGGGGTGGGTTTA TCTTGCTCTGCACTGCACTGTATTCTT GGCAAGGGTAGTAGGGGCTGA TCTTGCTCTGCACTGTATTCTT TGCATTGCTTTCCAGACAAGGTGTGA
ABPA1	AK032261		TGCAAAGCTGAACTTAAAGGA TCGTTTCGTTATCGGAATTAACC
NPY	NM_023456		TACTACTCCGCTCTGCGACA GATGAGGGTGGAACTTGGA
POMC	NM_008895		TTCTGCTACAGTCGCTCAGG- CAGTCAGGGGCTGTTTCATCT
STAT-3	U08378		TCTCCTTCTGGGTCTGGCTA GTCCTTTTCCACCCAAGTGA
SOCS-1	NM_009896		CTTAACCCGGTACTCCGTGA GAGGTCTCCAGCCAGAAGTG

t-test and the Mann–Whitney test, a *P* value of <0.05 being considered significant.

Identification of full-length Urop11 mRNA and cDNA library screening

The original Urop11-351 bp clone identified by SSH was labeled with [α -³²P]dCTP (3000 Ci/mmol, NonaPrimer kit, Gibco) and the labeled clone used to probe a λ Triplex mouse brain cDNA library (ClonTech). The single positive 880 bp clone isolated was sequenced. RACE-PCR was then performed on mouse hypothalamus total RNA (GeneRacer; ClonTech), using the manufacturer's protocol. For 5' end analysis, the U11 GSP, U11-L, designed from the original SSH sequence, and U11-P3r located near the 5' end of the previously isolated Urop11 triplex sequence, were used for nested amplification. The 3' end of the Urop11 mRNA was identified by 3'RACE using the U11-Es primer as the GSP. All PCR products were cloned (XL TOPO cloning kit; Invitrogen) and sequenced (GenomExpress).

Bioinformatic analysis

The GenBank (NCBI), Ensembl (Wellcome Trust Sanger Institute), and EST (RIKEN, TIGR) libraries were used. ORF motifs were identified using ORF finder (National Center for Biotechnology Information (NCBI)), ATGpr (HRI of Japan), and NetStart 1.0 (Centre de Biochimie Structurale (CBS), Montpellier, France). Polymerase II (Pol II) promoter sites were predicted using Promoter 2.0 Prediction Server (CBS), Neural Network Promoter Prediction (NNPP2.1), and Dragon Promoter Finder1.3. Transcription factor binding sites were localized using MATCH (TRANSFAC Professional 5.1 database). Protein motifs were identified using InterProScan (EMBL) and PSORT II. Post-translational modifications were predicted using Prosite patterns (Expasy), NetOGlyc 2.0 server, and NetPhos 2.0 server (CBS).

In situ hybridization

Two purified Urop11 PCR products (T7-Er/Es or Er/T7-Es, 253bp; Table 1) were separately transcribed to obtain sense and reverse 11dig-UTP-labeled cRNAs (DIG RNA labeling kit; Roche Diagnostic). Brain sections were incubated with each probe separately at 59 °C overnight (500 ng/slide in: 50% formamide, 195 mM NaCl, 10 mM Tris–HCl (pH 7.5), 5 mM NaH₂PO₄, 2H₂O, 5 mM Na₂HPO₄, 5 mM EDTA, 1× Denhart solution, and 125 mg/ml yeast tRNA), then sequentially washed in buffer (1× SSC (0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)), 50% formamide, and 0.1% Tween 20) and maleic acid, NaCl, Tween (MABT) solution (150 mM NaCl, 100 mM maleic acid,

190 mM NaOH, and 0.1% Tween 20 (pH 7.5)). Sections were then incubated overnight at room temperature with alkaline phosphatase-conjugated anti-dig antibodies (Roche Diagnostic), diluted in the ratio of 1:1000 in blocking solution, and extensively washed at room temperature in MABT solution and NaCl, Tris, Magnesium, Tween (NTMT) solution (100 mM NaCl, 100 mM Tris–HCl (pH 9.5), 50 mM MgCl₂, and 0.1% Tween 20). Bound antibodies were detected by incubation with 45 μ g/ml NBT and 175 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) at 37 °C in the dark for 20 min–24 h.

Immunocytochemistry

Cells expressing Urop11 protein were identified using polyclonal antibodies against Urop11 obtained by intradermal injection of a rabbit with the predicted antigenic peptide ¹⁷NH₂-CSE-NNT-LFH-LPR-YRN-CONH₂³² (Covalab, Villeurbanne, France) coupled to KLH on days 1, 21, and 42, followed by s.c. injection on day 63. Blood samples taken on days 0, 32, 54, 73, 94, and 111 were tested for antibody by ELISA (CovaTest). IgG was purified from the day 111 sample using a protein A column. Coronal mouse brain sections (16 μ m thick) were treated to eliminate non-specific labeling due to endogenous biotin (Blocking kit, Vector, Systems Inc., Richardson, TX, USA), extensively washed with PBS, 0.3% Triton X-100, and incubated for 3 days at 4 °C with an optimal dilution of the protein A–protein purified IgG (1:500 in PBS, 0.1% Triton X-100, and 1% BSA: PBS-TBSA). After several washes, the sections were sequentially incubated at room temperature with biotinylated anti-rabbit IgG antibodies (Jackson Immuno Research Laboratories, Suffolk, UK; 1:5000 in PBS-TBSA; 2 h), avidin–biotin-peroxidase complex (ABC, Vectastain Elite kit, Vector; 1:500 in phosphate buffer saline-Tween-Bovine Serum Albumin (PBS-TBSA); 30 min), and DAB staining solution (2 mg in 10 ml 50 mM Tris (pH 7.6), 0.02% H₂O₂). Pre-immune serum from the immunized rabbit was used as the control. The NiE1115 and GT1-1 cell lines were fixed in cold acetone, and then treated as above.

Western blotting of neural tissues for Urop11-p110 expression

Dissected hypothalamus and spinal cords or neural cell pellets were sonicated (100 Hz, 5–10×10 s) on ice in lysis buffer (20 mM Tris (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10% sucrose, and a protease inhibitor cocktail (Complete 1×; from Roche Diagnostics)), samples (20–40 μ g protein estimated using the Bio-Rad DC protein assay) were then heated for 5 min at 95 °C in 0.125 M Tris (pH 6.8), 2% SDS, 20% glycerol, 0.02% bromophenol blue, 0.2 M 1,4 dithio-DL-treitol (DDT),

subjected to SDS-PAGE (10–15%; 2 h/100 V), and electro transferred (50 min/100 mA) to a nitrocellulose sheet (Schleicher & Schuell, Brentford, UK). The blots were blocked (1 h/room temperature in 5% defatted milk in PBS (pH 7.4), containing 0.1% Tween 20), then incubated overnight at 4 °C with anti-Urop11 antibodies diluted in TPBS buffer (150 mM NaCl, 12 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH 7.4), and 0.1% Tween 20), washed twice with TPBS buffer, and incubated for 1 h at room temperature with anti-rabbit IgG antibodies (Jackson) diluted in the ratio of 1:50 000 in TPBS buffer. The blots were then developed using the ECL⁺ detection system (CovaLight reagent, Covalab) according to the manufacturer's protocol and exposed to a photographic film (βmax, Amersham). For the adsorption experiment, anti-Urop11 antibodies (1:800) were incubated for 1 h at room temperature with 100–500 mg/ml Urop11-p110 peptide prior to use.

Cell fractionation was performed according to the manufacturer's recommendations (ProteoExtract Sub-cellular Proteome Extraction Kit; Calbiochem), and the various cell extracts treated as above.

(si)-RNA-mediated gene silencing

Three 21-oligonucleotide small interfering (si)-RNA duplexes (1-CCA GCA GCA GCA GAA TTA-G(d)TT; 2-TCA GCA ACC AGG AAT GCA-U(d)TT; and 3-TTA GAA CTG TGA GTC TCA -A(d)TT; Eurogentec) were designed to target the coding sequence of Urop11. Mouse neural cells (1·10⁶ cells in 100 µl Nucleofector solution V) were gently mixed with 2 µg (si)-RNA or an (si)-RNA negative control duplex (OR-0030-NEG05), then nucleoporation was performed following the manufacturer's recommendations (Amaza Biosystems, Gaithersburg, MD, USA) after optimizing the reaction conditions. One milliliter of pre-warmed Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS) and 100 µg/ml streptomycin/penicillin was added to the transfected cells, which were then incubated for 24 h at 37 °C in a 5% CO₂ atmosphere. Urop11 expression was analyzed in total cell lysates.

Results

Identification of *Urop11* as a new gene that is upregulated in the hypothalamus of obese CDV-infected mice

Selection of Urop11 by subtractive analysis

Eighty cDNA clones, identified by SSH analysis of hypothalamic mRNA from infected obese (tester) and infected lean (driver) Swiss mice, both belonging to the same set of infection experiment, were gridded onto several nylon filters and successively probed with labeled

aRNA from both original populations (mRNA from either obese or lean mice). Figure 1A shows a representative result for filters spotted with clone numbers 11–36, while Fig. 1B shows the obese mRNA/lean mRNA band ratio for the same clones. Several cDNAs were found to be expressed at least twice as strongly in the obese hypothalamus (Fig. 1B). Of these, five cDNAs for Upregulated Obese Products, Urops 11, 12, 14, 26, and 32, which were upregulated respectively, 20-, 3-, 12-, 6-, and 8-fold in the obese hypothalamus, were sequenced and the sequences deposited in the NCBI library as AJ441054, AJ0001700, BE949470, AJ457095, and AJ457094 respectively. Urop12 was identified as neuroserpin mRNA and Urop14 matched a mouse expressed sequence tags (EST), but the other three did not match known genes. Because of the strong upregulation and the novelty of the sequence, we focused on the Urop11 cDNA. To characterize the gene and its products, we used molecular biological and computational methods to obtain the full-length sequence, localize consensus sites, and predict the protein sequence.

Cloning of full-length Urop11 by cDNA library screening, RNA ligase mediated-rapid amplification of cDNA ends (RLM-RACE) analysis, and computational analysis

Screening of the mouse brain Triplex library with the Urop11 sequence obtained by SSH (331 bp) identified a longer sequence of 880 bp perfectly overlapping the original clone. As no open reading frame (ORF) was identifiable, we performed RACE-PCR using U11-gene-specific primer (GSP) to clearly identify the 5' and 3'

Figure 1 SSH screening and reverse northern blot (RNB). (A) The amplicons isolated following SSH analysis were gridded in the same order onto two nylon membranes, which were then hybridized with ³²P-labeled aRNA from either an obese-infected mouse (left panel) or a lean-infected mice (right panel). The clone number and arrow indicate the position of the clones. 18S RNA and cyclophilin were used as controls for normalization. (B) Densitometry analysis of the hybridized membranes (image Quant). Clone expression was normalized to that of 18S RNA and cyclophilin and defined as upregulated in the obese mouse when expression was increased by at least twofold. The five clones showing greater upregulation (11, 12, 14, 26, and 32) were selected and named Urop (upregulated obese product).

extremities of U11 mRNA. Sequencing of the longest 5'RACE-PCR product in combination with the overlapping sequence isolated by 3'RACE gave a cDNA sequence of 4309 bp (Accession AJ558021). Two

Urop11-GSP primers, U11-Zr and Urop11-Lr (Table 1), localized at each end of this sequence were designed and used to amplify the complete sequence (Fig. 2). This cDNA contained two polyadenylation sites

Figure 2 Urop11 cDNA, Urop11-p110 protein, and predicted promoter sites. The sequence of the Urop11 mRNA (AJ558021) is shown in the bottom panel and promoter predictions using NNPP.1.0, Dragon, and Promoter 1.0 software are shown in the top panel. In the top panel, potential promoters are shown in shaded boxes, the putative transcriptional start site (+1) closest to the Urop11-Zs primer (upstream sequence isolated by RACE-PCR) is indicated by an arrow, potential CAAT boxes are double underlined, and transcriptional factor binding sites (MATCH) are shown boxed. In the bottom panel, the Urop11-p110 protein sequence is given and the synthetic peptide used to immunize the rabbit is shown boxed. Predicted post-translational sites are underlined and indicated as follows: N-glycosylation (triangle), protein kinase phosphorylation (star), cAMP phosphorylation (double stars), casein kinase II (dot), and N-myristoylation (square). Predicted 'hot loops' for disordered protein are shown in italics. The cRNA probe used for ISH, obtained using the Urop11 primer pair Es-Er, is shown in italics. The polyadenylation site is shown in bold underlined.

located 25 and 290 nucleotides from the predicted polyA tail. Computational analysis identified a continuous ORF coding for a 110 amino acid protein (Urop110) with an estimated molecular mass of 12 291 Da (ORF Finder, ATGpr, NetStart 1.0; accuracy >95%). Several possible post-translational modification sites (*N*-glycosylation, cAMP phosphorylation, protein kinase C phosphorylation, CK2 phosphorylation, and myristylation) were identified, as well as linear motifs for disordered protein ('hot loops'). A search using the basic local alignment search tool (BLAST) program revealed no significant nucleotide homology with any other genes or EST from available public databases. Surprisingly, using public database libraries and computational tools, we found a perfect match between the entire Urop11 sequence and the sequence of the fourth intron of the mouse amyloid β 4 precursor-binding protein A1 gene (*ABPA1*, Accession number AK032261) consisting of 12 exons covering 43 kb on mouse chromosome 19. The Urop11 messenger located inside the *ABPA1* genomic sequence was expressed independent of *ABPA1* mRNA (RT-PCR experiments, data not shown). As RACE-PCR was performed on genomic-free total RNA checked for absence of DNA, we assumed that the Urop11 sequence was not genomic contamination. To strengthen the accuracy of the ORF prediction, we analyzed the genomic sequence of the fourth intron of *ABPA1* between the sequence upstream of the Urop11 cDNA and the *ABPA1* exon number 3 in order to identify polymerase II promoter and transcription factor binding sites. Neural Network Promoter Prediction software (Pedersen & Nielsen 1997, Reese 2001) clearly identified two 51-nucleotide initiation consensus sequences starting 1971 and 2612 nucleotides upstream of the Urop11-p110 ORF (99% accuracy) and 186 nucleotides upstream of the 5' end sequence isolated by RACE-PCR. A similar prediction was obtained using Promoter 2.0 (Knudsen 1999) and Dragon Promoter Prediction (Bajic *et al.* 2002) software (65 and 98% accuracy respectively), which picked out two RNA Pol II promoters between the predicted NPPP promoter positions (Fig. 2). Three weak signals (1, 2, and 5 kb) on northern blots (not shown) suggested the existence of Urop11 messengers of different sizes.

Identification of Urop11-p110 protein

As the bioinformatic analysis suggested a putative Urop11 protein (Urop11-p110), we investigated this by western blotting using a rabbit antiserum raised against a synthetic peptide designed from the deduced sequence. A protein larger than expected (about 35 kDa) was detected in the mouse hypothalamus and spinal cord (Fig. 3A) and, to a lesser extent, in other

brain structures (data not shown). Incubation of the antiserum with the Urop11-p110 synthetic peptide prior immunodetection resulted in a dramatic decreased signal, showing that binding was peptide-specific (Fig. 3A). The data obtained using immunoprecipitation (IP) emphasized the accuracy of Urop11 protein (Fig. 3B). In order to verify the relation between Urop11 protein and mRNA, we performed western blot analysis after (si)-RNA-mediated silencing of the *Urop11* gene in the GT1-7 hypothalamic cell line. As shown in Fig. 3C, (si)-RNA1 and (si)-RNA2 reduced Urop11 protein expression by up to 40% (Image Quant analysis) when compared with the negative control duplex (scramble), and this was associated with reduced mRNA expression (Fig. 3D). Surprisingly, the molecular weight of Urop11 in cell culture (panels 1–5) was larger than the expected size (panel 6, 75 kDa apparent molecular weight versus 35 kDa in tissue). The detection of the 70–75 kDa to a lesser extent (IP data Fig. 3B) points out its conformational complexity. In an attempt to address the function of Urop11, we next performed a subcellular fractionation, which demonstrated the presence of the Urop11 protein mainly in the cytosolic and membrane/organelle fractions. A very weak expression was also seen in the nucleic fraction, whereas the cytoskeleton extract was devoid of the Urop11 protein (Fig. 3E).

Localization of Urop11

To understand the function of Urop11, we investigated its localization in the brain by ISH using an anti sense riboprobe and by ICC using Urop11-p110-specific antibodies. Labeling was seen in numerous hypothalamic nuclei, being especially strong in the arcuate nuclei (Fig. 4A2), and the labeled neural cells were symmetrically distributed in both hemispheres (Fig. 4A1). In addition to neurons, oligodendrocytes were identified according to their shape and location in brain structures (ZL, *corpus callosum*) known to contain mostly oligodendrocytes as body cells (Fig. 4A5). The distribution of Urop11 protein was in agreement with the mRNA results, as a subset of hypothalamic neurons being strongly stained (Fig. 4B1); higher magnification showed staining of the cytoplasm and processes (Fig. 4B2). No labeling was seen using either the sense riboprobe or the pre-immune rabbit serum (Fig. 4A3 and B3–B4). We further demonstrated the presence of Urop11 protein at the cellular level using ICC, labeling being mainly seen in the cytoplasm and perinuclear area of GT1-7 cells (Fig. 3F). This cellular localization was also confirmed using the fractionation experiment. It is of interest to note that Urop11 mRNA is also constitutively expressed in mouse extra-hypothalamic structures,

Figure 3 Identification of Urop11 protein. (A) Samples of hypothalamic and spinal cord cell lysates were subjected to SDS-PAGE and western blotting, with anti-Urop11-p110 antibodies (lanes 1 and 2 respectively). Lanes 3 and 4 show identical blots after pre-incubation of the antibody with the immunizing peptide, as described in Materials and methods. GAPDH was used as the internal control using an anti-GAPDH antiserum (Chemicon Temecula, CA, USA; 1:50 000 dilution). (B) Hypothalamic protein lysates were immunopurified (IP) using the rabbit polyclonal antibodies in non-denaturized conditions and the complex further purified onto agarose–protein G beads. The IP was then run on SDS gel; a major band at about 35 kDa and a weak one at 75 kDa could be visualized. (C) Detection of Urop11 mRNAs from mouse GT1-7 hypothalamic cells incubated with si-RNA silencing primers (1, 2, 3, see Materials and methods) when compared with the control primer (EtBr detection). The Urop11 mRNA product detected in the mouse brain exhibited the same size as in GT1-7 cells. (D) Western blot of cell lysates from GT1-7 hypothalamic cells incubated with (si)-RNAs targeted at the Urop11 coding sequence or a negative control duplex (scramble). The details for the three (si)-RNA duplexes (indicated as 1–3) are given in Materials and methods. (E) Western blot of Urop11 subcellular distribution. The extraction kit enables the differential extraction of proteins according to their localization: cytosolic proteins, membranes and organelles, solubilized nucleic proteins and cytoskeleton components (extracts 1, 2, 3, and 4 respectively), same protein amounts being loaded onto the gel. ‘S’ corresponds to the hypothalamus from sham-inoculated mouse and ‘I’ to the CDV-infected mouse 14 days after inoculation. (F) Urop11 protein at the cellular level using ICC, labeling being seen mainly in the cytoplasm and perinuclear area of GT1-7 cells. (G) Urop 11 mRNA expression analyzed in several peripheral tissues from Swiss mouse (PCR of 35 cycles (94 °C, 2 min/60 °C, 30 s/72 °C, 2 min)).

both central (spinal cord (see Fig. 5), olfactory bulb, and hippocampus (not shown)) and peripheral, such as spleen, liver, and adipocytes (Fig. 3G), as well as in the 3T3 mouse adipocyte cell line at both the immature

and the mature states (data not shown). Thus, Urop11 expressed both by nervous, immune, and endocrine systems may represent a new actor in the crosstalk between these three systems.

Figure 4 ISH and ICC brain localization of Urop11 mRNA and protein. (A) Urop11 mRNA was detected by ISH in neurons of the paraventricular (PVN) and arcuate (Arc) hypothalamic nuclei (A1, A2, and A4), mainly at the cytoplasmic level, while the cRNA sense sequence gave no signal (A3). An intense hybridization signal was also observed in cells with an oligodendrocyte phenotype (A5). (B) Labeling of hypothalamic neurons (B1) by rabbit anti-Urop11-p110 antibodies; a subset of neurons showed strong labeling (B2). Pre-immune serum gave no labeling (B3, B4).

***In vivo* and *in vitro* Urop11 regulation**

Q-RT-PCR of hypothalamic mRNA of three infected obese mice (at 4, 5, and 9 mpi) showed markedly higher Urop11 mRNA levels when compared with infected lean matched mice (200, 2000, and 50% higher respectively, Fig. 5A). However, when compared with sham-inoculated mice, a significant decrease in Urop11 expression was seen in infected mice during the acute stage of infection in the hypothalamus (-58% , $P < 0.05$ at 14 dpi and -66% , $P < 0.05$ at 16 dpi) and spinal cord (-76% , $P < 0.01$ at 16 dpi; Fig. 5B). Interestingly, no change in Urop11 mRNA levels was seen before the beginning of viral replication (6 dpi, not shown),

emphasizing the regulatory role of CDV infection in Urop11 expression. The relevance of Urop11 expression to obesity was also examined in the hypothalamus of non-virus induced obese mice *ob/ob*, *db/db*, and diet-induced obese mice (HFD; Moraes *et al.* 2003). Furthermore, the effect of leptin on Urop11 expression was assessed after leptin treatment of HFD mice and their controls (chow fed). Similar Urop11 mRNA levels were seen in untreated HFD mice ($n = 5$) and their age-matched controls ($n = 7$; Fig. 5C) and leptin treatment resulted in a significant increase in Urop11 expression both in HFD mice and their controls ($+94$ and $+85\%$ respectively, $P < 0.05$; Fig. 5C), showing a positive regulatory effect of

exogenous leptin on Urop11 expression, whereas in leptin-deficient *ob/ob* ($n=3$) and leptin receptor-deficient *db/db* mice ($n=5$), Urop11 expression seems to be identical to that in control mice ($n=7$). Whereas, no change of neuropeptide Y (NPY) expression was observed in the hypothalamus from treated-HFD mice when compared with untreated HFD mice, we found that exogenous leptin is able to upregulate pro-opiomelanocortin (POMC) mRNA in treated-HFD mice (more than twofold; Fig. 5D). We also demonstrated that signal transducer and activator of transcription (STAT)-3 and suppressor of cytokine (SOC-1) expression were statistically upexpressed in the hypothalamus of leptin-treated HFD mice (Q-RT-PCR, results expressed as relative units normalized to the housekeeping gene *Cyp*, i.e. 0.98 ± 0.02 vs 0.70 ± 0.09 , and 2.38 ± 0.03 vs 1.81 ± 0.2 respectively; $*P<0.05$). Our observations argue for the integrity of the leptin network in these mice and strongly support the action of the exogenous leptin on Urop11 expression and other genes located within the leptin cascade, even in mice exhibiting high endogenous leptin levels.

Given the likely *in vivo* regulation of *Urop11* gene expression by leptin, *in vitro* analysis of Urop11 mRNA expression was performed on the N1E115 neuroblastoma cell line after 24–96 h of leptin treatment. As shown in Fig. 5E, Urop11 mRNA expression was increased at both concentrations of leptin used (100 or 250 ng/ml), the increase being much greater at the higher dose (up to 36-fold when compared with no leptin treatment). Similar modulation of Urop11 mRNA expression was observed in the hypothalamic neuronal cell line GT1-7, with a 60% increase following treatment with 100 ng/ml leptin (data not shown). In

both cell lines, an increase was also seen at the protein level (shown for GT1-7 in Fig. 5F). In summary, several *in vitro* experiments were performed using analyzing Urop11 mRNA and protein expression in N1E115 and GT1-7 cell lines (Fig. 5), as well as C8S (an astrocytoma cell line), 3T3 cell line, and hypothalamic slice cultures under leptin treatment. We found that leptin was able to upregulate Urop11 in a dose-dependent manner in these cell cultures. All together these *in vitro* and *in vivo* data (mice $n=11$; i.e. seven chow fed and four HFD mice), undoubtedly highlighted that leptin acts onto Urop11 expression.

These results suggest (i) direct or indirect regulation of Urop11 mRNA expression either by CDV or by the inflammatory process occurring during the acute stage of infection (Khuth *et al.* 2001), (ii) the association of Urop11 mRNA expression with the virally induced obesity status and aging, (iii) the possibility that Urop11 mRNA might be used as a CDV-induced obesity marker in asymptomatic mice which have survived encephalitic disease and are on the way to becoming obese.

Urop11 mRNA was also detected in human peripheral blood leukocytes (PBL) and lymphoid cell lines (C8166, C91PL, MT4, CEM), and at especially high levels in Dev cells, a human neural precursor cell line (RT-PCR; Fig. 6A), the same T_m for the mouse and human products demonstrated the similitude between these two amplified transcripts (Fig. 6B). Anti-Urop11-p110 antibodies revealed a single band in human samples, but the difference between Urop11 molecular weight in the mouse hypothalamus (35 kDa) and that in human samples (75 kDa, apparent molecular weight), also observed in mouse cell cultures, is to date unexplained.

Figure 5 *In vivo* and *in vitro* regulation of Urop11 expression. (A) Q-RT-PCR analysis performed on hypothalamic RNA of three obese mice and infected-lean mice confirmed the upregulation of Urop11 mRNA expression seen by RNB (200, 2000, and 50% increase normalized to cyclophilin (*Cyp*) mRNA levels in experiment 65, 35, or 58 respectively). (B) Q-RT-PCR analysis of Urop11 mRNA expression during the acute stage of CDV infection on hypothalamus of sham-inoculated and CDV-infected mice (14 dpi) and hypothalamus and spinal cord of sham-inoculated and CDV-infected mice (16 dpi), expression being normalized to *Cyp* mRNA levels. Downregulation of Urop11 mRNA was observed in the hypothalamus of CDV-infected mice during the acute stage of infection when compared with sham-inoculated mice at 14 dpi (58%, $P<0.05$, $n=8$) and 16 dpi (66%, $P<0.05$, $n=7$; control $n=6$ in both) and in the spinal cord at 16 dpi (76%, $P<0.01$, $n=8$ versus control $n=6$). (C) Urop11 mRNA expression in the hypothalamus of untreated control, HFD ($n=7$ in both), leptin deficiency *ob/ob* ($n=3$), and leptin receptor-deficient *db/db* ($n=4$) mice and of leptin-treated control and HFD mice ($n=4$ in both). The numbers indicate the percentage upregulation. No difference was observed between Urop11 mRNA levels in the hypothalamus of control and HFD mice by Q-RT-PCR analysis, expression being strongly upregulated in controls (+85%; $P<0.05$) or HFD mice (+94%, $P<0.05$) after leptin treatment. No change was seen in the hypothalamus of leptin deficiency obese *ob/ob* mice ($n=3$) and leptin receptor-deficient *db/db* mice ($n=4$). (D) NPY and POMC mRNA expression analysis were performed on hypothalamus from untreated and leptin-treated HFD mice (see Materials and methods) using Q-RT-PCR. Results are expressed as relative units normalized to the housekeeping gene *Cyp*. PCR was realized on the same RT product used for Urop 11 expression analysis (statistical Student's *t*-test). (E) Effect of the leptin treatment on Urop11 mRNA levels in N1E115 cells: N1E115 cells were incubated with recombinant leptin (100 or 250 ng/ml) and Urop11 mRNA analyzed using Q-RT-PCR. Urop11 was upregulated as early as 24-h culture with 250 ng/ml leptin, upregulation being stronger at 48 h (3600%). Moderate upregulation was also seen with a lower dose of leptin. (F) Effect of the leptin treatment on Urop11 protein expression in GT1-7 hypothalamic cells. Western blot analysis was performed onto cell lysates (40 μ g in each lane) using rabbit antibodies anti Urop 11 (1:400) and peroxidase-conjugated goat anti-rabbit IgG (1:50 000). (G) Urop11 mRNA expression is strongly upregulated in adipocyte from obese mice (4 and 13 mpi) when compared with sham-inoculated animals (roughly 300%), indicating a modification of Urop11 expression in tissue other than in brain.

Figure 6 Urop11 expression in human samples. (A) End-point PCR was performed in several human T lymphocyte cell lines and neural cell line (Dev) and amplicons hybridized with an internal ^{32}P -labeled probe. A strong signal of the expected size was seen in Dev cells and, to a lesser extent, in PBL and the T cell lines, C8166, C91PL, CEM, and MT2. (B) Identification of Urop11 mRNA was assessed by Q-RT-PCR as T_m of DNA products from human samples and mouse hypothalamus (83.8°C) was similar. (C) Accuracy of Urop 11 protein was proved in T human cell lines by western blots ($20\text{ }\mu\text{g}$ proteins) using rabbit antibodies against Urop11 (1:1000) and peroxidase-conjugated goat anti-rabbit IgG (1:50 000). A single 75 kDa band was seen; the strongest labeling being observed in activated cell lines (MT2, C91), and to a lesser extent in quiescent cell lines (C8666 and E12). Similar loading was demonstrated by detection of GAPDH (monoclonal antibodies 1:50 000) and peroxidase-conjugated anti mouse serum (1:20 000) on the same membrane.

Urop11 expression in human cell lines seemed to correlate with the state of activation, being very high in activated T lymphocytes (C91PL and MT2), low in quiescent cell lines (CEM and T lymphocytes), and intermediate in weakly activated cell lines (Jurkat, C8166, and E12).

In conclusion, the molecular data for Urop11, its presence both in neurons, oligodendrocytes, lymphoid cells, and adipocytes, and its regulation by leptin, argue for a specific role of this newly identified protein in the neuro-endocrino-immune pathway.

Discussion

We previously reported that, during the early stage of infection, CDV predominantly targets neurons in the mouse hypothalamus (Bernard *et al.* 1993) and we hypothesized that obesity syndrome associated with persistence of a low level of virus in the hypothalamus could be due to specific neuronal loss and/or epigenetic alteration of hypothalamic genes involved in food intake, energy control, and basal metabolism. Our mouse model of obesity allowed us to demonstrate

that a virus could induce marked disturbance of gene expression in the neural network responsible for energy homeostasis (Harrold 2004), emphasizing the overlooked possibility of viral infection in the etiology of obesity (Dhurandhar 2001). To discover new candidate genes of central energy homeostasis that are altered in CDV-induced obesity, we used SSH in order to characterize the pattern of cellular gene responses to CDV infection and identified several differentially expressed cDNAs. Of these, we focused on a new gene associated with mouse obesity, which we named Urop11, and determined its full-length nucleotide sequence, its predicted protein sequence, and chromosome localization, and demonstrated its functional relevance in the leptin network.

Urop11 is a new gene that is dramatically upregulated in the hypothalamus of virus-induced obese mice

In this study, we cloned *Urop11*, a gene strongly expressed in the hypothalamus of infected obese mice. Whatever the library used, we did not find any similarities with known messengers, anonymous sequences (EST), or computational predicted genes, suggesting that Urop11 could be the product of a hitherto unknown gene. A Blast search against the mouse genome localized the complete Urop11 sequence on chromosome 19. Alignment of the longest isolated Urop11 sequence with the mouse genome mining data led to its localization in the fourth intron of the predicted gene, inside the fourth intron of the *ABPA1* gene. Even if we cannot exclude the possibility that the Urop11 gene is composed of several exons, we know that the coding part of Urop11 mRNA is located in a single unspliced exon, as the isolated sequence perfectly matched the genomic sequence of the *Abp11* intron. Since Urop11 cDNA expression was still detected after efficient DNase treatment, we assumed that it did not represent genomic contamination, but rather a true messenger with a weak transcriptional level, as northern blot analysis showed very weak labeling. Several additional lines of evidence suggested the existence of Urop11 mRNA. First, two polyadenylation sites were identified close to the 3' end of the isolated cDNA sequence, and bioinformatic analysis identified a 110 amino acid ORF with a partial Kozak consensus sequence. Expression of this ORF was verified through the identification, in the hypothalamus and several other brain structures, of a major protein of 35 kDa on western blots, labeling of which was abolished when the anti-Urop11 antibodies were pre-adsorbed with the immunizing peptide or following (si)-RNAs interference. Secondly, bioinformatic analysis of the genomic sequence slightly

upstream of the 5' end of the Urop11 cDNA allowed the localization, with high probability, of several transcriptional starting points for Pol II, several contigs-assembly and annotation tool (CAAT) boxes, and many transcriptional factor consensus sequences in the same limited genomic area. Functional characterization of the Urop11 promoter sequence by sequential deletion will be the subject of future studies, to definitely locate the transcriptional start point and to determine the functional role of Urop11. As with Urop11 mRNA, the deduced Urop11 protein sequence showed no similarity with any protein in protein data libraries. Computational structural analysis revealed several possible post-translational modification sites (phosphorylation, myristoylation, and glycosylation sequences), which probably account for the larger apparent molecular weight of the expressed protein.

Interestingly, using the computational tool, DisEMBL, we were able to detect three disordered regions ('hot loops') inside the Urop11-p110 sequence. Although little is known about the cellular and structural meaning of intrinsically disordered proteins, they are thought to become ordered only when they are bound by another molecule or as a result of changes in the biochemical environment, this disordered state allowing more interaction with partners and an increase in protein functions without affecting the genome size (Dunker *et al.* 2002, Gunasekaran *et al.* 2003, 2004). It has also recently been demonstrated that protein disorder plays a central role in biology and diseases caused by protein mis-folding and aggregation (Kaplan *et al.* 2003). It is now well established that the eukaryotic genome codes for a high proportion of intrinsically unstructured proteins that are often involved in signaling processes and implicated in severe neurodegenerative diseases. In our study, we show that Urop11 is expressed in several central and peripheral tissues, and in numerous cell types, suggesting several possible physiological roles. Regulation of its expression exclusively in hypothalamic neurons and adipocytes from obese mice emphasized the link between the Urop11 and the pathological process. Further studies will be needed to understand these different roles and the hypothetical relationship of Urop11-p110 with ABPA1 protein.

The Urop11 hypothalamic gene is involved in the leptin response network

Both *in vivo* and *in vitro* analyses showed that Urop11 expression was regulated by leptin. Urop11 mRNA expression showed a strong dose-dependent increase in leptin-treated mice and in leptin-treated cultured neural cells. Leptin is an adipokine (Zhang *et al.* 1997) that conveys information on energy availability, influences energy homeostasis, and regulates

neuroendocrine function primarily in states of energy deficiency through the functional leptin receptor (Ob-Rb; Mercer *et al.* 1996, Elmquist *et al.* 1998). Ob-Rb, which belongs to the class I cytokine receptor family (Baumann *et al.* 1996), acts via both the Janus kinase/STAT pathway and the mitogen activated protein kinase (MAPK) pathway (Fruhbeck 2006). Although Ob-Rb mediates leptin action, access to this receptor on target cells is also influenced by the truncated leptin receptor isoforms, Ob-Ra and Ob-Re (Smith *et al.* 2005). These recent data suggest that the actions of leptin depend not only on its synthesis in adipose tissue and Ob-Rb expression in target cells, but also on factors that regulate tissue expression of Ob-Re, and thus leptin transport in the plasma (Hileman *et al.* 2002). Intriguingly, exogenous leptin upregulated Urop11 in the hyperleptinemic HFD mice. Nevertheless, we can assert the integrity of the leptin network as the expression of genes located downstream of the leptin receptor action are modified, such as POMC, STAT-3, and SOC-1 known to be leptin targets (Ghilardi *et al.* 1996, Vaisse *et al.* 1996). The absence of NPY responsiveness in leptin-treated HFD mice could be explained by an alteration of the loop system between NPY and leptin in obese mice, as suggested by Rohner-Jeanraud *et al.* (1996). On the other hand, the dramatic upregulation of Urop11 expression in the hypothalamus of obese CDV-infected mice, in which the Ob-Rb is markedly strongly downregulated (Bernard *et al.* 1999), suggests direct negative control of Ob-Rb on Urop11 gene expression; it is probable that a functional leptin receptor is not absolutely required for this effect. Urop11 might therefore represent a new intermediate element in the leptin pathway. Further studies are required to determine whether it plays a positive or a negative role, like Socs-3, a negative leptin regulator (Howard *et al.* 2004), and to determine whether regulation of Ob-Rb and Urop11 occurs in the same cell. The expression of Urop11 mRNA and protein in a subset of hypothalamic neurons indicates that this novel gene might be involved in the regulation of the energy homeostasis network. Moreover, the expression of Urop11 in mouse adipocytes and lymphocytes suggests it may be involved in neural/endocrine/immune system crosstalk. The identification at the molecular level of the mechanisms by which Urop11 functions might provide new approaches to pharmaceutical targeting aimed at modulating the intracellular effects of leptin.

Acknowledgements

We thank Professor Jean Paul Riou for advice and stimulating discussion. We are grateful to Tom Barkas for critical evaluation of the English.

Funding

This work was supported by grants from INSERM-INRA, The Rhone Alpes Region and Inserm funds. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 10 October 2006
Accepted 18 October 2006