0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(5):2121–2131

doi: 10.1210/en.2002-221037

Role of Signal Transducer and Activator of Transcription 3 in Regulation of Hypothalamic *Proopiomelanocortin* Gene Expression by Leptin

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Leptin acts on the brain to regulate body weight and neuroendocrine function. Proopiomelanocortin (POMC) neurons in the hypothalamus are important targets of leptin. These cells express the leptin receptor ObRb, and leptin can regulate POMC mRNA levels, but the cellular mechanisms by which this occurs is unknown. Here we show evidence that leptin stimulates *pomc* gene transcription via activation of intracellular signal transducer and activator of transcription 3 (STAT3) proteins. In *pomc*-promoter assays using transfected cells, leptin induces *pomc* promoter activity. Expression of dominant negative STAT3 strongly suppresses this effect. Furthermore, maximal activation requires the presence of the STAT3-binding site, tyrosine 1138, of ObRb. Mutational analysis identifies a 30-bp promoter element that is required for regulation by leptin. In rats, robust leptin-dependent induction of STAT3 phosphorylation is demonstrated in hypothalamic POMC neurons using double immunohistochemistry. In total, approximately 37% of POMC cells are positive for phospho-STAT3 after leptin treatment. Furthermore, leptin-responsive POMC neurons are concentrated in the rostral region of the hypothalamus. Combined, our data show that a subpopulation of POMC neurons is leptin-responsive and suggest that stimulation of hypothalamic *pomc* gene expression in these cells requires STAT3 activation. We speculate that STAT3 is critical for leptin-dependent effects on energy homeostasis that are mediated by the central melanocortin system. (*Endocrinology* 144: 2121–2131, 2003)

CENTRAL PATHWAYS CONTROL the regulation of body weight and food intake by the fat-derived hormone leptin (1, 2). Herein, the hypothalamus plays an important part in which several different leptin-responsive populations of neurons can be distinguished (3, 4). Of these, the proopiomelanocortin (POMC) neurons in the arcuate nucleus (ARC), producing the anorectic peptide α-MSH via endoproteolytic processing of the POMC polypeptide precursor (5), are key mediators of leptin action. A separate population of cells in the ARC coexpresses the melanocortin-receptor antagonist, agouti-related-protein and neuropeptide Y (NPY; Refs. 3, 6, and 7). These cells and the POMC neurons express the long form of the leptin receptor, ObRb, and are thus likely to be direct targets of circulating leptin (8–12). Consistent with this are recent findings that POMC neurons respond rapidly to leptin by increasing axonal firing rates and by decreasing membrane potentials, likely leading to release of neurotransmitters and neuropeptides, including α-MSH (13). The critical role of the melanocortin system in body weight regulation is evident from pharmacological experiments (14–17), as well as studies of mutations in the *pomc* gene (18, 19) and in the gene encoding the melanocortin-4 receptor, both leading to severe obesity in rodents and in humans (20–22). Indeed, the lack of only one functional copy of either gene results in increased fat mass and body weight, although to a less severe degree compared with loss of both alleles (22, 23). Combined, this suggests that each step in the central melanocortin system is tightly regulated to maintain normal energy balance.

Further evidence for the regulation and importance of the melanocortin system in leptin action stems from early findings that mice lacking functional leptin or leptin receptors are morbidly obese and have significantly reduced *pomc* mRNA levels in the hypothalamus (24–26). In addition, fasting of mice and rats for 2–3 d leads to a reduction in circulating leptin concentrations that is accompanied by a fall in *pomc* mRNA that can be prevented by administration of recombinant leptin during the fasting period (26, 27). The molecular mechanisms controlling this regulation by leptin have not been reported, and this question is the focus of the present study.

Transfection studies show that the long signaling form of the leptin receptor, ObRb, is rapidly phosphorylated on tyrosine residues in response to leptin (28). This phosphorylation is mediated by activation of Janus-activated kinase (JAK)2 proteins that are constitutively associated with conserved intracellular sequences of ObRb, located proximal to the transmembrane domain (28–31). ObRb contains three intracellular tyrosine residues, located at positions 985, 1077,

Abbreviations: ARC, Arcuate nucleus; DAB, diaminobenzidine; DMH, dorsomedial hypothalamus; DN, dominant negative; DR, dorsal raphae; ICC, immunocytochemistry; IHC, immunohistochemistry; ISHH, *in situ* hybridization histochemistry; JAK, Janus-activated kinase; LHA, lateral hypothalamic area; LIF, leukemia-inhibitory factor; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; PAG, periaquaductal gray; POMC, proopiomelanocortin; P-STAT3, phospho-STAT3; PVN, paraventricular nucleus; SOCS-3, suppressor of cytokine signaling-3; STAT3, signal transducer and activator of transcription 3; VMH, ventromedial hypothalamus; WT, wild-type.

and 1138 (32). Tyrosine 1138 is located three amino acids N terminal to a glutamine residue (YXXQ), generating a consensus signal transducer and activator of transcription 3 (STAT3)-binding motif (33). Indeed, cytoplasmic STAT proteins that bind to the phosphorylated Y1138 residue become tyrosine-phosphorylated by JAK2, then dimerize and translocate to the nucleus to regulate gene transcription (34–37). STAT3 nuclear translocation and STAT3 DNA-binding activity in response to leptin in the hypothalamus have been reported using STAT3-immunohistochemical techniques and gel-shift assays, respectively (38–41), but the nature of the cells in which this occurs and the biological importance of this event are unknown. We therefore hypothesized that leptin affects *pomc* promoter activity via activation of STAT3 in POMC neurons.

We here demonstrate that leptin induces STAT3 phosphorylation in nuclei of POMC neurons in the hypothalamus of rodents. Furthermore, *in vitro* studies show that leptin can directly activate the proximal *pomc* promoter in cells expressing the leptin receptor and that this requires STAT3 phosphorylation. We conclude that STAT3 is likely to be critical for mediating genomic effects of leptin to regulate *pomc* gene expression in the hypothalamus.

Materials and Methods

Materials

Recombinant mouse leptin was obtained from Dr. E. Parlow (National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program, Torrance, CA). The expression vector encoding the long form of the murine leptin receptor (ObRb WT) was described earlier (28), and its mutant with replacement of tyrosine 1138 to serine (ObRb Y1138S) was generated by site-directed mutagenesis (CLONTECH Laboratories, Inc., Palo Alto, CA) by Dr. M. Myers (Joslin Diabetes Center, Boston, MA). Dr. S. Melmed [University of California-Los Angeles (UCLA), Los Angeles, CA] and Dr. T. Hirano (Osaka University, Osaka, Japan) provided the wild-type (WT) STAT3 and dominant negative (DN) STAT3 (Y705F) expression vectors. A human (−879/+6) and a rat (−706/+64) *pomc* promoter-luciferase construct were described earlier (42, 43) and kindly given by Dr. S. Melmed. All reagents for transfection were from Invitrogen (Carlsbad, CA). Buffer supply for immunohistochemistry (IHC) and immunocytochemistry (ICC) was purchased from Sigma (St. Louis, MO), ABC Vectastain was from Vector Laboratories Inc. (Burlingame, CA), and diaminobenzidine (DAB) developing solution was from Roche (Basel, Germany). Phospho-specific-(Y705)-STAT3 antibodies were purchased from New England Biolabs, Inc. (Beverly, MA); sheep-anti-α-MSH polyclonal antibody from Chemicon (Temecula, CA); normal donkey serum, biotinylated donkey-antisheep, and goat-antirabbit from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); and normal goat serum from Invitrogen.

Generation of deletions of the rat and human pomc promoters

Deletions of the human *pomc* promoter were made from the −879 to +6 fragment originally cloned into pGL3-basic vector (provided by Dr. S. Melmed). Specifically, deletions were generated by applying PCR and using the mentioned parental human *pomc*-promoter plasmid as template, and with specific primers to amplify the desired fragments of the promoter (−761/+6, −361/+6, −161/+6, −91/+6, and −61/+6) and using Pfx polymerase (Invitrogen). In addition, restriction sites for *Kpn*I and *Hin*dIII were added to the appropriate ends of the PCR primers. To clone the PCR products into the pGL2-Basic vector (Promega Corp., Madison, WI), both the PCR products and the pGL2-Basic vector were digested with *Kpn*I and *Hin*dIII (New England Biolabs, Inc.) and then finally ligated using T4 DNA ligase (Promega Corp.). After that, the constructs were transformed into competent cells, and positive clones were purified with MiniPrep Kit (QIAGEN, Valencia, CA). A comparable construct of the rat promoter (−704/+64) cloned into pGL2-Basic was also made by PCR as described above for the human promoter, but using rat-specific primers and the rat construct (−706/+64) from Dr. S. Melmed as template. Integrity of all plasmids was confirmed by DNA sequencing and by restriction enzyme digests.

Cell culture and transient transfection

The 293T and AtT-20 cells were grown in DMEM with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (10 mg/ml) added and incubated at 37 C in 5% CO_2. After growing to approximately 70% confluence, cells were transfected with LipofectAMINE (Invitrogen) according to the recommendations of the manufacturer. All stimulations were done 12–18 h post transfection. Hormone concentrations and stimulation times are indicated in the figure legends.

Luciferase and β-galactosidase assays

Cells were lysed, and aliquots were used for luciferase assay as described earlier (44). Briefly, luciferin (Molecular Probes, Inc., Eugene, OR) and assay buffer were added simultaneously to the cell lysate, and luciferin was measured for 20 sec in a Luminometer (LB 9501, EG&G Berthold, Bad Wildbad, Germany). Using Galacton (Tropix Inc., Bedford, MA), β-galactosidase activities were determined as described by the manufacturer, and samples were measured in the Luminometer for 5 sec.

ICC

The 293T cells were plated on poly-D-lysine (20 μg/ml; Sigma) -coated four-chamber glass Lab-Tek (Nunc Inc., Naperville, IL) slides and transfected with ObRb and STAT3 vectors as described above. Cells were stimulated with and without leptin for 20 min. Stimulation medium was rapidly removed, and cells were washed with ice-cold PBS, followed by fixation in precooled methanol for 10 min at −20 C. After three washing steps (Tris-buffered saline/0.1% Triton X-100), unspecific binding sites were blocked for 1 h at room temperature (5% normal goat serum in 0.1% Triton X-100). After that, cells were incubated with phospho-STAT3 (P-STAT3) antibody (1:3000 in blocking solution) overnight at 4 C. The next day, cells were washed in Tris-buffered saline and incubated with an Alexa 579 conjugated antirabbit antibody (1:1000 in blocking solution) for 2 h. After a final wash, the chambers were removed from the slides and cover-slipped with Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA) containing diamidinophenolindole for nuclear counterstain. Results were visualized on a fluorescence microscope (Axioscope2, Carl Zeiss, Thornwood, NY), and pictures were taken with a digital camera (AxioCam, Carl Zeiss).

IHC

Male Sprague Dawley rats, 4–5 wk of age, were purchased from Jackson ImmunoResearch Laboratories, Inc. (Bar Harbor, ME). The animals and procedures used were in accordance with the guidelines and approval of the Harvard Medical School and Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committees. Rats were injected ip with leptin (1.0 mg/kg body weight) or vehicle (PBS). Animals were then deeply anesthetized with ketamine (100 μg/kg body weight) and xylazine (10 μg/kg body weight), the heart was uncovered, and the circulation was flushed with 0.9% saline for 5 min via the left ventricle, followed by 10% neutral buffered formalin solution for 30 min. After that, the brain was carefully removed, postfixed for 12–15 h in formalin solution, and finally cryoprotected in 20% sucrose solution. Brains were frozen in dry ice and cut in 25-μm coronal sections on a microtome, collected in five series, and stored in 0.02% sodium azide containing PBS at 4 C until further use. From the five series of brain sections, one was used for single P-STAT3 IHC, another for single α-MSH IHC, a third series was used for double staining, and a forth series was driven to Nissl stain to determine the morphology of the sections. For IHC, free-floating tissue sections were used. For α-MSH IHC, sections were incubated in 0.3% H_2O_2 to block endogenous peroxide, blocked in 3% normal donkey serum in PBS/0.25% Triton X-100/0.02% sodium azide for 1 h, and incubated overnight at room temper-

ature with the primary antibody (sheep anti-α-MSH, 1:60,000) diluted in the same blocking solution as described above. On the next day, sections were washed, incubated with a biotinylated secondary donkey antisheep antibody for 1 h (1:1000, in blocking solution without sodium azide), and then treated with ABC solution for 1 h; finally, the signal was developed by DAB solution, giving a brown precipitate. For P-STAT3 IHC, the tissue needed to be pretreated with 1% NaOH and 1% H_2O_2 in H_2O for 20 min, 0.3% glycine for 10 min, and 0.03% sodium dodecyl sulfate for 10 min. After that, sections were blocked for 1 h with 3% normal goat serum in PBS/0.25% Triton X-100/0.2% sodium azide, P-STAT3 antibody was added (rabbit anti-P-STAT3, 1:3000 in blocking solution), and finally incubated overnight at 4 C. On the next day, the procedure was performed as described for α-MSH IHC, except that the secondary antibody was a biotinylated antirabbit antibody (1:1000 in blocking solution without sodium azide). For double IHC, both procedures were performed consecutively as described above (first α-MSH, and then P-STAT3), except that P-STAT3 DAB stain was performed by addition of 0.1% cobalt chloride and 0.1% nickel sulfate resulting in a dark blue precipitate. Pictures were taken as described above with a digital camera and a brightfield microscope.

Cell counting and quantification

For cell counting, one of five series was used from leptin-treated rats. All sections containing POMC cell bodies in the mediobasal hypothalamus were organized systematically in a rostral-to-caudal manner according to the rat brain atlas (45) and then counted for α-MSH-positive cells, P-STAT3-positive cells, and double-labeled cells. To estimate the total cell number in this brain region, we multiplied all numbers by five to account for the five series. This was done in a total of three animals.

Statistical analysis

One-way ANOVA (between subjects) and Fisher's projected least significant difference test for *post hoc* analysis have been used for statistical analysis of cell counting and quantification results.

Results

Leptin activates the proximal rat and human pomc promoters in transfected cells

It has been shown earlier that leptin can increase hypothalamic POMC mRNA levels in the leptin-sensitive *ob/ob* mice, as well as in normal mice and rats (24, 26, 27). Because the long form of the leptin receptor (ObRb) is expressed in POMC neurons (46), we speculated that this effect of leptin is mediated by activation of receptor-dependent intracellular signaling pathways, leading to increased transcription of the *pomc* promoter. To investigate this possibility, we generated reporter plasmids containing proximal sequences of the rat (−704 to +64) and human (−761 to +6) *pomc* promoters located upstream of the luciferase reporter gene in the promoterless pGL2-Basic vector. Regulation of luciferase activities was first studied in the human embryonic kidney cell line, 293T, because these cells have previously proven capable of activating intracellular signaling in response to leptin when transfected with ObRb-expression vectors and of stimulating the *TRH*-promoter (47). Cells were transiently cotransfected with ObRb expression vectors together with *pomc* promoter constructs. Approximately 16 h after transfection, cells were left untreated or treated with 40 nM leptin for 6 h. Measurements of luciferase activities in cell lysates demonstrated stimulation of the rat and the human *pomc* promoters by approximately 3-fold and approximately 6-fold, respectively, whereas the promoterless vector showed no response (Fig. 1A). We also demonstrated a leptin-dependent stimulation of both promoters in the pituitary-derived AtT-20 cell

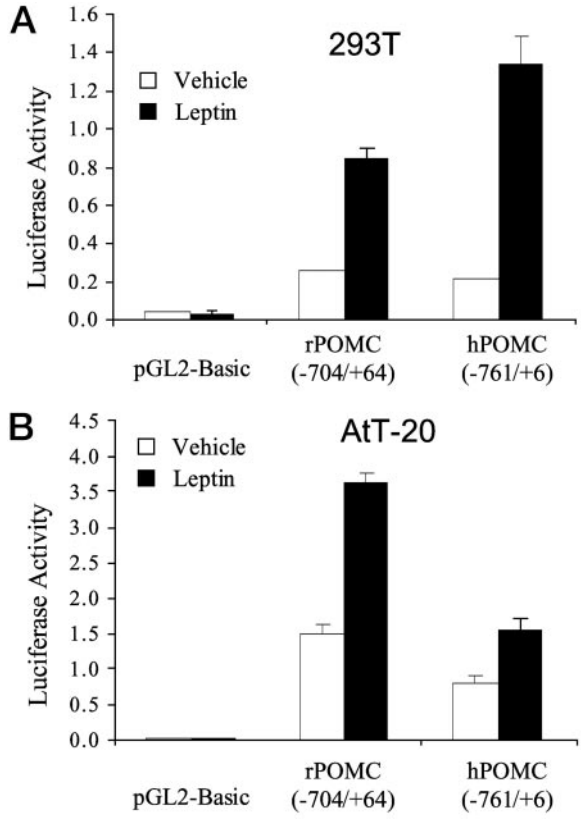

FIG. 1. Leptin activates rat and human proximal *pomc* promoters in transfected cells. Human embryonic kidney cells (293T) (A) or AtT-20 cells (B) were transiently transfected with mammalian expression vectors encoding the murine long form of the leptin receptor (ObRb), together with the promoterless pGL-2-Basic luciferase-vector, rat-POMC promoter (−704/+64), or human-POMC (hPOMC) promoter (−761/+6) plasmids. After transfection, cells were left untreated (*white bars*) or were treated with 40 nM leptin for 6 h (*black bars*). A CMV-lacZ control vector was also cotransfected into the cells to normalize luciferase activities. Transfections and treatments were done in triplicate. Shown is one representative experiment. Data are means ± SEM.

line (Fig. 1B), but the responses were significantly less (~2-fold) compared with those obtained in 293T cells. We do not know the cause of this difference in responsiveness between the two cell lines, but speculate that this relates to limiting levels of positive signaling factors or higher levels of negative signaling components in the AtT-20 cells. We conclude, however, that leptin has the capacity to directly stimulate proximal *pomc* promoter activities in cells expressing ObRb.

STAT3 is required for maximal activation of the pomc promoter by leptin in 293T cells

ObRb belongs to the cytokine receptor superfamily (29, 30) and stimulates the JAK-STAT3 signaling pathway *in vitro* and *in vivo* (23, 28, 35, 41). We therefore investigated the potential role of the STAT3 transcription factor in regulation of the *pomc* proximal promoter by leptin in the transfection system. The human *pomc* promoter-luciferase construct (−761/+6) was cotransfected with ObRb plasmids, together with either WT or DN STAT3 (Y705F) expression vectors, and the cells were treated for 6 h as described above. As shown

in Fig. 2A (*left*), expression of DN STAT3 strongly attenuates (70%) the leptin response. To directly show activation of STAT3 phosphorylation on tyrosine 705 in response to leptin in the same cell system and inhibition of this effect by DN STAT3, we applied fluorescence ICC using an antibody specific to the phosphorylated form of STAT3 (Fig. 2A, *right*). As shown, leptin treatment of cells expressing ObRb and WT STAT3 for 20 min resulted in strong fluorescence in both the cytoplasm and the nucleus of numerous cells. In contrast, a dramatic reduction in STAT3 phosphorylation was observed in leptin-treated cells expressing DN STAT3. These results demonstrate that functional STAT3 proteins are required for maximal activation of the *pomc* promoter by leptin in this system.

Y1138 of ObRb is required for maximal activation of the human pomc promoter in 293T cells

The intracellular domain of the long form of the murine leptin receptor, ObRb, contains three tyrosine residues of which the C-terminal Y1138 is known to mediate leptin-dependent binding and activation of STAT3 (32, 36, 37). To support the finding that STAT3 is critical for regulation of *pomc* promoter activity by leptin, we therefore tested the significance of Y1138 for this effect. Cells were transfected with the human *pomc* promoter plasmid, together with either the WT or mutated (Y1138S) ObRb plasmid, and treated or not treated with leptin for 6 h before measurement of luciferase activities. As shown in Fig. 2B (*left*), cells expressing leptin receptors lacking Y1138 showed a marked reduction (~60%) in promoter activity in response to leptin, compared with cells expressing the WT ObRb. As expected, this was accompanied by a significant reduction in the number of cells exhibiting activation of STAT3 phosphorylation by mutant ObRb, as demonstrated by ICC (Fig. 2B, *right*), although a few cells partly responded to leptin. This latter result may suggest that other tyrosine residues of ObRb have some capacity to activate STAT3, and it is consistent with the observed residual activation of the *pomc* promoter by the Y1138S receptor. Combined, however, these results show that Y1138 of ObRb is required for full stimulation of STAT3 phosphorylation and of *pomc*-promoter activities in response to leptin.

An element of the proximal pomc promoter is required for STAT3-dependent regulation of pomc transcription by leptin in transfected cells

To begin to identify promoter elements responsible for the regulation of the *pomc* promoter by leptin, we generated a number of progressive deletions of the promoter. As shown in Fig. 3A, the human promoter construct containing 91 base pairs upstream of the transcriptional start site (−91/+6)

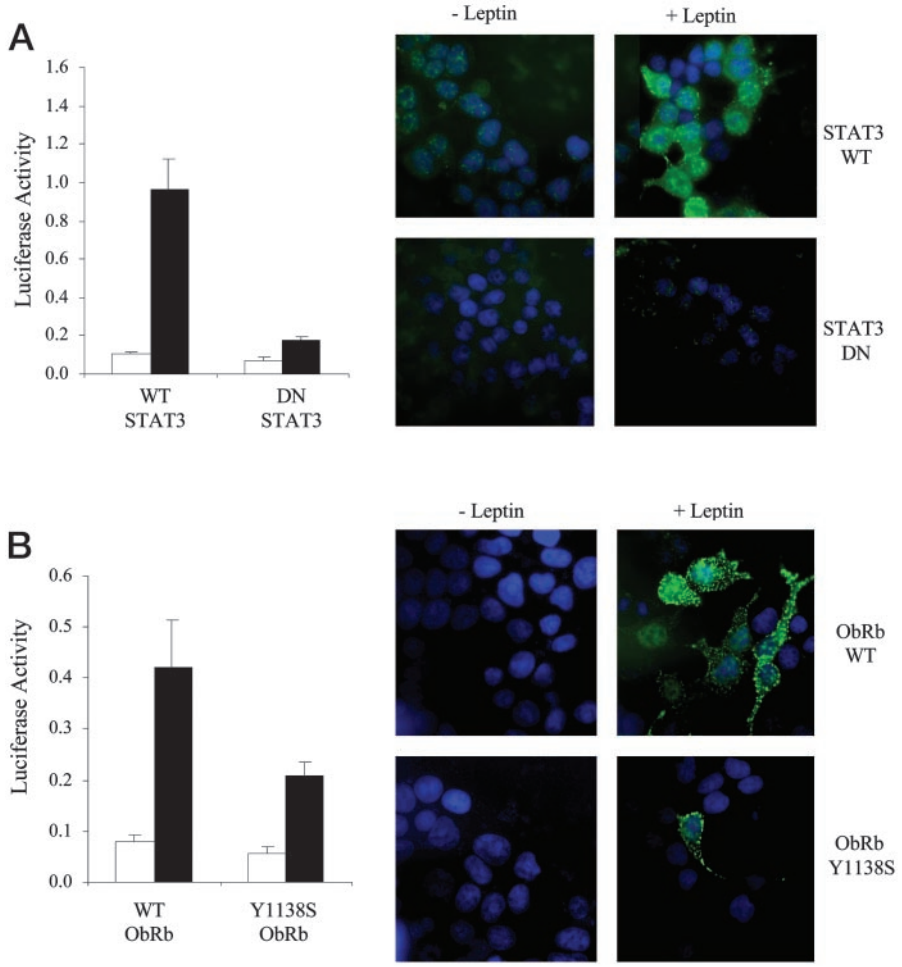

FIG. 2. Y1138 of the leptin receptor and STAT3 are required for maximal activation of the *pomc* promoter by leptin in 293T cells. A, *Left*, 293T cells were transfected with ObRb and the human *pomc* promoter constructs. Vectors encoding WT STAT3 or DN Y705F-STAT3 were also cotransfected into the cells. Transfections and treatments were performed in triplicate. Shown is one representative experiment. Data are means ± SEM. *Black bars* depict luciferase activities in lysates from leptin-treated cells (6 h, 40 nM). *Right*, Cells were grown on lab-tech slides, transfected with ObRb together with WT STAT3 or DN STAT3 plasmids, and subsequently treated or not treated with 40 nM leptin for 20 min. Cells were subjected to fluorescence ICC with anti-pY-705-STAT3-antiserum (*green*). *Blue color* shows diamidinophenolindole nuclear counter staining. Shown are representative fields of each transfection and treatment after capturing with a digital camera mounted on a fluorescence microscope. B, *Left*, 293T cells were transfected with ObRb or ObRb lacking tyrosine 1138 (Y1138S) vectors, together with the human *pomc* promoter construct. Transfections and treatments (6 h, 40 nM) were performed in triplicate. Shown are luciferase activities. *Right*, Cells were transfected as above, treated with 40 nM leptin for 20 min, and subjected to fluorescence ICC, except that WT STAT3 vectors were included and *pomc*-luc plasmids were omitted in the transfections. Representative fields of each transfection and treatment are shown.

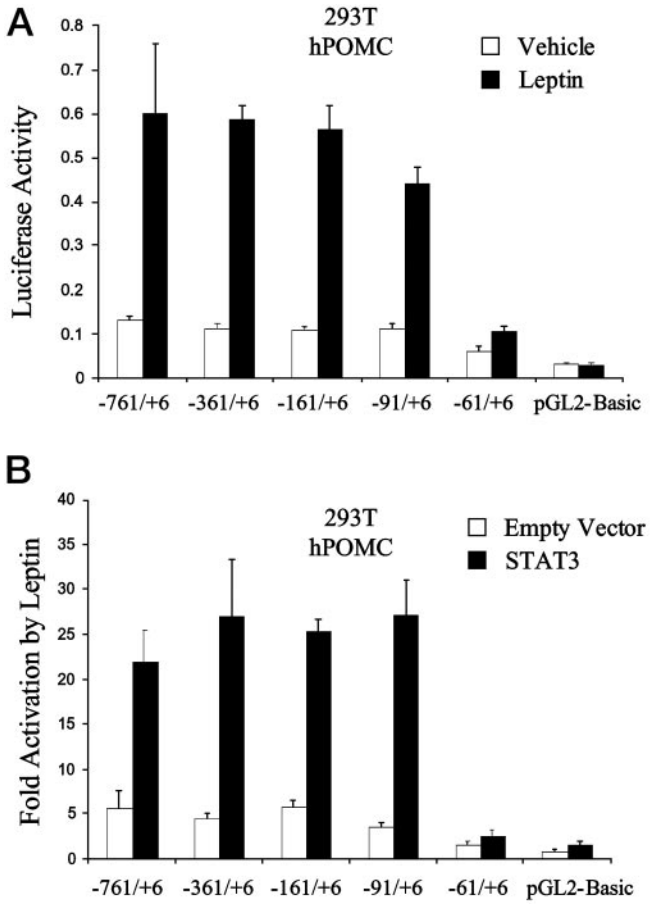

FIG. 3. Identification of a 30-bp element of the human *pomc* promoter that is required for leptin and STAT3 responsiveness in 293T cells. A, Cells were transfected with ObRb plasmids together with progressive deletion plasmids of the human *pomc* promoter. Data are means ± SEM. *Black bars* depict luciferase activities in leptin-treated cells (6 h, 40 nM), and *white bars* represent vehicle-treated cells. B, Cells were transfected as above, except that WT STAT3 vectors were cotransfected or not. Data are depicted as fold activation without coexpression (*white bars*) or with coexpression of STAT3 (*black bars*). Data are means ± SEM.

exhibited full responsiveness to leptin, whereas deletion of further 30 bases (−61) resulted in a near-complete loss of activation. Figure 3B shows fold-responses to leptin in cells coexpressing STAT3 or an empty control vector. Coexpression of WT STAT3 enhanced the leptin responsiveness of the −91/+6 construct from approximately 5-fold to approximately 30-fold, but the −61/+6 construct was almost nonresponsive to leptin under both of these conditions. These results clearly demonstrate that removal of the promoter sequence between −91 and −61 results in complete loss of STAT3-mediated activation of the human *pomc* promoter by leptin.

We examined the human promoter region encompassing the 30-bp sequence for possible STAT DNA-binding elements [*TT*(N)$_5$*AA*; Refs. 48 and 49). One putative site in the 3′-5′ orientation was identified at bases −73 to −65. This sequence (*T*GCCGGG*AA*) is not a canonical STAT-DNA binding element, but is highly conserved and identical among the rat and murine *pomc* promoters (TGCCAGGAA). However, with EMSA, we could not detect STAT3 binding to human, rat, or mouse DNA probes, using nuclear extracts from leptin-treated 293T cells, transiently expressing ObRb and STAT3 proteins (data not shown). In parallel experiments using the same nuclear extracts, we did however demonstrate strong leptin-dependent binding of STAT3 to an oligonucleotide probe containing a known STAT inducible element (SIE–m67; Ref. 50), thus proving the functionality of the assay. Combined, these data show that the −91 to −61 region of the human *pomc* promoter is required for STAT3-dependent activation by leptin in 293T cells, and suggests that promoter regulation may be mediated via a more complex and indirect STAT3-dependent mechanism that has yet to be identified.

Leptin-dependent STAT3 phosphorylation in the rat brain

To further support our evidence for a role of STAT3 in *pomc* gene regulation by leptin, we next wanted to determine whether STAT3 is phosphorylated in hypothalamic POMC neurons in leptin-treated rats. For this purpose, we first developed immunohistochemical methods to detect phosphorylated STAT3 in brain sections from rats. Sprague Dawley rats were given a single ip injection of recombinant leptin (1.0 mg/kg body weight) or vehicle (PBS) and killed 45 min later. Coronal brain sections (25-μm thick) were subjected to IHC using the same phospho-specific-STAT3 (Y705) antiserum as described in Fig. 2. Overall, we found very few P-STAT3-positive cells in the hypothalamus from PBS-treated animals (Fig. 4, A and D). In striking contrast, dense populations of cells with nuclear staining were seen throughout the ARC and in subregions of the ventromedial nucleus of hypothalamus (VMH) and the dorsomedial (DMH) region, similar to findings reported by Hubschle *et al.* (38). Representative photomicrographs of sections from two levels of the mediobasal hypothalamus of a leptin-treated rat are shown in Fig. 4, B and E. Matched brain sections were used to identify exact locations of individual hypothalamic nuclei by applying the Nissl-stain (Fig. 4, C and F). Combined, these results demonstrate rapid induction of STAT3 phosphorylation in regions of the hypothalamus known to express high levels of the leptin receptor (12).

Other regions of the hypothalamus containing numerous cells demonstrating leptin-dependent STAT3 phosphorylation are the paraventricular nucleus (PVN; Fig. 5, A–C) and the lateral hypothalamic area (LHA; Fig. 5, D–F). Strong staining in processes of cells in the LHA could also be identified. Outside the hypothalamus, a distinct population of cells in regions of the periaquaductal gray (PAG) and the dorsal raphae (DR; Fig. 5, G–I) were activated in response to leptin administration. Finally, we also found a dense group of cells that were positive for P-STAT3 in the caudal brain stem, more specifically, in regions of the nucleus of the solitary tract (NTS) and possibly parts of the dorsal motor nucleus of the vagus nerve (Fig. 5, J–L). These results suggest that leptin is acting directly at these regions and imply that neurons located at these sites are important for the biological effects of leptin.

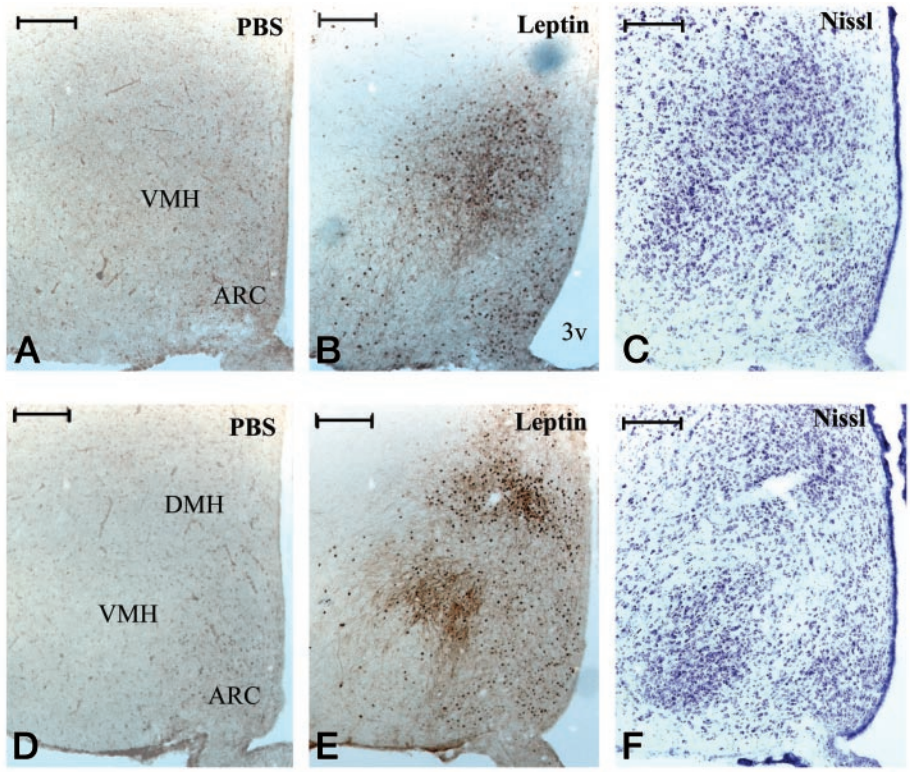

FIG. 4. Leptin rapidly stimulates STAT3 phosphorylation in the mediobasal hypothalamus. Rats were given a single ip injection of recombinant leptin (1.0 mg/kg; B and E) or vehicle (PBS; A and D) and killed 45 min later. Coronal brain sections were obtained and subjected to IHC using anti-pY-STAT3 antiserum (A, B, D, and E) or to Nissl staining (C and F) as described in *Materials and Methods*. Shown are microphotographs of two matched series of sections from the hypothalamus (A–C, bregma −2.30) and (D–F, bregma −3.30). 3v, Third ventricle. *Scale bars*, 200 μm.

Leptin induces STAT3 phosphorylation in POMC neurons in rats

The major site within the brain containing POMC neurons resides in the ARC of the hypothalamus (51–54). To directly determine whether STAT3 is phosphorylated in these cells in response to leptin and thus support our transfection results, we developed a double-IHC method for detecting P-STAT3 and the POMC-derived melanocortin peptide, α-MSH, as presented in Fig. 6. *Brown* represents staining for α-MSH, and *blue* represents P-STAT3. A representative, low-magnification microphotograph of a section from a PBS-treated rat is shown in Fig. 6A. Consistent with earlier reports, we found cell bodies containing α-MSH in the ARC of the hypothalamus. As expected, α-MSH was detected in the cytoplasm of these cells, as shown in a high-magnification microphotograph from the same section (Fig. 6B). Cells positive for P-STAT3 were not found in this section, consistent with the lack of STAT3 phosphorylation in the mediobasal hypothalamus from PBS-injected animals (Fig. 4). Dense concentrations of nerve fibers containing α-MSH could be seen in the ARC, DMH, LHA, and many extrahypothalamic brain regions (data not shown), as previously reported (55). In contrast to brain sections from the control animals, sections from rats killed 45 min after leptin injection demonstrated numerous POMC neurons in the ARC that were positive for nuclear P-STAT3 immunoreactivity. An example of a field containing several double-labeled cells is shown in Fig. 6C. As expected, cells positive for P-STAT3 (*blue*), but negative for α-MSH, were also identified, some of which may be agouti-related-protein and NPY neurons. Together, these data show for the first time induction of STAT3 phosphorylation by leptin in POMC neurons located in the mediobasal hypothalamus, consistent with reports showing expression of leptin receptors in these cells (46) and thus supporting our data from above suggesting a role of STAT3 in regulation of *pomc* gene expression by leptin.

Leptin-responsive POMC neurons are concentrated in the rostral region of the mediobasal hypothalamus

It has been reported that POMC neurons located in the rostral part of the ARC are more responsive to leptin when measuring POMC mRNA by *in situ* hybridization, compared with those in the more caudal region (24, 27). We therefore counted double-labeled cells throughout the mediobasal hypothalamus in a systematic and rostral-to-caudal manner in three leptin-treated rats. The results from analysis of one rat are presented in Fig. 7. In a representative section from the rostral and caudal regions, we found that 76% and 28% of the α-MSH-positive cell bodies, respectively, were also positive for P-STAT3 (Fig. 7A). Results from all sections containing POMC neurons of the animal are depicted in Fig. 7B. Altogether, 1134 cells were counted positive for α-MSH, and 408 (37%) of these were colocalized with P-STAT3. Because we only counted one of five series, a total of approximately 5670 POMC cells and approximately 2040 doubles are predicted in the rat brain. Furthermore, in the most rostral sections, 70–90% were double labeled, whereas only 20–30% were counted in the caudal sections (Fig. 7C). Very similar results were obtained from analysis and counting of sections from two other leptin-treated rats. Combined, these immunohistochemical studies show for the first time that STAT3 is rapidly phosphorylated in POMC neurons *in vivo* in response to exogenous leptin administration. In addition, our data suggest that only a subset (~37%) of POMC neurons is

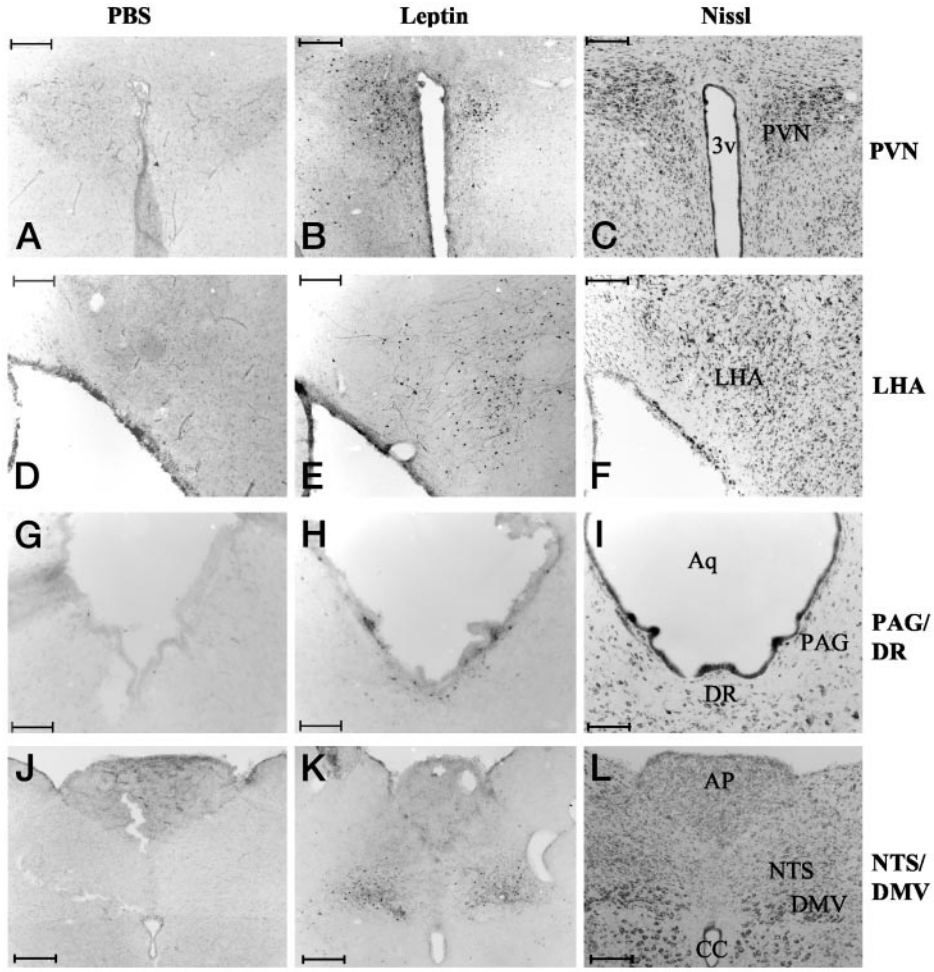

FIG. 5. Activation of STAT3 phosphorylation by leptin in hypothalamic and extrahypothalamic brain regions of the rat. Animals were treated, and brain sections were obtained as described for Fig. 4. Shown are microphotographs of four matched series of sections from the PVN (A–C), LHA (D–F), PAG (G–I), and NTS (J–L). DMV, Dorsal motor nucleus of the vagus; 3v, third ventricle; CC, central canal; AP, area postrema; Aq, aquaduct. Nissl staining is shown of corresponding sections and fields to each brain region (C, F, I, and L). *Scale bars*, A–F and J–L, 200 μm; G–I, 100 μm.

leptin-responsive and that these cells are concentrated in the rostral part of the mediobasal hypothalamus.

Discussion

Powerful genetic and pharmacological data demonstrate that the central melanocortin system is critical for the normal regulation of energy homeostasis and strongly implicates hypothalamic POMC neurons as key mediators of leptin action to affect body weight and regulate neuroendocrine function (56). It is known that these cells express leptin receptors (46), and several studies have shown regulation of POMC mRNA levels by leptin in rodents (24, 26, 27), but the mechanism underlying this effect is unknown. Here, we present strong evidence suggesting a critical role of the STAT3 transcription factor to stimulate hypothalamic *pomc* gene transcription when activated by leptin receptors expressed in POMC neurons.

Using a heterologous transfection system in mammalian cells, we have determined that the STAT3-binding site, tyrosine 1138, of the leptin receptor and tyrosine residue 705 of STAT3 are obligate components of an intracellular signaling pathway to maximally induce transcriptional activation of the proximal *pomc* promoter by leptin. We did, however, detect some residual promoter activation in leptin-treated cells expressing DN STAT3 proteins lacking Y705 and in cells expressing leptin receptors lacking Y1138. This may suggest that leptin-regulated pathways other than STAT3 signaling are also important for *pomc* promoter activation. For example, leptin-dependent activation and the presence of phosphoinositol-3-kinase (57–59), phosphodiesterase-3B (59), and the MAPK-ERK pathway (60, 61) have been reported in the mediobasal hypothalamus. But whether these enzymes and the down-stream pathways are directly activated in POMC neurons by leptin and whether they play a role in regulation of the *pomc* gene is presently unknown and requires further studies.

Previous studies have identified STAT3-responsive elements in the proximal *pomc* promoter (42). Specifically, two juxtaposed sequences located at position −399 to −379 of the rat promoter were shown to cooperatively bind STAT3 in nuclear extracts from pituitary-derived AtT-20 cells treated with leukemia-inhibitory factor (LIF). Furthermore, mutation of these putative STAT3 binding sites revealed that these were required for full stimulation of rat *pomc* promoter activities by LIF, suggesting a role of these elements in pituitary-adrenal responses to LIF during inflammation. Because the LIF-receptor system and the leptin receptor have similar signaling characteristics, we made progressive deletions of the promoter to test whether the leptin receptor signaling acts via these same elements. In contrast to the findings in the

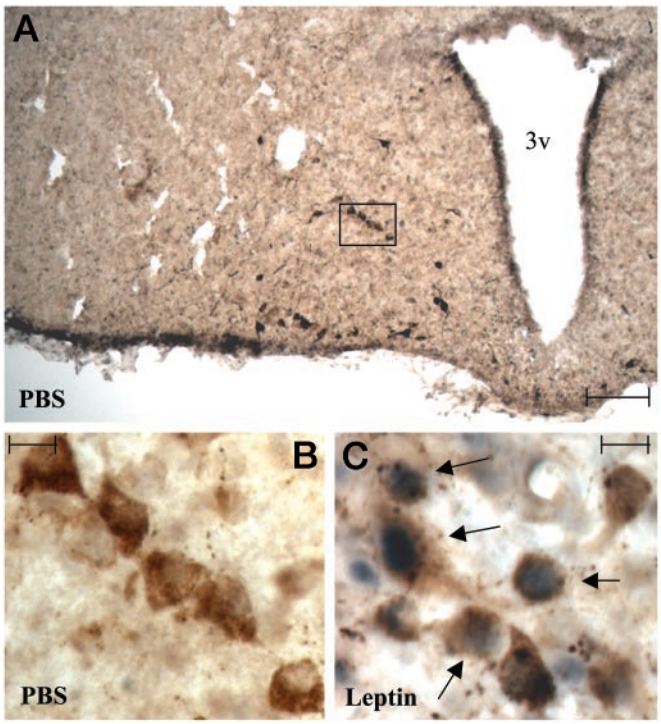

FIG. 6. Activation of STAT3 phosphorylation by leptin in POMC neurons. Rats were given a single ip injection of recombinant leptin (1.0 mg/kg; C) or PBS (A and B) and killed 45 min later. Coronal brain sections were subjected to double IHC using anti-α-MSH (*brown* staining) and anti-pY-STAT3 (*blue* staining) antiserum as described in *Materials and Methods*. Shown are examples of microphotographs of fields from sections of the basal hypothalamus of a PBS-treated rat (A, low magnification; B, high magnification of area indicated in A) and a leptin-treated rat (C, high magnification). 3v, Third ventricle. *Arrows* indicate double-labeled cells. *Scale bars*, A, 100 μm; B and C, 10 μm.

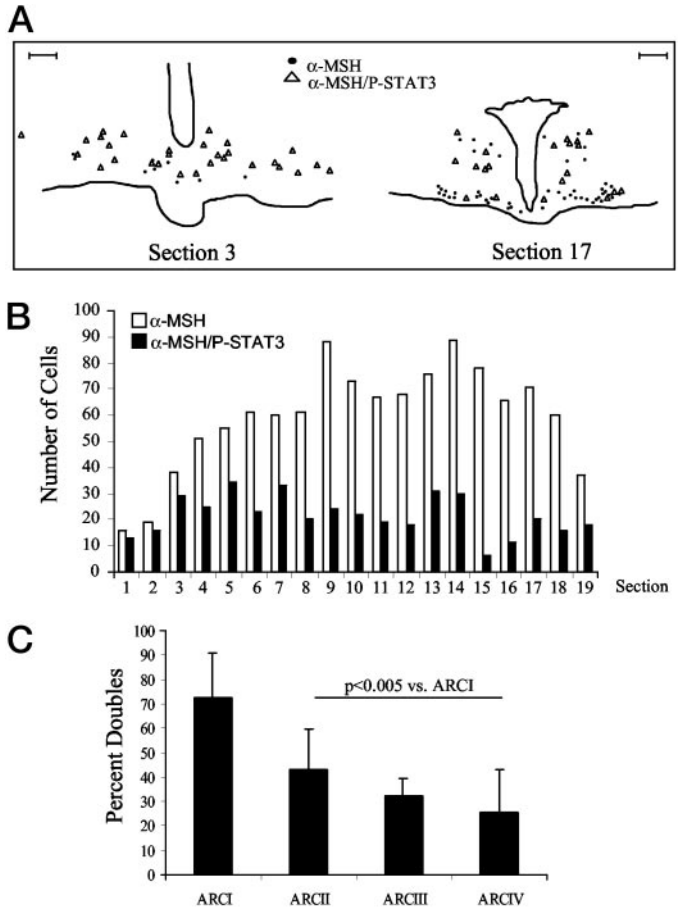

FIG. 7. Leptin-responsive POMC neurons are concentrated in the rostral region of the mediobasal hypothalamus. Animals were treated and brain sections obtained as described for Fig. 4. A, Example of results from double-labeling analysis and counting of a rostral and a caudal section from a leptin-treated rat (sections correspond to B). *Triangles* and *dots* represent double- (α-MSH/P-STAT3) and single- (α-MSH) labeled cells, respectively. B, Total results from counting of all sections (n = 19) containing α-MSH-positive cells (*white bars*) and double-labeled cells (*black bars*) in a rostral-to-caudal manner from one series of sections from a leptin-treated rat. C, Percentage of doubled-labeled cells after grouping and statistical analysis of the results depicted in B. ARC I, sections 1–4; ARC II, sections 5–9; ARC III, sections 10–14; and ARC IV, sections 15–19. The statistically significant differences between the rostral (ARC I) and caudal sections (ARC II–IV) were confirmed by one-way-ANOVA (between subjects), and Fisher's projected least significant difference test was used for *post hoc* analysis. Similar results were obtained from independent analysis of sections from two additional leptin-treated animals.

pituitary *pomc* system in response to LIF, we found that a 30-bp sequence located between −91 and −61 of the human proximal *pomc* promoter was required for STAT3-dependent activation by leptin. Surprisingly, we could not detect binding of STAT3 to this minimal promoter region using EMSA. This suggests that STAT3 may act indirectly via activation of other transcription factors that ultimately regulate *pomc* transcription via the −91/−61 element. Indeed, interactions of STAT molecules with other transcription factor systems, including the glucocorticoid receptor (62), SP1 (63, 64), C/EBP (63), and c-Fos and JunB (42, 65), have been described, but further studies are required to determine whether these proteins or other mechanisms are important for the observed effects. We conclude that STAT3 and proximal promoter sequences are likely to play key roles in the stimulation of the hypothalamic *pomc* promoter by leptin. To directly demonstrate this *in vivo*, more complex studies are required. Specifically, transgenic expression of DN STAT3 proteins in POMC neurons or conditional deletion of STAT3 in the same neurons could determine this and thus conclusively connect our transfection results and *in vivo* findings.

Several methods have been applied to identify nuclei and cells in the brain that are leptin-responsive. These include c-Fos IHC (3, 32), ObRb *in situ* hybridization histochemistry (ISHH; Ref. 12), suppressor of cytokine signaling-3 (SOCS-3) ISHH (44), and STAT3 translocation by IHC (38). Although results from these studies have clearly increased our understanding of leptin action in the brain, some issues remain to make these techniques optimal tools. For example, c-Fos is only activated in a subset of leptin-sensitive neurons (3), the ObRb and SOCS-3 ISHHs are not as sensitive as desired, and the STAT3 translocation assay may have a relatively high background due to the presence of STAT3 in the cytoplasm. We conclude that the P-STAT3 IHC is a particularly useful and sensitive method to identify leptin-responsive cells in the brain.

As expected from the previous studies of ObRb mRNA expression and of SOCS-3 mRNA induction by leptin, we

found that the predominant leptin-responsive nuclei in the brain are the ARC, VMH, and DMH. Careful mappings of leptin-sensitive sites in the hypothalamus by Hubschle *et al.* (38) showed very similar results using a STAT3-translocation assay in rats. We also found moderate populations of positive cells in the PVN, which may constitute hypophysiotrophic TRH neurons (66), and in regions of the LHA, which may represent populations of orexin and/or melanin-concentrating hormone-expressing neurons (67). Double-labeling studies are required to examine these possibilities. The PVN and LHA have not consistently been identified with the ISHH methods, but the LHA was clearly mapped using the STAT3-translocation assay in the rat and recently using P-STAT3 IHC in the mouse (39). Altogether, this latter study reported quite similar results in the mouse as we present here in the rat, but the authors did not mention the PVN or the DMH as leptin-responsive sites. On the other hand, we did not identify the parabrachial nucleus in our studies of the rat. The reason for these differences is unclear, but may in part relate to the different species studied and/or to the time-points used. On the basis of the good correlation with previous ISHH and IHC studies listed above and earlier *in vitro* receptor-signaling studies, we conclude that P-STAT3 positive cells identified in brains of leptin-treated animals are likely to represent cells that express functional long-form leptin receptors and are accessible to circulating leptin, thus representing direct targets of leptin.

Outside the hypothalamus, we found a moderate number of P-STAT3 cells in the PAG and DR of leptin-treated rats. The identity and role of these cells in leptin action are unknown. Finally, a dense group of cells was observed in the caudal brain stem, specifically in the NTS, as also reported by Hosoi *et al.* (39). The nature of these cells has not been determined, but they could represent POMC neurons, as a small population of POMC neurons is known to exist at this site (68). Because of low expression of α-MSH in these neurons compared with the hypothalamic POMC cells, detection requires colchicine treatment (68), which we did not perform in this study, and a possible colocalization could thus not be determined. Other potential candidates include GLP-1 neurons because leptin receptor mRNA (12) and leptin-dependent induction of c-Fos have been colocalized to these cells in the NTS (69). Furthermore, our results are consistent with reports showing inhibitory effects of low doses of leptin on feeding when injected directly into the NTS (70). Finally, a recent paper concludes that leptin has the ability to directly affect the firing rate of NTS neurons that are activated in response to gastric loadings (71). In conclusion, our data strongly suggest that leptin acts directly in the caudal brain stem and imply that leptin may affect afferent satiety signals that are transmitted via the vagus nerve from the stomach and gut to the NTS (72). Such effects of leptin may ultimately lead to regulation of meal size, and future studies will determine the validity of these interesting possibilities.

Using double-labeling IHC, we show here for the first time activation of STAT3 phosphorylation by leptin in hypothalamic cells known to express leptin receptors, specifically the POMC neurons. This result strongly supports our transfection studies suggesting a critical role of the STAT3 pathway in regulation of *pomc*-gene expression by leptin. By counting double-labeled cells in the ARC, we found that 37% of POMC neurons contained phosphorylated STAT3 in leptin-treated rats. We cannot, however, exclude the possibility that additional cells will be activated at earlier or later time-points compared with the 45 min used in this study. We also counted all α-MSH-positive cells in sections from two rats and calculated a total of approximately 5600 POMC neurons in the basal hypothalamus per brain, slightly higher than the approximately 3000 cells reported in the mouse (13). We calculated that approximately 2000 cells of the POMC neurons are P-STAT3 positive after leptin administration, thus suggesting that only a subpopulation of POMC neurons in the rat is responsive to leptin.

Studies indicate that regulation of POMC mRNA in response to fasting and leptin treatment is mainly confined to the rostral region of the ARC (27). Furthermore, Elias *et al.* (3) have reported that c-Fos immunoreactivity in POMC neurons of leptin-treated rats is concentrated in the rostral part of the mediobasal hypothalamus. Finally, Thornton *et al.* (24) report that the number of detectable POMC neurons is increased in the rostral part of the ARC, but not in the caudal ARC, after leptin injections in *ob/ob* mice. To further evaluate this possibility using P-STAT3 IHC, we determined regional differences in responsiveness to leptin in POMC neurons by systematically counting the number and calculating the percentage of P-STAT3-positive POMC neurons in different regions of the ARC. Indeed, we found approximately 75% in the rostral ARC and about a third (~25%) in the caudal region of the ARC. Our data are thus consistent with previous studies demonstrating the presence of leptin-responsive POMC neurons throughout the ARC, but at the same time showing concentration of these cells in the rostral region. Similar conclusions from studies of leptin-responsive subpopulations of NPY neurons in the ARC have also been reported (6). POMC neurons are thus likely to be a heterogeneous population of cells in which subsets may serve different functions. The meaning of these findings and the specific functions of individual POMC populations in melanocortin action clearly deserve further examination.

In conclusion, our studies suggest that STAT3 activation is critical for regulation of hypothalamic *pomc* gene expression by leptin and that this occurs by leptin acting directly via leptin receptors expressed on POMC neurons. Consistent with a key role of this transcription factor in leptin action, recent data demonstrate that removal of the STAT3-binding site (Y1138) of the leptin receptor in mice results in leptin resistance, extreme obesity, and decreased hypothalamic POMC mRNA levels (73). Combined, these findings underscore the importance of transcriptional events in leptin action and imply that STAT3 is likely to be critical for regulation of the melanocortin pathway to maintain energy balance by leptin.

Acknowledgments

We thank Dr. J. S. Flier (Boston, MA) for valuable input and support. We are also very thankful for advice from Dr. J. Elmquist (Boston, MA), Dr. J. Tatro (Boston, MA), C. Lee (Boston, MA), N. Trentacosta (Providence, RI), and E. Csizmadia (Boston, MA) with regard to the immunohistochemical studies.

Received October 25, 2002. Accepted January 16, 2003.

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This work was supported by grants from the National Institutes of Health (RO1-DK-60673, to C.B.) and the Joslin Diabetes Center (5-P30-DK-36836, to C.B.) and by an Emmy-Noether grant from the Deutsche Forschungsgemeinschaft (MU 1662/2-1, to H.M.).

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