Cig30, a Mouse Member of a Novel Membrane Protein Gene Family, Is Involved in the Recruitment of Brown Adipose Tissue*

(Received for publication, July 2, 1997, and in revised form, August 27, 1997)

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We have identified a previously uncharacterized gene that is implicated in the thermogenic function of brown adipose tissue of mice. This gene, termed Cig30, is the first mammalian member of a novel gene family comprising several nematode and yeast genes, such as SUR4 and FEN1, mutation of which is associated with highly pleiotropic phenotypes. It codes for a 30-kDa plasma membrane glycoprotein with five putative transmembrane domains. The Cig30 mRNA was readily detected only in brown fat and liver. When animals were exposed to a 3-day cold stress, the Cig30 expression was selectively elevated in brown fat more than 200-fold. Similar increases were brought about in two other conditions of brown fat recruitment, namely during perinatal development and after cafeteria diet. The magnitude of Cig30 mRNA induction in the cold could be mimicked by chronic norepinephrine treatment in vivo. However, in primary cultures of brown adipocytes, a synergistic action of norepinephrine and dexamethasone was required for full expression of the gene, indicating that both catecholamines and glucocorticoids are required for the induction of Cig30. We propose that the CIG30 protein is involved in a pathway connected with brown fat hyperplasia.

Brown adipose tissue is a mammalian organ specialized for heat production in the process called nonshivering thermogenesis. The proton potential generated by respiration across the inner mitochondrial membrane is dissipated in brown adipocytes through a unique protein channel, the uncoupling protein (UCP), and the electrochemical energy is directly transformed into heat (1, 2). Thermogenic activity of brown fat is particularly important in small mammals, such as rodents, when challenged by low ambient temperatures, notably after birth (3) or when arousing from hibernation (4). Energy dissipation by brown fat can also be mimicked by injection of norepinephrine (5). Among the acute effects of norepinephrine is an increase in intracellular cAMP concentration and lipolysis of triglycerides to free fatty acids, which are the main substrate for thermogenesis (7). Catecholamines also enhance glucose transport in the brown adipocytes (8–10) and elicit significant changes in phospholipid composition (11–13). At the gene expression level, adrenergic stimulation of brown fat rapidly increases the transcription of the Ucp gene (14, 15) and elevates mRNA levels of several genes, such as those for LPL (16, 17), GPDH (18), and GLUT4 (19). Remarkably, norepinephrine can also induce tissue hyperplasia in brown fat. Increased mitotic activity observed in brown adipose tissue in animals acutely exposed to cold (20) leads to a 3–4-fold increase in DNA content in the tissue about 1 week later (21), and this induction of proliferation can be mimicked by injection of norepinephrine (21, 22).

Thus, brown adipose tissue is rather unique in responding to a simple, non-invasive environmental stimulus as low ambient temperature by undergoing the recruitment process, i.e. a program of concerted physiological changes leading to accelerated proliferation and differentiation. Also unique is the possibility of using white adipose tissue, which has similar biochemical equipment yet plays a nearly antagonistic role in energy storage and expenditure, as a suitable reference system in a search for genes involved in this recruitment process. Such an approach based on differential screening of cDNA libraries from white adipose tissue and from brown adipose tissue of warm- and cold-adapted animals was employed by Jacobsson et al. (15) to isolate the Ucp gene. The authors simultaneously selected several other so-called CIN clones that exhibited a high degree of brown fat specificity and inducibility by cold.

We have investigated here one of the CIN clones, the CIN-2. To this end, we cloned and characterized the corresponding full-length mRNA of CIN-2. It proved to be a novel mammalian gene, which we have termed Cig30 (cold-inducible glycoprotein of 30 kDa) and demonstrated that elevation of Cig30 expression is associated with recruitment of brown adipose tissue. We suggest that the CIG30 protein is involved in a membrane event related to cellular proliferation in brown adipose tissue.

EXPERIMENTAL PROCEDURES

Animals and Treatments—Gene expression studies were carried out with 6–8-week-old male mice of the NMRI strain purchased from a local supplier (Eklunds). After arrival, the mice were housed separately, preacclimated, and maintained at 28 °C, if not otherwise stated. In cold-stress experiments, the animals were exposed to 4 °C for specified times, whereas controls remained at 28 °C. Mice kept on a cafeteria diet received, besides the standard chow, cookies, chocolates, and cheeses of various kinds for 1 week. For chronic norepinephrine treatment, the mice were implanted subcutaneously (in the interscapular region) with microosmotic pumps (Alzet, model 1003D) delivering 6 µg...
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(18.8 nmol) of norepinephrine in saline with 0.1 mM ascorbate per hour for 3 days (i.e. 430 µg (1.55 µmol) of norepinephrine totally). Control mice were treated with saline, 0.1 mM ascorbate only. Pregnant NMRI females obtained for perinatal gene expression studies were kept at room temperature.

Cell Line and Primary Cell Cultures of Brown Adipocytes—The HIB-1B 3T3 fibroblasts (23) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Brown fat precursor cells were isolated from 3–4-week-old NMRI mice and cultured as described earlier (24, 25). The cells were seeded into six-well dishes and grown for 9 days, with medium exchange on days 1, 3, and 6. Upon confluence (day 6), the cells were chronically treated for 3 days with 0.1 µM norepinephrine, 1 µM dexamethasone or a combination of both. Control cells were left untreated. Dexamethasone was added in a single dose; freshly prepared norepinephrine was added every 12 h.

cDNA Cloning and Sequencing—The 3′-fragment of Cig30 cDNA was originally selected from a cDNA library, which was prepared from cold-stimulated brown fat and cloned into pBR322 (15, 26). We subcloned the insert following PCR amplification with primer 1 (CTGGT-TGGGATCCGATTAGATGTGG) into the pDIRECT plasmid used for the PCR-Direct Cloning System (CLONTECH). The entire insert was then subcloned into both M13mp18 and M13mp19 and sequenced. The missing part of cDNA was amplification with primer 1 (CTGGT-TGGGATCCGATTAGATGTGG) into the pDIRECT plasmid using the dideoxynucleotide chain termination method with Sequenase Version 2.0 (U. S. Biochemical Corp.). Reactions were labeled with [35S]dATP (Amersham) and electrophoresed on 5% LongRanger gels (AT Biochem). Dry gels were visualized on a PhosphorImager, and sequence readings were sized with the ImageQuant program, version 3.3. University of Wisconsin Genetics Computer Group package (27) was used for sequence data analyses and presentations.

In Vitro Transcription, Translation, and Glycosylation—The Cig30 putative open reading frame (173–985) was subcloned by PCR (using primers 6 and 7) into the pcDNA I vector (Invitrogen). The construct was linearized with HpaI and transcribed with T7 RNA polymerase using mCAP mRNA capping kit (Stratagene). Nucleate-treated rabbit reticu- locyte lysate (Promega) was then programmed with approximately 1 µg of resulting RNA in the presence of Retinu I-[3H]Smethionine (Amer- sham). Translations were carried out in standard 25-µl reactions with various amounts (0–2.4 µl) of canine pancreatic microsomal membranes (Promega). Aliquots were solubilized in SDS sample buffer (final concentra- tions, 1.5% SDS, 15% glycerol, 100 mM Tris-HCl, pH 6.8, 1% (w/v) captoethanol) for 30 min at 37 °C and separated on 12% SDS-PAGE along with the Rainbow 14C-methylated protein molecular weight markers (Amersham), followed by autoradiography on a PhosphorImager.

Immunoblotting and Polyclonal Antibodies—Plasma membranes from interscapular brown fat were prepared as in Ref. 28. Protein was measured with the BCA protein assay (Pierce). For Western blotting, 20 µg of protein were separated per well using the cationic lipid-mediated method with DOTAP (Boehringer Mannheim). For negative controls, the cells were transfected with the pcDNA-IRESneo vector only. 2 days after transfection, the cells were replated onto 10-cm Petri dishes with the selection medium containing 500 µg of G418/ml (Life Technologies, Inc.). After 1–2 weeks, resistant colonies were isolated, expanded, and checked for Cig30 mRNA. Six clones with highest levels of the Cig30 mRNA and three clones of the negative controls were used in further experiments.

mRNA Expression Analysis and cDNA Probes—Total RNA was pre- pared from tissues and cultured cells using the Ultraspec RNA isolation protocol (Biotechnics Laboratories). RNA aliquots (5–20 µg) were denatured, separated on 1.2% formaldehyde gels, and blotted onto Hybond N membranes (Amersham). Prehybridization and hybridization were carried out in 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 50 mM sodium phosphate, pH 6.5, 0.5% SDS, 20 µg/ml salmon sperm DNA at 45 °C overnight. Membranes were washed twice in 2 × SSC, 0.1% SDS at room temperature for 15 min and once in 0.1 × SSC, 0.1% SDS at 50 °C for 15–30 min. Following exposure to DuPont Cronex x-ray films or PhosphorImager screens, membranes were stripped in 0.1% SDS for 15 min at 85 °C and reprobed. A 980-bp MoeII fragment from 5′-end of the Cig30 cDNA was used as a cig30 hybridization probe.

pcDNA-cig30GFP Construction, Pronuclear Microinjection, and Microscopy—The gfp gene was cut out from the pcGFP-N2 plasmid (CLON- TECH) with HindIII and BclI and ligated in HindIII-BamHI sites of the pcDNA I vector, creating pcDNA-GFP. The Cig30 cDNA was PCR amplified with primer 6 and primer 8 (CGGATCCTTGGCTCCTTC-TGGTCAAGCT), which replaces the Cig30 stop codon with a BamHI site. The PCR product was subsequently digested with SalI and BamHI and ligated in XhoI-BamHI sites of the pcDNA-GFP construct resulting in a fusion gene Cig30-gfp, driven by an enhancer/promoter derived from human cytomegalovirus. The CIG30 and GFP polypeptides are connected through a short space of 8 amino acids (RIHRPGAV). The expected fusion gene product was verified by immunoblotting with anti-gfp antiserum (CLONTECH) following in vitro translation of the construct.

Embryonic Stem Cell Lines Expressing the Cig30 Gene—A dicistronic expression vector pcIGIRESneo was constructed as follows: First, the internal ribosomal entry site (IRES) from the pcIPIRE-4a vector (Novagen) was amplified by PCR with primer 9 (CGGCGCCGCG-GAATTATCGGTTTTTTCATTTTCCCAACCA) and primer 10 (GCTGGAAT-TGGACGATCATGCAAGACCA) and ligated into the pcDNA I vector, creating pcDNA-GFP. The neo gene with a synthetic poly(A) signal was PCR amplified from the pcneo vector (pcDNA I) using primer 11 (CCGATCATGACGATCATGCAAGA) and primer 12 (CGGATCCCTCATGATGCAAGA) and ligated into NdeI and BamHI sites of the pcI-neo vector (Promega), replacing the neo gene and generating pcI-IRES. Second, the neo gene with a synthetic poly(A) signal was PCR amplified from the pcI-neo vector using primer 13 (CGGATCCATGACGATCATGCAAGA) and primer 14 (CGGATCCGCTATGACGATCATGCAAGA) and ligated into NdeI and BamHI sites in the pcI-neo vector using the ApaI-BamHI fragment from pcI-neo into the ApaI-BamHI sites of pcI-IRES, which resulted in the pcI-neo-IRES neo vector. The pcI-neo-IRES neo vector was then amplified with primers 6 and 7, ligated with SalI and XhoI, and ligated as the first cistron into the XhoI and XbaI sites in the pcI-IRESneo vector. The final construct, pcI-Cig30:IRESNeo, was linearized with AhdI before transfections. The HIB-1B cells were grown in 35-mm wells to 50–75% confluence. Thereafter, 2.5 µg of pcI-cig30: IRESneo was transfected per well using the cationic lipid-mediated method with DOTAP (Boehringer Mannheim). For negative controls, the cells were transfected with the pcI-IRESneo vector only. 2 days after transfection, the cells were replated onto 10-cm Petri dishes with the selection medium containing 500 µg of G418/ml (Life Technologies, Inc.). After 1–2 weeks, resistant colonies were isolated, expanded, and checked for Cig30 mRNA. Six clones with highest levels of the Cig30 mRNA and three clones of the negative controls were used in further experiments.
The UCP and β-actin probes were as described previously (30); the LPL probe was as described in Ref. 17. The GLUT4 was obtained from Dr. M. Mueckler, and cDNA was excised with EcoRI. The probes were labeled using random-primed DNA labeling kit (Boehringer Mannheim) with [32P]dCTP.

**Phospholipid Synthesis and Glucose Uptake Measurements**—Phospholipid synthesis was measured in stably transfected HIB-1B cells as the incorporation of [32P]Pi into the phospholipid fraction. Pre confluent cells cultured in 10-cm Petri dishes were incubated in 5 ml of Krebs-Ringer bicarbonate buffer with 2% fatty acid-free bovine serum albumin in the presence of 100 mCi of [32P]Pi at 37 °C and 8% CO2 for 1 h. The buffer was then quickly removed, the cells were scraped into 3 ml of phosphate-buffered saline, and the phospholipids were extracted as described earlier (31). Final samples were dissolved in 20 ml of chloroform, 6 ml of which were applied as 10-mm streaks on 10 × 10 cm HPTLC Silica gel 60 F254 plates (Merck). The plates were developed according to Ref. 32 in methyl acetate-2-propanol-chloroform-methanol-0.25% KCl (25:25:25:10:9, v/v), dried, and exposed to a PhosphorImager screen. For glucose uptake measurements, primary cultures of brown adipocytes or cultures of HIB-1B cell lines were grown on 35-mm dishes. Where indicated, the cells were treated with 1 mM dexamethasone or 0.1 mM norepinephrine (or both) for 3 days and with 0.1 mM insulin for 1 h before assay, and 2-deoxy-[3H]glucose transport was measured according to Ref. 33. Harvested cells were thoroughly sonicated in 300 ml of phosphate-buffered saline and counted on a Beckman liquid scintillation system 3801. The net uptake of 2-deoxy-D-glucose was expressed in pmol/min/mg protein ± S.E.

**RESULTS**

**Cloning and Analysis of a Full-length Cig30 cDNA**—The CIN-2 clone was initially isolated from a brown adipose tissue cDNA library (26) by differential screening for cold-inducible genes (15). By DNA sequencing, we found a poly(A) tract in this cDNA, but no open reading frame, implying that the clone covered the 3′-untranslated region. To obtain the full-length cDNA, we used the 5′-RACE method as described under “Experimental Procedures.” The resulting 1.9-kb cDNA, corresponding to a novel gene named Cig30, was sequenced (Fig. 1A). Sequence analysis revealed a single open reading frame of 813 bp, flanked by a 172-bp 5′-untranslated region and a 900-bp 3′-untranslated region.
The inferred polypeptide was compared with those in protein banks using the BLAST network service (35). Nine proteins were found to have significant homology with CIG30, six of which are hypothetical gene products from Caenorhabditis elegans and the other three are yeast proteins. Yeast mutant strains exist for all three of these genes (36), but complex phenotypes make determination of their primary function very difficult. Among the reported defects are modified phospholipid composition (37), altered localization of sterol synthesis, particularly to fenopropimorph (40), and a large reduction in 1,3-β-glucan synthase activity (41). Some physical properties of these proteins are summarized in Table I. This group emerges as a novel protein family (36) with remarkable similarities from yeast to mice (Fig. 1, C and D).

The presence of hydrophobic stretches indicated that CIG30 might be a membrane protein. As shown in Fig. 2A, a hydropathy plot based on the GES algorithm (42) predicts five transmembrane helices in the CIG30 protein. In good agreement with the GES algorithm, the TMAP program (43) also predicts five transmembrane regions (Figs. 1A and 2A). An arbitrary stretch of 35 amino acid residues in the middle part of the CIG30 polypeptide shares 88% identity with the C40H1.4 protein and 31% identity throughout the protein family (Fig. 1A, underlined with a dotted line, and evident also in Fig. 1C). This region of highest homology lies in a putative loop and seems to be rather amphiphilic in an α-helical projection with conserved positions on the opposite sides of the helix (Fig. 2B).

In positions 6–9 of the CIG30 protein, we found a consensus sequence for an N-glycosylation site (N-F-S-R). Examination of the sequence for an N-terminal cleavable precursor gave negative results. A perfect consensus for a leucine zipper was found in the fourth transmembrane region. The protein also contains potential phosphorylation sites for protein kinase C (amino acid positions 58–60, 83–85, and 187–189) and casein kinase II (amino acid positions 23–26 and 292–295) and three N-myristoylation consensus sites (amino acid positions 98–103, 214–219, and 229–234).

**Glycoprotein Nature**—To experimentally demonstrate the existence of the open reading frame in the Cig30 cDNA, we subcloned various fragments of the cDNA (nt 1–1173, 43–1173, 148–1129, 162–1056) in the pcDNA I vector. The corresponding constructs were in vitro transcribed, translated in rabbit reticulocyte lysate in the presence of [35S]methionine, and analyzed by SDS-PAGE. All clones gave rise to a 28-kDa protein product, and the shortest clone was translated with highest efficiency (data not shown). For further study, we therefore chose the 162–1056 cDNA fragment covering the putative open reading frame (nt 173–985). When translated in rabbit reticulocyte lysate with canine pancreatic microsomal membranes, the apparent size of the CIG30 polypeptide was shifted from 28 kDa to 30 kDa, which is proposed to be due to a glycosylation event (Fig. 3A), thus implying that CIG30 is a glycoprotein. We assume that the start codon is located at nt 173 of the Cig30 cDNA, both because it is the first ATG codon and because it is embedded in a minimal Kozak consensus sequence ((A/G)NNATGG) (44).

**Intracellular Localization**—To address the question of the intracellular localization of the CIG30 protein, we constructed a fusion gene, in which the green fluorescent protein (GFP) was attached to the C terminus of the CIG30. The Cig30-gfp gene was cloned downstream of the enhancer/promoter from human cytomegalovirus and assayed by transient expression in mouse preimplantation embryos as described under “Experimental Procedures.” Despite the relatively weak fluorescence of the wild type GFP protein, this expression system allowed us to demonstrate that the CIG30 fusion protein is primarily located to the plasma membrane. Nonetheless, we cannot exclude that CIG30 alone is also localized to other membranes, such as those of the endoplasmic reticulum (Fig. 3B).

Polyclonal antibodies were raised against a synthetic peptide corresponding to the C-terminal part of the protein. In plasma
membrane fractions prepared from brown fat of warm-acclimated, 1-week, and 1-month cold-acclimated mice, the antibody detected a faint band of 30 kDa in stimulated tissues. This band was of similar size to the in vitro translated and glycosylated CIG30 (Fig. 3C). No signal was found in total homogenates (data not shown). As an additional indication that the protein is a membrane protein, we noticed that CIG30 tended to aggregate, especially when boiled, and had to be solubilized at low temperatures.

**HIB-1B Cells Stably Transfected with the Cig30 Gene Have neither Altered Phospholipid Synthesis nor Abnormal Glucose Uptake**—We tested the HIB-1B immortalized cell line derived from brown adipose tissue (23) for the presence of the Cig30 mRNA and found no signal under various experimental conditions (not shown). We proposed that overexpression of Cig30 in these model cells could affect some cellular processes in which the corresponding yeast mutants were defective, e.g. phospholipid synthesis (37) or glucose uptake (38). To study these parameters in vitro, we established several lines of HIB-1B cells stably transfected with the Cig30 gene. These lines expressed high levels of the Cig30 mRNA, whereas control HIB-1B cells showed no detectable Cig30 expression (Fig. 4A).

Phospholipid synthesis was measured as incorporation of [32P]Pi into phospholipids for 1 h. The phospholipid fraction was then isolated and separated on TLC. However, as shown in Fig. 4B, the pattern of radioactivity incorporation into phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol was unchanged between Cig30-transfected and control cell lines. Data presented in Table II indicate that expression of the
Cig30 gene did not alter glucose transport into HIB-1B cells in the basal state, nor did it significantly enhance their responsiveness to insulin. Thus, the CIG30 protein is unlikely to be directly involved in the transport of glucose into mammalian cells.

**Increase of Cig30 Expression Coincides with the Recruitment of Brown Fat**—We examined expression of the Cig30 gene in 12 tissues of mice kept either at 28 °C or at 4 °C for 3 days (Fig. 5A). By Northern blotting of total RNA from warm-acclimated animals, we detected Cig30 mRNA in brown fat and liver, whereas no detectable signal was seen in white fat, heart, lung, kidney, testis, muscle, spleen, brain, thymus, and intestine.

**Glucose uptake in Cig30-transfected IIIB-1B cells**

2-Deoxyglucose uptake was measured in HIB-1B cells stably transfected with either pCI-Cig30:IRESneo (HIB-CIG30) or pCI-IRESneo (HIB control) following a treatment with 0.1 μg insulin for 1 h (+insulin) or no treatment (-insulin). Data are given in pmol/min/mg protein, and expressed as means ± S.E. from triplicate measurements. *p < 0.05.

|           | +Insulin | -Insulin |
|-----------|----------|----------|
| HIB control | 0.91 ± 0.022 | 1.03 ± 0.017* |
| HIB-CIG30   | 0.87 ± 0.024 | 0.95 ± 0.020 |

**TABLE II**

| Insulin  | +Insulin | +Insulin |
|----------|----------|----------|
| HIB control | 0.91 ± 0.022 | 1.03 ± 0.017* |
| HIB-CIG30   | 0.87 ± 0.024 | 0.95 ± 0.020 |

Following overly prolonged autoradiography, a weak signal could be observed in kidney, white fat, heart, and skin (not shown). The Cig30 mRNA was consistently detected as two bands. The major hybridization band was about 1.9–2 kb; the size of the minor species was approximately 1.6–1.8 kb. The short mRNA form could be accounted for by an alternative use of the polyadenylation site at nt 1472 (see Fig. 1A). There is, however, no poly(A) consensus site in the 3′-end of the long Cig30 mRNA form.

Of particular interest was the fact that the Cig30 mRNA level in brown fat of cold-exposed animals was 200-fold elevated, whereas the liver Cig30 mRNA level remained unchanged. Therefore, we measured Cig30 mRNA in brown fat of mice exposed to cold for various periods of time. As shown in Fig. 5B, only a very small increase was seen after 4 h of cold exposure, and the signal peaked after 3 days. It then gradually declined to 50–60% of the maximum after 1 month, being, however, still about 100-fold increased above controls.

Brown adipose tissue is recruited not only during cold exposure but also during early postnatal development to meet increased heat production requirements. We therefore investigated whether the Cig30 gene was activated at this stage of development in mice. As shown in Fig. 5C, the Cig30 mRNA Increase of Cig30 Expression Coincides with the Recruitment of Brown Fat A, Northern blot of total RNA (10 μg per lane) from various mouse tissues. RNA samples were prepared from both thermoneutral mice (28 °C) and mice exposed to 4 °C for 3 days. The membrane with blotted RNAs was sequentially hybridized with a 32P-labeled cDNA probe corresponding to the protein coding region in the Cig30 gene in 12 tissues of mice kept either at 28 °C or at 4 °C for 3 days (Fig. 5A). By Northern blotting of total RNA from warm-acclimated animals, we detected Cig30 mRNA in brown fat and liver, whereas no detectable signal was seen in white fat, heart, lung, kidney, testis, muscle, spleen, brain, thymus, and intestine.

**FIG. 5.** Elevated expression of Cig30 in vivo coincides with the recruitment of brown adipose tissue. A, Northern blot of total RNA (10 μg per lane) from various mouse tissues. RNA samples were prepared from both thermoneutral mice (28 °C) and mice exposed to 4 °C for 3 days. The membrane with blotted RNAs was sequentially hybridized with a 32P-labeled cDNA probe corresponding to the protein coding region in the Cig30 mRNA and with the β-actin probe. Photograph of the ethidium bromide-stained gel with 18 S rRNA showed integrity and uniformity of RNA samples. Position of 18 S rRNA (18S samples). B, Northern blot analysis using the cig30 cDNA probe. 28 °C, thermoneutral controls; 4 °C, cold stimulation for 3 days; fasting, 24-h fasting; FAST, 24-h fasting followed by a 4-h cold exposure; FAST+REF, 24-h fasting followed by a 4-h refeeding. Membranes were stripped and rehybridized with the β-actin probe.
level was transiently elevated in brown fat in the perinatal period, reaching a maximum level immediately after birth and declining to basal levels before weaning. However, the stimulation was of much lower amplitude than that observed in brown fat of cold-exposed mature mice.

Cafeteria diet has also been recognized as a factor inducing brown fat recruitment. We found a significant effect of cafeteria diet on Cig30 expression in mice kept at 28 °C (Fig. 5D), although only very slightly when compared with a cold stimulus. Fasting did not have any stimulatory effect, neither did it prevent the early effect of cold. Refeeding after 24 h of fasting did not appreciably increase the Cig30 mRNA level, suggesting that neither glucose nor insulin is capable of eliciting Cig30 mRNA expression.

**Norepinephrine Is Sufficient for Induction of Cig30 mRNA in Vivo**—Recruitment of brown adipose tissue is brought about by norepinephrine released from sympathetic nerves. To investigate whether norepinephrine could mimic the effect of cold in terms of Cig30 expression, we treated mice kept at 28 °C with norepinephrine for 3 days. The hormone was administered continuously using microosmotic pumps placed subcutaneously in the interscapular region. The mice received either vehicle alone or norepinephrine for 72 h. As shown in Fig. 6, norepinephrine induced the Cig30 mRNA level by about two orders of magnitude, suggesting that the cold effect on Cig30 expression is mainly due to norepinephrine. The marginal induction of Cig30 expression in the saline controls was probably due to catecholamine release following the mechanical stress of the minipumps.

**Synergistic Action of Dexamethasone and Norepinephrine Is Required for Full Expression of Cig30 in Primary Cultures of Brown Adipocytes**—Brown adipocyte precursors isolated from the stromal vascular fraction of brown adipose tissue can differentiate in primary culture in vitro into morphologically and physiologically adequate mature cells (45). To investigate whether brown adipocytes were specifically the site of Cig30 expression and whether the induction of Cig30 expression in vivo was a primary or secondary effect of norepinephrine, we analyzed Cig30 mRNA levels in primary cultures of brown fat cells by Northern blotting. The results are presented in Fig. 7A.

The Cig30 mRNA was virtually absent in brown adipocytes spontaneously differentiated in standard culture medium. Chronic treatment with 0.1 μM norepinephrine for 3 days from day 6 resulted in appreciable expression, the level of which...
Cultured brown adipocytes were chronically treated with 0.1 μM norepinephrine (NE), 1 μM dexamethasone (DEX) or with a combination of both (NE + DEX) from day 6 to day 9. Thereafter, the cells were given 0.1 μM insulin for 1 h (+ insulin) or left untreated (− insulin) and assayed for 2-deoxyglucose uptake. Values are in pmol/min/mg protein and are expressed as means ± S.E. from triplicate measurements.

|          | −Insulin | +Insulin |
|----------|----------|----------|
| Control  | 1.47 ± 0.03 | 3.89 ± 0.16 |
| NE       | 1.94 ± 0.11 | 4.99 ± 0.18 |
| DEX      | 1.05 ± 0.02 | 4.62 ± 0.21 |
| NE + DEX | 2.18 ± 0.05 | 6.13 ± 0.22 |

*a Significantly higher than the corresponding treatment without insulin (p < 0.01).

*b Significantly higher than insulin-treated control (p < 0.01) or NE or DEX (p < 0.05).

we was, however, much lower than that in cold-stimulated brown adipose tissue. Because glucocorticoids have been implicated in the promotion of brown adipocyte differentiation (33), we added 1 μM dexamethasone to the cells and found a comparably small increase in Cig30 expression. Unexpectedly, when both hormones were present together for 3 days, the Cig30 expression was strongly potentiated, being 10-fold greater than either of the stimuli alone. Norepinephrine stimulation for 1 h after chronic treatment with dexamethasone had no additional effect, the stimuli alone. Norepinephrine stimulation for 1 h after was strongly potentiated, being 10-fold greater than either of the hormones alone. Norepinephrine in itself was about 2-fold less potent, and dexamethasone had virtually no effect. The genes for GLUT4 and LPL, both significantly higher than insulin-treated control (p < 0.01) or NE or DEX (p < 0.05).

Combined with the above results (Fig. 7A), the Cig30 gene required the simultaneous presence of norepinephrine and dexamethasone for full expression; either of the hormones alone was less than 20% as potent. The expression of the Ucp gene was also maximally stimulated in the norepinephrine plus dexamethasone-treated cells; norepinephrine in itself was about 2-fold less potent, and dexamethasone had virtually no effect. The genes for GLUT4 and LPL, both primarily involved in anabolic processes, exhibited a different expression profile, reaching a maximum level in the dexamethasone-treated cells, and a similar level in the cells treated with norepinephrine plus dexamethasone. Norepinephrine in itself was less potent. Taken together, these results indicate that the

**DISCUSSION**

In this report, we have described cloning and characterization of a cDNA from mouse brown adipose tissue that corresponds to a novel mammalian gene, which we have termed Cig30. The gene codes for a 30-kDa glycoprotein of yet unknown function. However, the data strongly suggest that this function is associated with recruitment and thermogenesis in brown adipose tissue.

All nine proteins homologous to CIG30 that are currently present in the protein banks are fairly well conserved both in being hydrophilic and basic, in the amino acid sequence of the central core (Table I and Fig. 1C), and probably in the overall topology. Similarly to the CIG30 protein, an N-linked glycosylation site has been reported in the N-terminal part of the Fen1p protein (41), and leucine zipper structures found in Sur4p and Fen1p proteins have been suggested to trigger dimerization in the plasma membrane (38, 41). The number of predicted transmembrane stretches reported for different proteins varies from four to six, which is probably the result of some ambiguity in the central part of the consensus. The core region is the most highly conserved amino acid sequence (Fig. 8A) implicating this segment as being crucial for protein function. We have noticed that the conserved region displays amphipathic properties (Fig. 2B), which might explain the uncertainty in estimating transmembrane topology. It is possible that this structure is associated with or inserted into the membrane producing an active site. In accordance with the structure published by El-Sherbeini and Clemas (41) for the yeast Gns1 protein (which is identical with Fen1p), we suggest that the common topology of this protein family is a five-transmembrane-spanning structure (Fig. 8B).

Using the Northern blotting approach, we have found that the most prominent feature of Cig30 gene regulation is its high inducibility in brown adipose tissue by cold (Fig. 4A). The time course of the cold-induced expression is delayed compared with genes directly involved in thermogenesis, such as Ucp and Lpl (14–17), and is more reminiscent of induction of the genes for GPDH (18) or GLUT4 (19). As the Cig30 mRNA peaks after 3 days in the cold (Fig. 5B), it is plausible that CIG30 is primarily related to hyperplastic changes in the tissue during cold acclima-
tion, rather than being involved in immediate heat production. This would be in line with the slow proliferation onset observed in the stimulated brown adipose tissue (20, 21).

In the present paper, we show that diet-induced thermogenesis is also associated with stimulation of Cig30 gene expression (Fig. 5D). There are two possible effects of a cafeteria diet on brown adipose tissue, either a central effect mediated via the hypothalamus or a direct effect of high blood glucose level on brown adipocytes. We were not able to distinguish these two pathways in our experiments. However, since refeding after 24-h fasting did not lead to a detectable stimulation of Cig30 mRNA, we speculate that neither glucose nor insulin is sufficient to induce Cig30 gene expression. Similarly, fasting could not prevent the early onset of Cig30 mRNA induction on cold exposure (Fig. 5D). The Cig30 mRNA level in liver was not significantly influenced by any treatment in this experiment (not shown). It may be concluded that the diet-induced increase of Cig30 gene expression is most likely due to norepinephrine release caused by stimulation of the hypothalamus. This is also in agreement with our results demonstrating that chronically administered norepinephrine is sufficient for marked induction of Cig30 expression in vivo (Fig. 6).

Following chronic treatment with norepinephrine and dexamethasone, we were able to detect high Cig30 mRNA levels in brown adipocytes differentiated in vitro (Fig. 7A). The requirement for a synergistic effect of these two agents for high expression in vitro was unexpected and did not fully conform with the in vivo results, which indicated that norepinephrine alone is a sufficient stimulus to greatly induce Cig30 gene expression. This discrepancy could perhaps be explained by endogenous glucocorticoids, the peak plasma levels of which are only about 31746. This discrepancy could perhaps be explained by endogenous glucocorticoids, the peak plasma levels of which are only about 31746.

In our hands, however, overexpression of Cig30 in HIB-1B cells did not elicit any significant changes in phospholipid synthesis (Fig. 4) nor did we see any marked abnormalities in tissued, there is a remarkable increase in turnover of specific phospholipids, particularly that of phosphatidylinositol (11). This would be in line with the slow proliferation onset observed in the stimulated brown adipose tissue (20, 21).

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