Selective Activation of Mitogen-Activated Protein (MAP) Kinase Kinase 3 and p38α MAP Kinase Is Essential for Cyclic AMP-Dependent UCP1 Expression in Adipocytes

Jacques Robidoux,1,3 Wenhong Cao,2,3 Hui Quan,3 Kiefer W. Daniel,1,3 Fatiha Moukdar,3 Xu Bai,1 Lisa M. Floering,1,3 and Sheila Collins1,2,3*

Department of Psychiatry and Behavioral Sciences1 and Division of Endocrinology, Department of Medicine,2 Duke University Medical Center, Durham, North Carolina 27710, and Endocrine Biology Program, Division of Biological Sciences, CIIT Centers for Health Research, Research Triangle Park, North Carolina 27709

Received 25 November 2004/Returned for modification 21 December 2004/Accepted 1 April 2005

The sympathetic nervous system regulates the activity and expression of uncoupling protein 1 (UCP1) through the three β-adrenergic receptor subtypes and their ability to raise intracellular cyclic AMP (cAMP) levels. Unexpectedly, we recently discovered that the cAMP-dependent regulation of multiple genes in brown adipocytes, including Ucp1, occurred through the p38 mitogen-activated protein kinases (MAPK) (W. Cao, K. W. Daniel, J. Robidoux, P. Puigserver, A. V. Medvedev, X. Bai, L. M. Floering, B. M. Spiegelman, and S. Collins, Mol. Cell. Biol. 24:3057–3067, 2004). However, no well-defined pathway linking cAMP accumulation or cAMP-dependent protein kinase (PKA) to p38 MAPK has been described. Therefore, in the present study using both in vivo and in vitro models, we have initiated a retrograde approach to define the required components, beginning with the p38 MAPK isoforms themselves and the MAP kinase kinase(s) that regulates them. Our strategy included ectopic expression of wild-type and mutant kinases as well as targeted inhibition of gene expression using small interfering RNA. The results indicate that the β-adrenergic receptors and PKA lead to a highly selective activation of the p38α isoform of MAPK, which in turn promotes Ucp1 gene transcription. In addition, this specific activation of p38α relies solely on the presence of MAP kinase kinase 3, despite the expression in brown fat of MKK3, -4, and -6. Finally, of the three scaffold proteins of the JIP family expressed in brown adipocytes, only JIP2 communoprecipitates p38α MAPK and MKK3. Therefore, in the brown adipocyte the recently described scaffold protein JIP2 assembles the required factors MKK3 and p38α MAPK linking PKA to the control of thermogenic gene expression.

Uncoupling protein 1 (UCP1) is essential for rodents and other small mammals to maintain their body temperatures, since it is the sole mediator of cold-induced nonshivering thermogenesis (4, 6, 48); UCP1 is also a key contributor to the regulation of diet-induced thermogenesis (6, 58). The UCP1 protein resides within the inner membrane of mitochondria, where it serves as a portal for dissipation of the proton gradient such that respiration is uncoupled from ATP production and generates heat (35, 49, 54). The UCP1 mRNA and protein are found in “brown” and to a lesser extent in “white” adipose tissue; however, its expression is confined to brown adipocytes (55). Similar brown adipocytes exist scattered within white adipose depots in adult humans (22, 37), but their contribution to thermogenesis is admittedly modest. Nevertheless, studies in animals or humans exposed to high catecholamine levels or treated with sympathomimetics show that brown adipocytes expressing UCP1 can be recruited within white adipose depots (10, 12, 13, 16, 29).

Brown adipose tissue (BAT) and white adipose tissue are innervated by sympathetic noradrenergic nerves (2, 3, 42, 50, 63). In response to cold exposure or diet, sympathetic nervous system activation leads to the release of norepinephrine to interact with adrenergic receptors (AR); in particular the family of βARs (39, 49, 55, 72). Catecholamine stimulation of the three βARs present in adipocytes promotes a series of events initiated by the production of cyclic AMP (cAMP) and the activation of cAMP-dependent protein kinase (PKA) (20, 56, 64). These events result in lipolysis and liberation of free fatty acids (FFA) from triglyceride stores (39). These FFA serve not only as substrates for oxidative respiration but also as allosteric activators of UCP1 function (24, 25, 60). βAR-mediated increases in cAMP also stimulate Ucp1 gene transcription. The cAMP response of the Ucp1 gene is achieved predominantly through an enhancer region (9, 15, 38). This enhancer, which is well conserved among species (11), confers specificity of expression to brown adipocytes as well as the cAMP response and contains at least two key elements: a peroxisome proliferator response element (PPRE) and a cAMP response element (CRE).

We have recently shown that the cAMP-dependent transcription of the Ucp1 gene is regulated through these two elements by p38 mitogen-activated protein kinase (MAPK) (7). The effect of p38 MAPK on these elements occurs in a coordinated fashion. First, p38 MAPK phosphorylates a protein called PGC-1α (7), which is a transcriptional coactivator and mediator of mitochondriogenesis (68), among other functions. This modification of PGC-1α enhances its activity as a nuclear coactivator of gene transcription in coordination with peroxi-
PKA ACTIVATION OF MKK3 AND \( \text{p38} \): MAPK

**Table 1. Sequences for the siRNAs used in these studies**

| Targeted gene | Sense sequence 5′–3′ | Antisense sequence 5′–3′ |
|---------------|----------------------|--------------------------|
| MKK3-a        | GGACUACGGUACCUUCCCTT  | GCAGGGAUUCUGCAGGUCCTT    |
| MKK3-b        | GGUGUGGAGAAGAACAUGTT  | CAUGUUCUCUUGGACCCCTT    |
| MKK3-c        | GGACCUGAUUGCUUGUUAAT  | UAACACGCAAUAGGGUCCTG     |
| MKKα          | GGCGUCAUUCAUUAUGGATT  | UCCAAUGAAAGGCAACCTT      |
| p38α MAPK-a   | GGUCACUGGAGAUAUCCATT  | UGAAUAUCUCCUGAGACCCTT    |
| p38α MAPK-b   | GGACCUCUUUAUGAGCUUUT  | AAAGCUUAUAUGGGUCCTT      |
| p38β MAPK     | GGUGUCUGUAAAGACUGGTT  | CAGCUCCUUAAACGCACCTT     |

*Note that for some of the targeted genes, two (p38α MAPK) or three (MKK3 3′) siRNA duplexes were used because they achieved >80% specific gene knockdown.*

some proliferator-activated receptor γ (PPARγ); PPARγ in turn binds to the UCP1 PPRE (7). Second, p38 directly stimulates expression of the Ucp1 gene through phosphorylation of the transcription factor ATF-2; ATF-2 binds to the CRE (7). Finally, the PGC-1α gene itself also possesses a CRE (28) but in the brown adipocyte is a target of p38-activated ATF-2 and not CREB (7). By increasing the overall amount of PGC-1α protein over time, p38 MAPK primes the cell for a sustained enhancement of UCP1 expression. Despite this new understanding of the role of p38 MAPK in the regulation of the Ucp1 and PGC-1α genes in brown fat, the cascade of signaling events downstream of PKA by which p38 MAPK becomes activated is completely unknown.

To begin to unravel this new pathway, we realized that it was necessary to tackle this problem in a “bottom-up” approach. Therefore, we reasoned that a strategy that would best serve this effort should first identify the actual p38 MAPK isoform(s) involved and proceed in a retrograde manner. The p38 MAPK group is composed of four isoforms: p38α (26, 41), p38β (32), p38γ (43), and p38θ (66). Among them, p38α and -β are sensitive to the pyrimidyl imidazoles SB202190 and SB203580 (14, 23). These two isoforms are expressed in adipocytes (36).

Depending on cell type and stimulus, p38 MAPK can be activated by MKK3 (17) or MKK6 (27, 46, 52, 61) or by both of them. In some cell types MKK4 can activate p38 MAPK (17, 44). However, depending upon the stimulus or physiological state, there are circumstances in which these MKKs can clearly display substrate preferences or noninterchangeable roles (62, 69). For example, MKK3 tends to prefer p38α while MKK6 is equally efficient at both p38α and p38θ (18). We also embarked on the current series of studies because defining the exact p38 isoform(s) and its immediate activator(s) in the control of UCP1 transcription may provide clear targets to modulate thermogenesis. Using a variety of experimental approaches, we show that p38α and its activator MKK3 are the sole players in the control of UCP1 gene transcription.

**MATERIALS AND METHODS**

**Chemicals and plasmids.** The plasmid BBCAT, containing the 3.74-kb mouse Ucp1 5′-flanking sequence was a gift from Jiahuai Han (26, 32). The mouse pCMV-SPORT6-p38 MAPK and p38α-actin-luciferase (pcDNA3-Act) expression plasmid, and the 3AR expression plasmid, and the pcDNA4/HisMax TOPO TA Expression vector were all gifts from Roger J. Davis, University of Massachusetts Medical School: MKK3, MKK4, MKK6, and its constitutively active form, MKK6E (17, 52). The expression vector pcDNA3-MKK7 was a gift from Josef M. Penninger, University of Toronto (70). The S-protein-tagged JLP was a gift from E. Premkumar Reddy, Temple University (5, 40). The full-length clones for JIP1, JIP2, and JIP3 were purchased from Open Biosystems (Huntsville, AL.) and were subcloned in pDNA4/HisMax TOPA TA Expression vector from Invitrogen (Carlsbad, Calif.).

The β3AR-selective agonist CL316,243 (CL) was a gift from Elliott Danforth, Jr. (American Cyanamid Co., Pearl River, NY). The anti-FLAG M2-agarose antibody, dobutamine, forskolin, H89, IGEPAI, isoproterenol, norepinephrine, and salbutamol were from Sigma (St. Louis, MO). Rp-cAMPS was from Biomol Research Laboratory (Plymouth Meeting, PA). SB202190 and SB203580 were from Calbiochem (La Jolla, CA). The nonradioactive PKA activity assay was from Promega (Madison, WI). The Rap1 activation assay was from Upstate (Charlottesville, VA). Specific antibodies to the following epitopes or individual proteins were from Cell Signaling Technologies Inc. (Beverly, MA): 6×-His, p38 MAPK, p38α MAPK, phospho-p38 MAPK, MKK3, phospho-MKK3, MKK4, phospho-MKK4, MKK7, phospho-MKK7, and glutathione S-transferase (GST)-ATF2. Antibodies specific for the p38α and p38θ MAPK isoforms and MKK6 were from Chemicon (Temecula, CA). For immunoprecipitation experiments, MKK3, MKK6, and the S-tag antibodies were obtained from Santa Cruz (Santa Cruz, CA). The S-protein agarose resin was from Santa Cruz. An additional antibody to phospho-p38 MAPK was from Zymed (South San Francisco, CA). The small interfering RNAs (siRNAs) presented in Table 1 and related reagents such as siPORT-Lipid, RNAiLatter, RNAaqua4PCR, and RNAaqua4MIDI RNA extraction kit were from Ambion (Austin, TX). The High Capacity cDNA Archive kit, reverse transcription-PCR (RT-PCR) primers, FAM- and VIC-labeled probes, and the TaqMan enzyme were from Applied Biosystems (Foster City, CA). Lipofectamine and precast Tris-glycine polyacrylamide gels were obtained from Invitrogen (Carlsbad, CA). The alkaline phosphatase-conjugated secondary antibodies, the alkaline phosphatase detection reagents, and glutathione Sepharose 4B were from Amersham Biosciences (Piscataway, NJ). Complete Protease Inhibitor Cocktail (CPIC) tablets, and protein G-agarose were from Roche Molecular Biochemicals (Indianapolis, IN). The ProQ Diamond Phosphoprotein Gel Stain and destaining solution were from Molecular Probes (Eugene, OR). Rosiglitazone was a gift from GlaxoWellcome Inc. (Research Triangle Park, NC). All other reagents were from the best available sources.

**Cell culture and transfection.** The HIB-1B brown preadipocytes (57) were maintained in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum. Cells in 6-well plates were transfected with a total amount of plasmid DNA up to 2.2 μg/well and 5 to 10 μl Lipofectamine. As needed, these DNA mixtures included pGAL-β-AR (0.5 μg), pcDNA3-kinesins (0.2 to 1 μg), UCP1 enhancer-TK-CAT (1.0 μg), and β-actin-luc (0.2 μg) or cytomegalovirus-β-galactosidase (CMV-β-GAL) (0.125 μg). Transfection with siRNAs (20 to 60 nM) were performed using siPORT (4 to 8 μl). In cotransfection experiments involving siRNAs, the siRNA was added to the well at the time of seeding the cells, and the plasmid transfection was performed 12 h later. In all cases, the pPARγ agonist rosiglitazone (1 μM) was added at the same time as the serum following the serum-free period of the transfection protocols. Where indicated, HIB-1B cells were treated for 1 h with 10 μM H89, 0.5 mM Rp-cAMPS, or 5 μM SB prior to treatment with CL (10 μM), Forsk (10 μM), dobutamine (10 μM), salbutamol (10 μM), isoproterenol (10 μM), or norepinephrine (1 μM), for which the time of incubation varied in accord to the assay needs. Also, for cells treated with norepinephrine, there was a preincubation phase for 30 min with yohimbine (10 μM) and prazosin (1 μM) in order to inhibit any activation of α-adrenergic receptors.

**Nonradioactive PKA assay.** HIB-1B cells were preincubated for 50 min with H89 (10 μM), Rp-cAMPS (0.1 mM), or SB (5 μM), followed by 10 min with 0.2 mM isobutylmethyoxanthine in order to inhibit phosphodiesterase activity. They
were then incubated for 5 min with either CL (10 μM) or Forsk (10 μM). Cells were then washed once with phosphate-buffered saline (PBS) containing 5 mM β-glycerophosphate and 1 mM sodium orthovanadate, followed by a lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 10 mM β-mercaptoethanol, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.5 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% IGEPAL, and 1 CPIC tablet per 10 ml) for 15 min. Five microliters of this cell lysate was incubated for 30 min at 30°C with 5 μl of the 5× PepTag PKA reaction buffer, 2 μg of PepTag A1 peptide (fluorescent keptide), and 1 ml of the peptide protection solution (all parts of the PKA assay kit were from Promega) in a 25-μl total volume. For the positive control, the sample has been replaced by 10 ng of PKA catalytic subunit, and for the negative only lysing buffer is added to the reaction mixture. The reaction was stopped by boiling the sample for 10 min, a final concentration of 3.2% glycerol was added, and 3.5 μl sample was loaded and resolved on a 6% agarose gel. Image acquisition was performed on a typhoon 9410 variable modes imager and analyzed using ImageQuant TL v2003.03 software.

**Rap1 activation assay using RafGDS-RBD.** Rap1 pull-down assays were performed essentially as described previously (19) using the reagents from Upstate (Charlottesville, VA). HIB-1B cells were seeded in 10-cm-diameter dishes. Cells were washed twice in cold PBS and lysed on ice in 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 2.5 mM MgCl₂, 1% NP-40, 10% glycerol, and 1 Complete Mini Antiprotease tablet per 10 ml of lysis buffer). Cell debris was removed by centrifugation at 13,200 × g for 10 min at 4°C. Fifty microliters of the GST-RafGDS-RBD-agarose slurry was added to each supernatant, and the mixture was incubated at 4°C for 45 min on a rotating wheel. Beads were washed three times with lysis buffer. Samples were denatured for 3 min at 95°C and subjected to sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed using the anti-Rap1 antibody included in the kit and an alkaline phosphatase-conjugated anti-rabbit secondary antibody and ECF detection kit from Amersham Biosciences. Image acquisition was performed on a typhoon 9410 variable modes imager and analyzed using ImageQuant TL v2003.03 software, both from GE healthcare (Piscataway, NJ).

**Protein kinase assay for p38 MAPK activity.** HIB-1B cells were transfected or not with FLAG-tagged p38 MAPK, and total cell p38 MAPK activity was assessed from cellular lysate while isofrom-specific activity was assessed using immunoprecipitation using M2 anti-FLAG agarose antibody. The cells were washed twice with PBS containing 5 mM β-glycerophosphate and 1 mM sodium orthovanadate and then lysed for 30 min in a 25 mM Tris-HCl buffer containing 150 mM NaCl, 5 mM β-glycerophosphate, 1 mM sodium orthovanadate, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.1% IGEPAL, and 1 CPIC tablet per 10 ml. Protein kinase assays in vitro were performed on whole-cell lysates or after immunoprecipitation. In the latter case, the lysate was incubated overnight with 40 μl of M2 anti-FLAG agarose antibody. Cell lysates or immune complexes were incubated at 30°C for 45 min with 2 μg GST-ATF-2I-109, 250 μM ATP (containing or not 10 μCi [γ-32P]ATP) in 40 μl of kinase reaction buffer (43). For the radioactive version of the protocol, an equal amount of ²¹[X]Laemmli sample buffer was added to terminate the reactions. In the nonradioactive version, 40 μl of glutathione Sepharose 4B was used to pull down the substrate. Protein complexes were resolved with 4% to 20% acrylamide gradient Tris-glycine gels. Protein phosphorylation was visualized either by autoradiography for the radioisotopic protocol or with the Pro-Q Diamond phosphor-protein stain for the nonradioactive method. In both cases image acquisition was performed on a Typhoon 9410 variable mode imager and analyzed using ImageQuant TL v2003.03 software.

**Western blot for MAPK phosphorylation.** MAPK phosphorylation was evaluated by Western blot using specific anti-phospho-MAPK and total MAPK antibodies (1:1,000 dilution) and secondary antibodies (1:10,000 for the Aamersham antibody and 1:2,500 for the Sigma antibodies). The alkaline phosphatase activity was determined using the ECF detection kit from Amersham Biosciences. Image acquisition was performed on a Typhoon 9410 variable modes imager and analyzed using ImageQuant TL v2003.03 software.

**CAT and luciferase assays.** Cells were harvested to assay UCP1 enhancer activity 48 h after transfection. CL316,243 or Forsk was added for the last 6 to 8 h of the transfection to stimulate cAMP production, after which cell extracts were prepared in lysis buffer from a CAT enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals). CAT and luciferase assays were performed as previously described (8).

**RNA isolation, reverse transcription-PCR, and real-time PCR.** Total mRNA was extracted from cultured cells using RNAaquaousPCR and from tissue using the Bio-Rad MNI RNA purification kit. For tissues, the samples were submerged in “RNAlater” prior to the extraction. These RNA reagents were from Ambion. cDNA was generated using the High Capacity cDNA Archive kit from Applied Biosystems exactly as described in the kit (although scaled down to a 50-μl total volume). Real-time PCR was performed using TaqMan probes from Applied Biosystems (Foster City, CA) on an ABI PRISM 7700 Sequence Detector from Perkin Elmer (Boston, MA) exactly as indicated by Applied Biosystems. For the quantification of the p38α and p38β MAPK mRNA, standard curves (0 to 4,000 amol) were generated using plasmids containing the cDNAs of the mouse genes. GAPDH was used as internal standard.

**Immunoprecipitation.** For immunoprecipitation experiments, the tissue or the cells were lysed with the same buffer as for the kinase assays. The lysate (1 to 2 mg of total protein) was precleared by a preincubation of 2 h with 40 μl protein G-agarose. The cleared lysate was then incubated for 3 h with the antibody (kitase assay) or overnight (coimmunoprecipitation) with antibodies. The G-protein agarose was added, and the mixture was incubated for an additional 3 h and washed once with the lysing buffer and five times with the washing buffer composed of 50 mM Tris-HCl, 150 mM NaCl, and antimyotrophins. The proteins are eluted from the resin in an ultrafree-mc 5-μm centrifugal filtration device by exposing the resin to sample buffer without reducing agent at room temperature for 10 min.

**RESULTS**

In previous studies we demonstrated that p38 MAPK activity is involved in the βAR- and cAMP-dependent induction of the Ucp1 gene in brown adipocytes (7, 8). However, neither the identity of the p38 MAPK nor the molecular intermediaries linking PKA to the activation of p38 MAPK are known. Therefore, we used a series of experiments designed to define the p38 MAPK isoforms and immediate upstream activators involved. In our earlier studies, we presumed that the elevated cAMP levels generated in response to β-agonist stimulation are activating PKA, concluding that this kinase is solely responsible for conveying the cAMP signal that leads to p38 MAPK activation and Ucp1 gene expression. This conclusion was based on the ability of two mechanistically different “inhibitors” of cAMP, the competitive antagonist Rp-cAMPS and the catalytic inhibitor H89, to suppress both p38 MAPK activation and transcription of the Ucp1 gene. This “signature” typically indicates involvement of PKA. However, in a variety of cell types, cAMP has been shown to activate the small G-protein Rap1 through its interaction with a family of guanine nucleotide exchange factors (GEFs) that include Epac (exchange protein directly activated by cAMP), G–FEP–I, and cAMP–GEF–I (1, 33, 65). The activities of these molecules are blocked by Rp-cAMPS but are unaffected by H89. Importantly, Rap1 has been shown to be an activator of p38 MAPK (30, 59). Therefore, as we began this series of studies it was necessary to unequivocally determine whether PKA or a GEF (or some combination of both) leads to stimulation of p38 MAPK and Ucp1 gene expression. We treated HIB-1B brown adipocytes with the βAR agonist, CL316,243 (CL), or the adenyl cyclase stimulator, forskolin (Forsk), in the presence of H89 or Rp-cAMPS or the p38 MAPK inhibitor SB202190 (SB). As shown in Fig. 1A, PKA was activated by either CL or Forsk. The response to both activators was blocked by H89 or Rp-cAMPS but not by SB. Therefore, these results indicate activation of PKA and additionally show that inhibition of p38 MAPK does not affect PKA. As shown in Fig. 1B, neither CL nor Forsk was able to elicit GTP loading to Rap1. Together these results strongly support the conclusion that p38 MAPK activation by cAMP does not depend upon a cAMP–GEF and Rap1 activation but, rather, solely requires PKA.

To confirm the role of both PKA and p38 MAPK in UCP1 induction, HIB-1B cells were pretreated with H89, Rp-cAMPS, or SB, followed by stimulation with either CL or Forsk. Activation of p38 MAPK was measured using glutathione-S-trans-
The image contains data from a scientific paper discussing the role of specific signaling pathways in the activation of the UCP1 enhancer. The figure illustrates the mechanisms by which β-arrenergic receptor (βAR) agonists or Forskolin (Forsk) promote uncoupling protein 1 (UCP1) enhancer activity, which involves protein kinase A (PKA) and p38 MAPK. HIB-1B cells were transfected and treated with various agents to measure PKA activity, Rap1 activation, p38 MAPK activity, and UCP1 enhancer activity. The results indicate that, irrespective of the stimulus, PKA is necessary for p38 activation and that, in turn, induction of UCP1 enhancer activity requires p38 MAPK activity.
Since all three βARs are expressed in brown adipocytes and can stimulate cAMP production (56), we proposed that all of them can activate p38 MAPK and UCP1 transcription. To test this hypothesis, we treated HIB-1B cells with specific agonists of these receptors. As shown in Fig. 1E, the β1AR-selective agonist, dobutamine, and the β2AR-selective agonist, salbutamol, both activated p38 MAPK in a PKA-dependent manner. The nonselective βAR activator isoproterenol and the natural adrenergic agonist norepinephrine also activated p38 MAPK in a PKA-dependent fashion (Fig. 1E). Furthermore, these four βAR agonists also induced UCP1 enhancer activation, which was blocked by p38 MAPK inhibition (Fig. 1F). These results show that all three βAR subtypes can stimulate p38 MAPK activity and subsequently Ucp1 gene transcription.

To determine whether β1AR stimulation leads to p38 MAPK activation and to a p38 MAPK-dependent Ucp1 gene expression in brown fat in vivo, SB (12.5 mg/kg of body weight) and CL (1 mg/kg) were administered to mice. Phosphorylation and activation of p38 MAPK and JNK was assessed by Western blotting and kinase assays and Ucp1 gene expression by real-time PCR. As shown in Fig. 2A, CL treatment induced phosphorylation of p38 MAPK by 2.5- ± 0.2-fold and p38 MAPK enzyme activity by 2.4- ± 0.1-fold. In contrast, following CL treatment, phosphorylation of JNK could not be detected (Fig. 2B). The ability of antibody to recognize phospho-JNK was confirmed by treating HIB-1B cells with 5 μg/ml anisomycin for 15 min (Fig. 2B). Under these same treatment conditions, CL injection stimulated Ucp1 gene expression, and this stimulation was largely prevented (70%) by prior p38 MAPK inhibition (Fig. 2C). Consistent with what we have previously reported (7), these results clearly show that selective β1AR agonist stimulation in vivo triggers p38 MAPK activity to regulation of Ucp1 gene transcription.

To identify the p38 MAPK isoforms(s) responsible for UCP1 enhancer activation, we first assessed which SB-sensitive isoforms were expressed in BAT and in the brown adipocyte cell line used to dissect the molecular pathway between PKA and Ucp1 gene expression. As shown in Fig. 3A, both p38α and -β mRNAs were expressed in BAT as well as in HIB-1B cells. Consistent with this finding, both proteins were detected by Western blot (Fig. 3B). We next overexpressed the p38α or -β isoforms in HIB-1B cells and measured UCP1 promoter activity. As shown in Fig. 3C, both isoforms could stimulate UCP1 enhancer activation equally (with a slight preference for the α isoform). Next, we coexpressed MKK6E (a constitutively active form of this kinase that can phosphorylate and activate p38 MAPK) with either p38α or -β in HIB-1B cells, followed by measurements of UCP1 enhancer activity. As shown in Fig. 3D, MKK6E could activate either of the p38 isoforms as measured by significant amplification of UCP1 enhancer activity, but there was a greater preference for p38α MAPK. Together, these results indicate that both p38α and -β isoforms are capable of stimulating UCP1 transcription and that under conditions of maximal stimulation p38α MAPK might couple more efficiently to UCP1 induction. However, these data do not indicate whether either or both isoforms play a role under adrenergic stimulation. To address this issue, we introduced FLAG-tagged p38α or -β MAPK into HIB-1B cells and subsequently treated the cells with CL or Forsk. As clearly shown in Fig. 4A and B, p38α MAPK but not p38β was activated by CL or Forsk. We also performed immunoprecipitation experiments of the endogenous p38 MAPK isoforms and confirmed that Forsk-induced p38 MAPK activity could be recovered only from the p38α MAPK immunoprecipitate (Fig. 4C). Interestingly, using our brown adipocyte model, transactivation of the UCP1 enhancer by CL or Forsk was potentiated only by p38α but not by the p38β isoform (Fig. 4D). In order to validate this selectivity in vivo, mice were either injected with 1 mg/kg CL or exposed to a 4°C environment. As shown in Fig. 4E and F, both manipulations led to the sole activation of the

![FIG. 2. β1AR agonist CL316,243 promotes uncoupling protein 1 (Ucp1) gene expression in vivo through p38 MAPK. Mice were pre-treated with two injections of saline (Control) or 12.5 mg/kg SB203580. The first injection was 25 h and the second 1 h prior to the saline (Basal) or CL injection (1 mg/kg). (A) After 30 min, the brown adipose tissue p38 MAPK activation was evaluated by Western blot using an anti-phospho-p38 MAPK antibody (top blot) or using GST-ATF2 as a substrate (middle blot). (B) After 30 min, JNK activation was evaluated by Western blot. (C) After 6 h, Ucp1 gene expression was evaluated by real-time PCR (triplicate samples per group) using Taqman probe: Basal (white bars) and CL (gray bars). The blots shown are the results of one of two experiments using two mice per each treatment group. For panels A and B, HIB-1B cells treated with anisomycin (5 μg/ml) were used as a positive control. The results presented in graphs are the means ± standard deviations of two independent experiments using two mice per treatment group.](image)
MAPK isoform in interscapular BAT. Altogether, these data establish that p38α MAPK but not p38β MAPK is activated during sympathetic nervous system stimulation of the thermogenic program and following exposure of BAT and brown adipocytes to sympathomimetic drugs.

Establishing that the α isoform of p38 MAPK is the one that is activated, we used siRNA gene silencing to demonstrate that p38α MAPK and not p38β MAPK was responsible for the induction of UCP1 expression. In these studies it was first necessary to demonstrate the efficacy of the siRNAs directed against either the p38α or -β isoforms. This was examined in HIB-1B cells. As shown in Fig. 5A, the siRNA against either form of p38 MAPK reduced the targeted protein level by more than 80% without affecting the other isoform. More importantly, as shown in Fig. 5B, using this approach we found that essentially all CL- and Forsk-promoted p38 activity can be attributed to the p38α MAPK isoform. Figure 5C further shows that the siRNA against p38α MAPK completely inhibited UCP1 enhancer activation, while the siRNA against p38β failed to do so. Similar results were obtained in experiments.

**FIG. 3.** p38α or p38β MAPK can promote UCP1 enhancer activity. The expression of p38α and p38β MAPKs was measured in BAT and HIB-1B cells by quantitative real-time PCR (A) and Western blotting with selective antibodies for each isoform (B). (C) HIB-1B cells were transfected with increasing concentration of FLAG-tagged p38α MAPK. Two days later, the kinases were immunoprecipitated and their expression was evaluated by Western blot using anti-p38 MAPK antibody (second and fourth blots); we measured their activity using GST-ATF2 as a substrate (first and third blots), and we evaluated UCP1 enhancer activity using a CAT assay (graph). (D) HIB-1B cells were transfected with FLAG-tagged p38 MAPK and/or FLAG-tagged MKK6E. Two days later, their kinases were immunoprecipitated and their expression was measured by Western blot using anti-p38 MAPK and anti-MKK6 antibodies (second, third, and fourth blots); p38 MAPK activity was measured using GST-ATF2 as substrate (first blot), and UCP1 enhancer activity was evaluated by CAT assay (graph). The results shown are means ± standard deviations of three independent experiments, each performed in triplicate, while the blots are from one of three experiments.
FIG. 4. p38α MAPK is selectively activated following β-adrenergic or forskolin stimulation. (A and B) HIB-1B cells were transfected with the β₁AR and with FLAG-tagged p38α MAPK (A) or FLAG-tagged p38β MAPK (B). (A and B) Cells were treated as detailed in Fig. 1A. Cells were lysed, the kinase was immunoprecipitated (Western blot, bottom blots in A and B), and activity was measured using GST-ATF2 as a substrate (top blots in A and B). The results shown are from one of three independent experiments. (C) HIB-1B cells were treated as shown, and p38α and p38β MAPKs were immunoprecipitated and kinase activity measured using GST-ATF2 as a substrate. (D) HIB-1B cells were transfected with the β₁AR and with FLAG-tagged p38α MAPK or FLAG-tagged p38β MAPK. Cells were treated as follows: Basal (white bars), CL (gray bars), and Forsk (black bar). For measurement of UCP1 enhancer activity cells were harvested after 6 h. The results shown are means ± standard deviations of three independent experiments, each performed in duplicate. For measurement of kinase activity, 20 min posttreatment the kinases were immunoprecipitated and activity was measured using GST-ATF2 as a substrate (upper blot). Relative amounts of the kinases in the assay are shown by Western blot using anti-FLAG antibody (lower blot). (E and F) Mice were either treated with CL (1 mg/kg intraperitoneally) for 30 min (E) or placed at 4°C for 1 h (F), and BAT was excised and processed for immunoprecipitation of p38α MAPK or p38β MAPK. Kinase activity and the protein levels of the p38 MAPK isoforms was measured as above.
employing dominant-negative constructs of p38α and p38β MAPK (not shown). Finally, it was rather remarkable to find that even the 60- to 80-fold induction of the endogenous Ucp1 gene in HIB-1B cells was totally eliminated by the p38α MAPK siRNA (Fig. 5C). Altogether these data leave little doubt about the highly specific activation of p38α MAPK by β3AR agonists and PKA and its essential role in the activation of the Ucp1 gene.

The next objective was to address the origin of p38α MAPK selectivity. For this purpose we explored which of the MKKs were activated by CL and Forsk. In Fig. 6A (middle panel), MKK3 and/or MKK6 was phosphorylated in a PKA-dependent manner following CL or Forsk. However, neither CL nor Forsk was able to promote the phosphorylation of MKK4 or MKK7 (Fig. 6B). These results are consistent with the fact that the JNK pathway is not activated in brown adipose tissue in vivo (Fig. 3B) or in brown adipocytes in vitro (Fig. 6B). However, the nonisoform selective nature of the MKK3/6 phospho-antibody required additional experimentation in order to address whether one (or both) of these two MKK isoforms was activated. We performed selective immunoprecipitation of MKK3 and MKK6 under basal and cAMP-stimulated conditions. As shown in Fig. 6C, Forsk-induced phospho-MKK3/6 immunoreactivity was detected only upon immunoprecipitation of MKK3. Confirmation of this exclusive activation of MKK3 versus MKK6 in vivo was obtained from BAT samples of mice treated with CL or placed in a 4°C environment. Collectively, these findings demonstrate a selective activation at the level of the direct upstream kinase within the p38 MAPK module, which might be an underlying mechanism of the above-described specific p38α MAPK activation.

MKK6 is a universal p38 MAPK activator, but the ability of MKK3 to activate p38β MAPK is modest at best (18). It was tempting to speculate that the specific involvement of p38α MAPK would be recapitulated at the level of MKK3. We therefore tested the functional impact of overexpressing MKK3, MKK4, MKK6, and MKK7 individually on UCP1 enhancer activity and p38 MAPK activity. The results clearly show that MKK3, and to a much lower extent MKK6, can potentiate the effects of CL and Forsk on both UCP1 expres-
sion (Fig. 7A) and p38 MAPK activity (Fig. 7B). The reciprocal experiments, using siRNAs that specifically downregulated MKK3 and MKK6 by more than 80% (Fig. 7C), show that only the selective siRNA directed against MKK3 could block CL and Forsk induction of p38 MAPK activity (Fig. 7D) as well as UCP1 expression (Fig. 7E). Finally, the siRNA that specifically targets MKK3 was the only one capable of completely interfering with the expression of the endogenous Ucp1 gene (Fig.
All together, these results unequivocally define the proximal steps in the cAMP- and PKA-dependent activation of the Ucp1 gene in brown fat as being mediated solely by MKK3 and p38α MAPK.

The MAP kinases are usually assembled together with their upstream MKKs into a signaling module that is coordinated by large scaffolding molecules such as the JIPs (JNK-interacting proteins) (21, 47). As a result, these scaffold proteins can
concentrate interacting signaling partners in the vicinity of an upstream activator in order to favor a particular pathway. Since there is no established link between PKA and p38 MAPK, we attempted to determine which, if any, of the known JIP family members in brown adipocytes might serve as the scaffold to specifically bind p38α MAPK and MKK3. The first objective was to determine the relative expression levels of the four known JIPs: JIP1, JIP2, JIP3, and JLP. As shown in Fig. 8A, there was no detectable expression of JIP1 in either BAT or the HIB-1B cell line (RT-PCR cycle, >45). However, JIP2, JIP3, and JLP were all found in both samples, with JIP2 being the least abundant at the mRNA level (Fig. 8A). Based on these results, each of these three JIPs was analyzed for its ability to interact specifically with MKK3 and p38α MAPK. His-tagged or S-protein-tagged constructs of JIP2, JIP3, and JLP were transfected into HIB-1B cells, followed by their immunoprecipitation in order to assess the identity of any interacting kinases. Figure 8B clearly shows that only JIP2 was able to specifically recover both MKK3 and p38α MAPK. It is noteworthy that neither MKK6 nor p38β MAPK was found under any circumstance, although all these molecules are clearly present as shown in the lysate.

**DISCUSSION**

Catecholamine regulation of brown fat thermogenesis has been clearly shown to involve increased transcription of the *Ucp1* gene as a result of stimulation of the βARs and cAMP production (see references 48 and 51 for reviews). Despite this, the details of the signaling cascade beyond this point have been ambiguous. It has been generally assumed that the elevated levels of cAMP lead to activation of PKA, which in turn phosphorylates the nuclear factor CREB, resulting in transcription of the various target genes in brown fat, including UCP1. In the last few years we have reported results from studies in white and brown adipocyte cell models and in vivo manipulations to show that p38 MAPK is activated in response to β-adrenergic stimuli (7, 8). This was a novel and unexpected link between cAMP and p38 MAPK, but one that relied heavily on the use of chemical inhibitors of p38 MAPK and PKA. Since there is no clearly delineated series of steps linking PKA to p38 MAPK in the literature, unequivocal identification of the individual molecules in this cascade requires the use of more stringent approaches.

The p38 MAPK group is composed of four isoforms, p38α (26, 41), p38β (32), p38γ (43), and p38δ (66). Among them, p38α and β are sensitive to the pyrimidyl imidazoles SB202190 and SB203580 (14, 23). Our earlier reports indicated that βAR and PKA stimulation of *Ucp1* expression in brown adipocytes was sensitive to SB (7, 8) and thus narrowed the scope of inquiry. The pyridinyl imidazole-sensitive p38 MAPK isoforms are often considered to be redundant enzymes, as their substrate specificities overlap significantly. However, in some cases these two isoforms have been shown to be able to discriminate between substrates, at least under conditions of forced overexpression of dominant-negative mutants (67, 73). In the studies that we report here, we used a combination of in vivo and in vitro approaches designed to distinguish between the p38 MAPK isoforms and to identify the upstream MKK enzyme(s) responsible for mediating the PKA signal to activate *Ucp1* gene expression. Our results clearly establish that p38α MAPK is a central obligatory component of this signaling cascade, with arguably no contribution from p38β MAPK, despite its presence. We do not at this point rule out the possibility that p38β MAPK might regulate other genes in the brown adipocyte that might be triggered by different stimuli.
For example, insulin has been reported to selectively activate p38β MAPK but not p38α in brown adipocytes, and this effect was associated with increased glucose transport (36).

Our studies also indicate that there is a unique requirement for MKK3 as the immediate upstream kinase for p38α. The selectivity of these two elements to be activated in succession is not particularly surprising, but nevertheless it does occur in spite of the existence in brown adipocytes of at least four MKKs, three of which have been demonstrated to activate p38α in other settings (17, 27, 44, 46, 52, 61). This tight coupling is most likely indicative of their existence in a multimolecular signaling complex, as is known to exist for the stress-activated kinases from yeast to mammals (21, 47). These signaling modules are maintained together by scaffolding proteins, such as KSR for the ERK pathway and the JIP proteins for the JNK pathway. A recent estimate of the size of this “functional family” of scaffolding proteins suggests more than 19 members (47). Clearly, this complexity necessitates more extensive investigation in order to assign individual scaffolds to specific kinase members but which will probably also depend upon the stimuli and the cell types in which they exist. Much is known about the role of these JIP scaffolding molecules for the JNK pathway (for which they were named), while in the case of p38 MAPK three members of the family (JIP2, JLP, and JIP4) have been proposed to be able to interact with p38 MAPK and/or MKK3 (5, 34, 40). When considering adipocytes it must be acknowledged that relatively little is known about these molecules aside from a recent report concerning a role for JIP1 in insulin-induced JNK activation (31). Nevertheless, since we have now established that PKA specifically utilizes MKK3 and p38α MAPK to regulate genes in the brown adipocyte, this information directed our quest for the scaffolding protein involved. Our results clearly show that at least three putative p38 MAPK scaffolds are expressed in brown adipose tissue. The absence of JIP1 and the presence of JIP2 was somewhat surprising, since JIP1 is clearly expressed at low levels in white adipose tissue (31) and JIP2 is expressed predominantly in neuronal tissue (71). However, as usual, adipose tissue was absent from the panel of tissue surveyed for JIP2 expression in this latter study. Since JIP3 and JLP are more widely expressed, their existence in adipose tissue was not unexpected. Interestingly, when JIP2 was immunoprecipitated, only MKK3 and p38α MAPK were recovered within the coprecipitate. Although not a definitive proof of the necessity of JIP2 for the PKA-dependent activation of p38α MAPK, it at least places these components within close proximity to each other. Also, during the revision of the manuscript JIP4 was cloned (34), therefore the role for JIP4 in parallel with JIP2 cannot be excluded.

From a signaling perspective, the adipocyte is unique in that all three known βAR subtypes are expressed there and each is coupled to the production of cAMP (56). Here we have also shown that all three βARs can stimulate p38 MAPK in the adipocyte to increase Ucp1 gene expression. Based on the present results and our earlier findings that cAMP-dependent activation of p38 MAPK elicits an orchestrated response to increasing the expression of PGC-1α, a master regulator of mitochondrial biogenesis (68), an interesting speculation arises. Since human adipocytes also express multiple βAR subtypes and the critical region of the Ucp1 gene that responds to cAMP and p38 MAPK is conserved between rodents and humans, there may well be a similar regulation of p38 MAPK and activation of UCP1 and PGC-1α expression in human adipocytes. This prospect will require careful examination from human visceral adipose samples, since this depot is the main location of brown adipocytes in adult humans and may potentially be a reservoir of quiescent cells capable of thermogenic activity upon appropriate stimulation.

ACKNOWLEDGMENTS

We thank Leslie P. Kozak, Jiahuai Han, Roger J. Davis, Josef M. Penninger, and E. Premkumar Reddy for their invaluable gifts of plasmids (listed in Materials and Methods). We also thank Alexander Medvedev for helpful discussions in the initial project. This work was supported by NIH awards R01 DK57698 and R01 DK53092 (S.C.) and a fellowship from Fonds de la Recherche en Santé du Québec (J.R.).

REFERENCES

1. Altschuler, D. L., S. N. Peterson, M. C. Ostrowski, and E. G. Lapetina. 1995. Cyclic AMP-dependent activation of Rap1b. J. Biol. Chem. 270:10373–10376.
2. Bannashad, M., V. T. Aoki, M. G. Adkison, W. S. Warren, and T. J. Bartness. 1999. Central nervous system origins of the sympathetic nervous system outflow to white adipose tissue. Am. J. Physiol. 275:R291–R299.
3. Bannashad, M., C. K. Song, and T. J. Bartness. 1999. CNS origins of the sympathetic nervous system outflow to brown adipose tissue. Am. J. Physiol. 276:R1569–R1578.
4. Bouillaud, F., D. Riquier, J. Thibault, and J. Weissbach. 1985. Molecular approach to thermogenesis in brown adipose tissue: cDNA cloning of the mitochondrial uncoupling protein. Proc. Natl. Acad. Sci. USA 82:445–448.
5. Buchsbaum, R. J., B. A. Connolly, and L. A. Feig. 2002. Interaction of Rac exchange factors Tiam1 and Ras-GRF1 with a scaffold for the p38 mitogen-activated protein kinase cascade. Mol. Cell. Biol. 22:4073–4085.
6. Cannon, B., and J. Nedergaard. 2004. Brown adipose tissue: function and physiological significance. Physiol. Rev. 84:277–359.
7. Cao, W., K. W. Daniel, J. Robidoux, P. Puigserver, A. V. Medvedev, X. Bai, L. M. Floering, B. M. Spiegelman, and S. Collins. 2004. p38 mitogen-activated protein kinase is the central regulator of cyclic AMP-dependent transcription of the brown fat uncoupling protein 1 gene. Mol. Cell. Biol. 24:3057–3067.
8. Cao, W., A. V. Medvedev, K. W. Daniel, and S. Collins. 2001. β-Adrenergic activation of p38 MAP kinase in adipocytes: cAMP induction of the uncoupling protein-1 (UCP1) gene requires p38 MAP kinase. J. Biol. Chem. 276:27071–27082.
9. Cassard-Douliert, A. M., C. Gelly, F. Bouillaud, and D. Ricquier. 1998. A 211-bp enhancer of the rat uncoupling protein-1 (UCP1) gene controls expression of the uncoupling protein-1 (UCP1) gene controls expression and regulated expression in brown adipose tissue. Biochem. J. 333:241–246.
10. Champigny, O., D. Riquier, O. Blondel, R. M. Mayer, G. Briscoe, and B. R. Holloway. 1991. β-Adrenergic receptor stimulation restores message and expression of brown-fat mitochondrial uncoupling protein in adult dogs. Proc. Natl. Acad. Sci. USA 88:10774–10777.
11. Collins, S., W. Cao, and J. Robidoux. 2004. Learning new tricks from old dogs: beta-adrenergic receptors teach new lessons on firing up adipose tissue metabolism. Mol. Endocrinol. 18:2123–2131.
12. Collins, S., K. W. Daniel, A. E. Petro, and R. S. Surwit. 1997. Strain-specific response to β-adrenergic receptor agonist treatment of diet-induced obesity in mice. Endocrinology 138:405–413.
13. Cousin, B., S. Cinti, M. Morrone, S. Raimbault, D. Ricquier, L. Penicaud, and L. Casteilla. 1997. Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (Rk/p38). EMBO J. 16:295–305.
14. del Mar Gonzalez-Barroso, M., C. Pecqueur, C. Gelly, D. Sanchis, M. C. Abes-Guerrera, F. Bouillaud, D. Ricquier, and A. M. Cassard-Douliert. 2000. Transcriptional activation of the human ucp1 gene in a rodent cell line, Synergism of retinoids, isoproterenol, and thiazolidinedione is mediated by a multipartite response element. J. Biol. Chem. 276:5341–5346.
15. Del Mar Gonzalez-Barroso, M., D. Riquier, and A. M. Cassard-Douliert. 2001. Brown adipose tissue: function and perspectives in obesity research. Obes. Rev. 2:161–72.
16. Derijard, B., J. Raingeaud, T. Barrett, I. H. Wu, J. Han, R. J. Ulevitch, and
Lever, J. D., R. T. Jung, J. O. Nnodim, P. J. Leslie, and D. Symons. 1993. Analysis of uncoupling protein and its mRNA in adipose tissue deposits of adult humans. Int. J. Obesity 16:383–390.

Moedert, M. A., C. Szendy, C. Czakon, R. J. Jones, and P. Cohen. 1997. Activation of the novel stress-activated protein kinase SAPK4 by cytokines and cellular stresses is mediated by SKK3 (MKK6); comparison of its substrate specificity with that of other SAP kinases. EMBO J. 16:5363–5371.

Gonzalez-Barroso, M. C., C. Fleury, F. Bouillaud, D. Nichols, and E. Riall. 1999. The uncoupling protein UCP1 does not increase the proton conductance of the inner mitochondrial membrane by functioning as a fatty acid anion transporter. J. Biol. Chem. 274:15528–15532.

Gonzalez-Barroso, M. M., C. Fleury, I. Arechaga, P. Zaragoza, C. Levi-Montalcini, S. Enerback, D. Ricquier, F. Bouillaud, and E. Riall. 1996. Activation of the uncoupling protein by fatty acids is modulated by mutations in the C-terminal region of the protein. Eur. J. Biochem. 239:445–450.

Han, J., J. D. Lee, L. Bibbs, and J. U. Ulevitch. 1994. A MAP kinase targeted by the tumor necrosis factor and cytokine family. Science 264:1247–1252.

Han, J., J. D. Lee, Y. Jiang, Z. Li, L. Feng, and J. R. Ulevitch. 1996. Characterization of the structure and function of a novel MAP kinase (MKK6) J. Biol. Chem. 271:2886–2891.

Herzig, S., F. Long, U. S. Jhala, S. Hedrick, R. Quinn, A. Bauer, D. Rudolph, G. Schutz, C. Yoon, P. Puigserver, B. Spiegelman, and M. Montminy. 2001. CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature 413:179–183.

Himes-Hagen, J., J. A. M. Melnyk, M. C. Zingaretti, E. Ceresi, G. Barbatelli, and R. J. Davis. 1992. Analysis of uncoupling protein and its flanking region. Nucleic Acids Res. 20:6277–6280.

Huang, C. C., J. L. You, M. Y. Wu, and K. S. Hsu. 2004. Rap1-induced p38 mitogen-activated protein kinase activation facilitates AMPA receptor trafficking via the GDI.Rab5 complex. Potential role in (S)-3,5-dihydroxyphenylalanine-induced neuronal cell death. J. Biol. Chem. 279:12286–12292.

Kroemer, G., and D. Ricquier. 1996. Mitochondria and their differential activation by catecholamines. Am. J. Physiol. 271:C670–C681.

Kroemer, G., and D. Ricquier. 2003. Regulation of AMPK/MAPK signaling modules by scaffold proteins in mammals. Annu. Rev. Cell Dev. Biol. 19:91–118.

Nedergaard, J., V. Golozoubouva, A. Matthias, A. Asad, A. Jacobsson, and B. Cannon. 2001. UCP1: the only protein able to mediate adaptive anti-shivering thermogenesis and metabolic inefficiency. Biochim. Biophys. Acta 1504:82–106.

Nicholls, D. G., and R. M. Locke. 1984. Thermogenic mechanisms in brown fat. Physiol. Rev. 64:62–144.

Noodin, J. O., and J. D. L. Mer. 1988. Neural and vascular provisions of rat interscapular brown adipose tissue. Am. J. Anat. 182:283–293.

Pecqueur, C., E. Couplan, F. Bouillaud, and D. Ricquier. 2001. Genetic and physiological analysis of the role of uncoupling proteins in human energy homeostasis. J. Mol. Cell. Endocrinol. 175:45–56.

Raingeaud, J., A. J. Whitemarsh, T. Barrett, B. Derijard, and R. J. Davis. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol. Cell. Biol. 16:2515–2523.

Ricquier, D., and F. Bouillaud. 2000. Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. J. Physiol. 529:3–10.

Ricquier, D., and F. Bouillaud. 2000. The uncoupling protein homologues: UCP1, UCP3, UCP4 and ATUCP. Biochem. J. 345:S161–S179.

Robidoux, J. T., L. L. Martin, and S. Collins. 2004. Beta-adrenergic receptors and regulation of energy expenditure: a family affair. Annu. Rev. Pharmacol. Toxicol. 44:297–323.

Roberts, E. M., K. W. Daniel, R. T. Premont, L. P. Kozak, and S. Collins. 1995. Regulation of the uncoupling protein gene (Ucp) by β3, β4, β-adrenergic receptor subtypes in immortalized brown adipose cell lines. J. Biol. Chem. 270:10723–10732.

Rosen, R., L. Choy, B. A. Graves, N. Fox, V. Solejeva, S. Klaus, D. Ricquier, and B. M. Spiegelman. 1992. Hibernoma formation in transgenic mice and isolation of a brown adipocyte cell line expressing the uncoupling protein gene. Proc. Natl. Acad. Sci. USA 89:7561–7565.

Rothwell, N. J., and M. J. Stock. 1979. A role for brown adipose tissue in diet-induced thermogenesis. Nature 281:31–35.

Sawada, Y., K. Nakamura, K. Doi, K. Takeda, K. Tobiume, S. Morita, I. Komuro, K. De Vos, M. Sheet, and H. Ichijo. 2001. Rap1 is involved in cell stretching modulation of p38 but not ERK or JNK MAP kinase. J. Cell Sci. 114:1221–1227.

Shabalina, L. G., A. Jacobsson, B. Cannon, and J. Nedergaard. 2004. Native UCP1 displays simple competitive kinetics between the regulators purine nucleotides and fatty acids. J. Biol. Chem. 279:38225–38230.

Slootman, E. J., S. Brady, M. A. Persson, and M. S. Barbosa, 1996. Cloning and characterization of MEK6, a novel member of the mitogen-activated protein kinase cascade. J. Biol. Chem. 271:11427–11433.

Tanaka, N., M. Kamanaka, H. Ensell, C. Dong, M. Wys, R. J. Davis, and R. A. Flavell. 2002. Differential involvement of p38 mitogen-activated protein kinase genes MKK3 and MKK6 in T-cell apoptosis. EMBO Rep. 3:785–791.

Thomas, S. A., and R. D. Palmiter. 1997. Thermoregulatory and metabolic phenotypes of mice lacking nonfetaladrenaline and noradrenaline. Nature 387:94–97.

van Liefde, I., A. van Witzenberg, and G. Vauquelin. 1992. Multiple beta adrenergic receptor subclases mediate the l-isoproterenol-induced lipolytic response in rat adipocytes. J. Pharmacol. Exp. Ther. 262:552–558.

Voeller, M. R., H. Yao, D. M. Gan, C. S. R. Pim, and H. Stork. 1997. CAMP activates MAP kinase and Elk-1 through a B-Raf- and Ral-dependent pathway. Cell 89:399–409.

Wang, X. S., K. Dienert, C. L. MantHEY, S. Wang, B. Rosenzweig, J. Bray, J. Delaney, C. N. Cole, P. Y. Chan-Hui, N. Mantly, H. S. Lichenstein, M. Zukowski, and Z. Yao. 1997. Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase gene. J. Biol. Chem. 272:23664–23674.

Wang, Y., B. Su, V. P. Sah, J. H. Brown, J. Han, and K. R. Chien. 1998. Carboxy-terminal for the nonmitogenic activating protein kinase gene 7: a specific activator for e-Nit2-lacZ transgene fusion in ventricular myocyte cells. J. Biol. Chem. 273:5423–5426.

Wu, Z., P. Puigserver, U. Andersson, C. Zhang, G. Adelmant, V. Mootha, A. Troy, S. Cinti, B. Lowell, R. C. Scarpulla, and B. M. Spiegelman. 1999. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98:115–124.

Wys, M., D. D. Yang, H. T. Lu, R. A. Flavell, and R. J. Davis. 1999.
Requirement of mitogen-activated protein kinase kinase 3 (MKK3) for tumor necrosis factor-induced cytokine expression. Proc. Natl. Acad. Sci. USA 96:3763–3768.

70. Yao, Z., K. Diener, X. S. Wang, M. Zukowski, G. Matsumoto, G. Zhou, R. Mu, T. Sasaki, H. Nishina, C. C. Hui, T. H. Tan, J. P. Woodgett, and J. M. Penninger. 1997. Activation of stress-activated protein kinases/c-Jun N-terminal protein kinases (SAPKs/JNKs) by a novel mitogen-activated protein kinase kinase. J. Biol. Chem. 272:32378–32383.

71. Yasuda, J., A. J. Whitmarsh, J. Cavanagh, M. Sharma, and R. J. Davis. 1999. The JIP group of mitogen-activated protein kinase scaffold proteins. Mol. Cell. Biol. 19:7245–7254.

72. Young, J. B., E. Saville, N. J. Rothwell, M. J. Stock, and L. Landsberg. 1982. Effect of diet and cold exposure on norepinephrine turnover in brown adipose tissue of the rat. J. Clin. Investig. 69:1061–1071.

73. Yu, J., D. Bian, C. Mahanivong, R. K. Cheng, W. Zhou, and S. Huang. 2004. p38 mitogen-activated protein kinase regulation of endothelial cell migration depends on urokinase plasminogen activator expression. J. Biol. Chem. 279:50446–50454.