THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 39, Issue of September 24, pp. 40946–40953, 2004
Printed in U.S.A.

EPAS1 Promotes Adipose Differentiation in 3T3-L1 Cells*

Received for publication, January 26, 2004, and in revised form, June 16, 2004
Published, JBC Papers in Press, July 15, 2004, DOI 10.1074/jbc.M400840200

Shigeki Shimba‡, Taira Wada‡, Shuntaro Hara§, and Masakatsu Tezuka‡¶

From the ‡Department of Health Science, College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba 274-8555, Japan and the §Department of Public Health and Molecular Toxicology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan

Adipose differentiation is regulated by several transcription factors, such as the CAAT/enhancer-binding protein family and peroxisome proliferator activator (PPAR) γ2. Several recent studies have shown that the basic helix-loop-helix-PAS superfamily is also involved in the regulation of adipose differentiation. In this study, we investigated the roles played by EPAS1 (endothelial PAS domain protein 1) in adipogenesis. EPAS1, also referred to as hypoxia-inducible factor 2α, is a transcription factor known to play essential roles in catecholamine homeostasis, vascular remodeling, and the maintenance of reactive oxygen species, and so forth. During adipose differentiation in 3T3-L1 cells, the level of EPAS1 mRNA began to increase 6 days after the induction, and EPAS1 was highly expressed in differentiated cells. To examine whether EPAS1 is involved in adipogenesis, we first isolated stable clones from 3T3-L1 cells in which we could induce the expression of an EPAS1 C-terminal deletion mutant (designated EPAS1-(1–485)) with the insect hormone. The induction of EPAS1-(1–485) allowed the cells to accumulate only minimum amounts of intracellular lipid droplets. Consistent with the morphological observations, a minimum amount of aP2 and PPARγ2 mRNA was induced in the EPAS1-(1–485) cells. We then examined whether or not EPAS1 was able to promote adipogenesis in NIH 3T3 cells, a relatively nonadipogenic cell line. Overexpression of EPAS1 in NIH 3T3 cells induced a significant amount of lipid accumulation compared with that of the control cells in the presence of the PPARγ ligand. The results were also confirmed by measuring the expression of adipocyte-related genes. Adenovirus-mediated EPAS1-(1–485) expression resulted in the reduction of basal and insulin-dependent glucose transport in 3T3-L1 adipocytes. The mechanism involved the transcriptional regulation of GLUT1, GLUT4, and IRS3 expression by EPAS1. Taken together, these results suggest that EPAS1 plays several supporting roles in maintaining specific aspects of adipogenesis and adipocyte function including regulation of glucose uptake followed by lipid synthesis.

Adipose differentiation is a complex process by which fibroblast-like undifferentiated cells are converted into cells that accumulate lipid droplets. Recent studies using DNA arrays revealed that expression of several hundred genes is altered in this process (1). This complex differentiation process is driven by the coordinated expression of various transcription factors, including the CAAT/enhancer-binding protein (C/EBP)[1] family and peroxisome proliferator activator (PPAR) γ2 (2–4). In preadipocytes, the Wnt signaling pathway is part of the machinery, which maintains the cells in undifferentiated states (5). Adipogenesis begins on the treatment of preadipocytes with fetal calf serum, insulin, dexamethasone, and an inducer of intracellular cyclic AMP such as isobuthylmethylxanthine. A rapid and transient increase in transcription and expression of C/EBPβ and C/EBPδ is observed in the early stages of differentiation. These factors have been shown to promote adipogenesis, presumably through induction of C/EBPα and PPARγ2 (2–4). The enforced expression of PPARγ2 or C/EBPα has been shown to stimulate adipogenesis in NIH 3T3 fibroblasts (6–8), suggesting the essential roles played by these factors in regulating adipogenesis *in vitro*. Cells lacking PPARγ2 had a greatly reduced level of C/EBPα (9–11). Similarly, cells lacking C/EBPα have reduced adipogenic potential and expression of PPARγ2 (12). Importantly, adding PPARγ2 back to C/EBPα-null cells has been shown to restore their ability to accumulate lipids and activate markers of adipose differentiation (12). Recent studies have demonstrated that ectopic expression of C/EBPα failed to rescue the ability to differentiate in PPARγ-null mouse embryo fibroblasts (13). Therefore, rather than being an equal co-director of the adipogenesis program, PPARγ2 plays a leading role in the adipogenic hierarchy. On the other hand, C/EBPα is critical in the establishment of insulin sensitivity (12). This effect of C/EBPα is mediated in part by the direct transcriptional induction of both the insulin receptor and IRS1 (12). Consequently, C/EBPα is influential in maintaining the expression of PPARγ2 and in promoting full insulin sensitivity.

Several recent studies have shown that the basic helix-loop-helix (bHLH)-PAS superfamily is also involved in regulation of adipose differentiation (14–17). The bHLH-PAS proteins are characterized by the PAS domain, which is composed of two imperfect 50-amino acid repeats and a bHLH domain. The term "PAS" is derived from the first three members of the family: the period gene, the aryl hydrocarbon receptor nuclear translocator gene (ARNT), and the single-minded (SIM) gene. Using the PAS domain and the bHLH domain, proteins of this family form heterodimers that bind to a target gene through the basic region and govern the functions of that gene. The PAS domain

* This work was supported in part by a grant from the Ministry of Education, Sciences, Sports, and Culture of Japan (to S. S. and M. T.); a General Individual Research Grant from Nihon University (to S. S.); a grant from the Ministry of Education, Culture, Sports, Science, and Technology to promote multidisciplinary research projects; and an Interdisciplinary General Joint Research Grant from Nihon University (to M. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 81-47-465-5694; Fax: 81-47-465-5637; E-mail: tezukam@pha.nihon-u.ac.jp.

[1] The abbreviations used are: C/EBP, CAAT/enhancer-binding protein; PPAR, peroxisome proliferator activator; bHLH, basic helix-loop-helix; HIF, hypoxia-inducible factor; PonA, ponasterone A.

is found in a variety of proteins that play roles in development and adaptation to the environment, such as neurogenesis (18), circadian rhythms (19), hypoxia (20), and xenobiotic metabolism (21). Thus, members of bHLH-PAS superfamily are thought to serve as sensors of environmental and developmental signals. We recently demonstrated that the aryl hydrocarbon receptor, a ligand-dependent transcription factor, is involved in the negative regulation of adipogenesis in 3T3-L1 cells (14). The proposed mechanism of action in this case is that the aryl hydrocarbon receptor inhibits the PPARγ2 activation pathway by stimulating p107 expression and p42/p44 mitogen-activated protein kinase, and the inhibition of the PPARγ2 signaling pathway results in a lower level of morphological differentiation in aryl hydrocarbon receptor-activated cells (14). Hypoxia inhibits adipogenesis in a hypoxia-inducible factor (HIF)-1α-dependent manner (15). The putative mechanism for this process is that HIF-1α-regulated gene Dec 1/Stra 13 represses PPARγ2 promoter activity (15). Clinical studies have shown that disruption of the Sim1 gene by a *de novo* balanced translocation between chromosomes results in obesity (16). Also, Sim1 heterozygous mice develop early-onset obesity (17). Consequently, several bHLH-PAS proteins are thought to participate in the regulation of adipose differentiation.

Among the proteins of bHLH-PAS family, we have focused here on the roles played by EPAS1 (endothelial PAS domain protein 1) in adipogenesis. EPAS1, also referred to as HIF-2α, HLF, or MOP2, is a transcription factor predominantly expressed in endothelial cells and the organ of ZuckerlandI (22–25). Studies using EPAS1-null mice revealed that EPAS1 is essential for catecholamine homeostasis and vascular remodeling (25, 26). More recently, Scortegagna *et al.* (27) revealed that EPAS1 plays a role in the maintenance of reactive oxygen species. In addition to these roles, a contribution of EPAS1 to the regulation of adipogenesis has been suggested for the following reasons. First, several growth factors, including insulin and insulin-like growth factor, induce and activate EPAS1 activity in a phosphatidylinositol 3-kinase-dependent or related manner (28). Also, HIF-1α, which is closely related to EPAS1, mediates insulin signaling in hepatocytes (29). More importantly, EPAS1 is highly expressed in white adipose tissue (this study). We show here that overexpression of a dominant negative form of EPAS1 in 3T3-L1 preadipocyte cells resulted in the suppression of morphological differentiation, as well as in the induction of adipocyte-related genes. The mechanism involves the direct transcriptional regulation of GLUT1, GLUT4, and IRS3 genes by EPAS1. We also examined here the ability of EPAS1 to induce adipose differentiation in nonadipogenic cells. Ectopic expression of wild-type EPAS1 in NIH 3T3 cells induced morphological differentiation and the expression of adipocyte-related genes. These results suggest that EPAS1 is an important transcription factor in the regulation of adipose differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture—3T3-L1 cells, obtained from the Human Science Research Resources Bank (Osaka, Japan), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. For induction of adipose differentiation, the cells were grown to confluence. The cells were then fed with differentiation medium (a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 containing 10% fetal bovine serum, 1.6 μM insulin, 0.0005% transferrin, 180 μM adenine, 20 pM triiodothyronine, 0.25 μM dexamethasone, and 500 μM isobutylmethylxanthine). After 3 days, the cells were refed with fresh differentiation medium without dexamethasone and isobutylmethylxanthine and maintained over the following days.

Oil Red O Staining—To judge the state of adipose differentiation by visual inspection, cultures were fixed with 4% formalin in phosphate-buffered saline for 2 h, rinsed three times with distilled water, and then air-dried. The fixed cells were stained with 0.5% Oil Red O solution for 1 h. After staining, the cultures were rinsed several times with 70% ethanol.

Immunoblot Analysis of EPAS1 Protein—The cells, grown in 60-mm dishes, were rinsed with ice-cold phosphate-buffered saline. The rinsed cells were scraped off the dish, placed in a microcentrifuge tube, and centrifuged at 5,000 × *g* for 1 min. The resulting pellets were suspended in lysis buffer (50 mM Hepes-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.0002% leupeptin, and 20% glycerol), vortex-mixed, and rocked at 4 °C for 60 min. The suspensions were centrifuged for 15 min at 10,000 × *g*, and the resulting supernatants were then frozen until further analysis. The protein concentration of the extracts was determined according to the method of Bradford, using bovine serum albumin as the standard (30). Protein samples were denatured by heating them to 90 °C in SDS-reducing buffer and resolved by electrophoresis on 10% SDS-polyacrylamide gels. After transfer of proteins to nitrocellulose membranes, the filters were probed with antibodies against EPAS1 (Novous) or the FLAG motif (Stratagene). Color visualization was performed with secondary antibodies conjugated with alkaline phosphatase and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate solution (Promega).

Ponasterone A (PonA)-inducible EPAS1-(1–485) Expression System—The PonA-inducible EPAS1-(1–485) expression system was constructed using complete control mammalian expression system (Stratagene) according to the manufacturer's instructions. Briefly, EPAS1-(1–485) cDNA was generated by PCR using full-length EPAS1 cDNA (31) and subcloned into expression vector pEGSH, which is under the control of the PonA-responsive regulatory plasmid pVgRXR. Cells stably transfected with regulatory plasmid pVgRXR were selected in conventional medium containing G418 (450 μg/ml). After selection and isolation, the cells were also transfected with pEGSH-EPAS1-(1–485) and allowed to grow in nonselective medium for 48 h. The cells were then cultured in medium containing hygromycin B (200 μg/ml). After 2–3 weeks, clones were isolated and expanded individually.

Analysis of RNA—Total RNA was extracted from the clones with TRIzol reagent (Invitrogen) according to the manufacturer's instructions and analyzed by Northern blotting. Probes were prepared by reverse transcription-PCR techniques.

Adenovirus Infection—Adenovirus expressing EPAS1-(1–485) tagged with the FLAG motif was prepared according to the protocol supplied by the manufacturer (Clontech). 3T3-L1 adipocytes were infected with the virus at a multiplicity of infection of ~50 in OPTI-MEM I (Invitrogen) for 24 h.

Promoter Analysis—The promoter region of GLUT1 gene (−3586 to +237), GLUT4 gene (−785 to −1), IRS3 gene (−1787 to +1), PPARγ2 gene (−615 to +67), and EPAS1 gene (−1169 to +3) was amplified by PCR from genomic DNA isolated from 3T3-L1 cells. The DNA fragments amplified were subcloned into pGL3 basic vector. HEK 293 cells were transiently transfected with the appropriate plasmids by lipofection, and the transfected cells were grown in conventional medium for 48 h. The cell lysates were prepared, and luciferase activities were assayed with the Dual Luciferase Reporter Assay System (Promega).

Determination of 2-Deoxyglucose Transport Activity—3T3-L1 adipocytes were deprived of insulin by incubation in Dulbecco's modified Eagle's medium for 24 h. The cells were then stimulated with 1 μM insulin in Krebs-Ringer-Hepes buffer (1.36 mM NaCl, 4.7 mM KCl, 1.25 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.2 mM KH_2PO_4, 20 mM Hepes-NaOH (pH 7.4), and 0.1% bovine serum albumin) for 30 min. The glucose uptake reaction was initiated by the addition of Krebs-Ringer-Hepes buffer containing 2-[^{14}C]deoxyglucose (1 mCi/mmol) to each well, and after 5 min, the reaction was terminated by washing the wells three times with ice-cold phosphate-buffered saline. The cells were then solubilized in 1 M NaOH, and the radioactivity was measured in a liquid scintillation counter (ALOKA).

Akt Phosphorylation—3T3-L1 adipocytes were stimulated with 1 μM insulin in serum-free Dulbecco's modified Eagle's medium for 30 min. Cell lysates were prepared in extraction buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na_3VO_4, 1 μg of leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The amount of phosphorylated Akt in cell lysates was determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Cell Signaling Technology).

RESULTS

The Level of EPAS1 Increased during Adipose Differentiation in 3T3-L1 Cells—In the first set of experiments, we examined

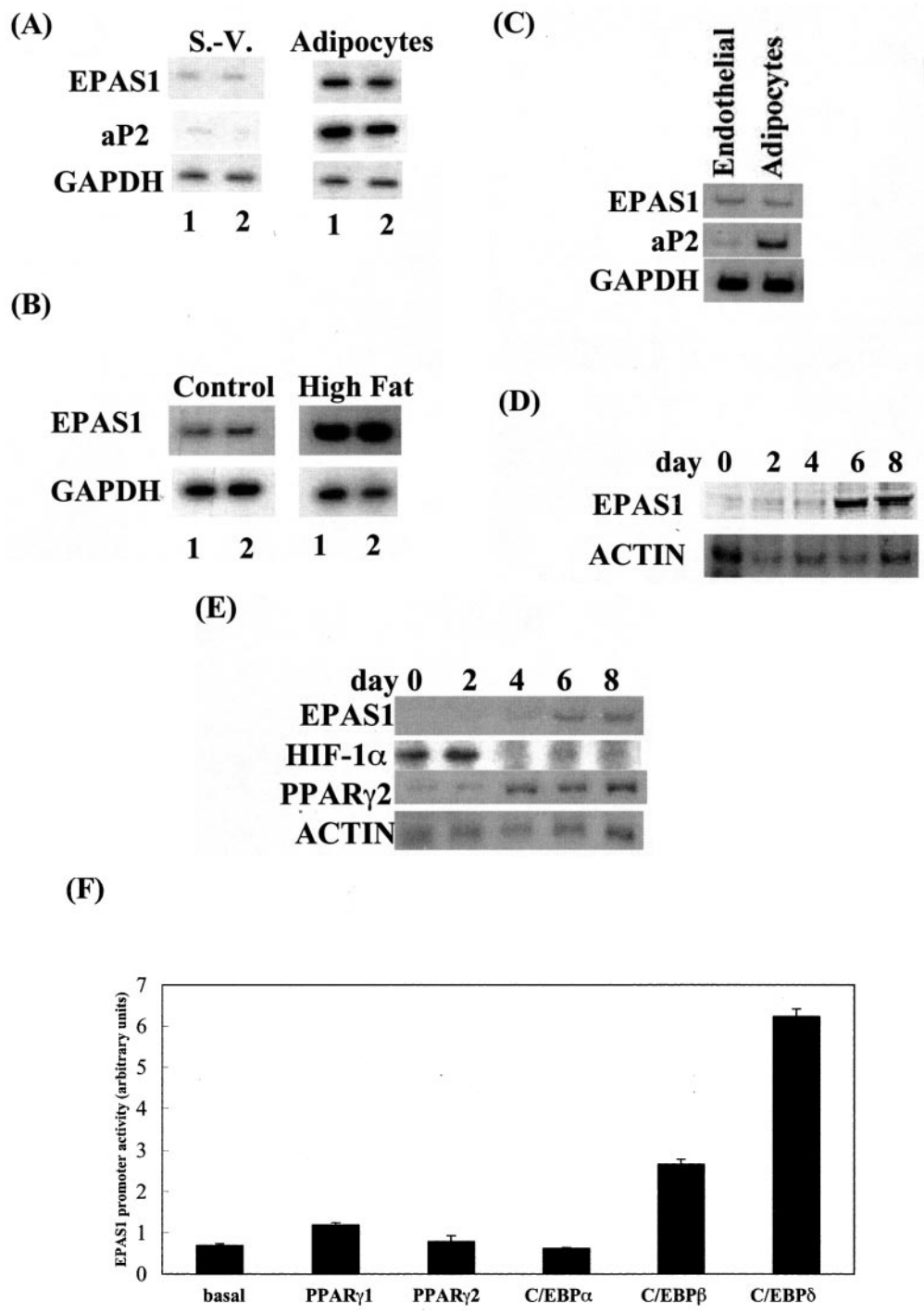

FIG. 1. **Expression of EPAS1 during adipose differentiation.** *A*, white adipose tissue was excised from two male C57BL/6J mice (6 weeks old), and the tissue was fractionated into adipocytes and a stromal-vascular (*S.-V.*) fraction. Total RNA was isolated, and the expression of EPAS1 mRNA was determined by Northern blot analysis. *Lanes 1* and *2* were run using samples from two distinct mice, respectively. *B*, obese C57BL/6J mice were generated by feeding the mice a high-fat diet for 4 weeks. White adipose tissue was excised from control mice and obese mice, and the expression of EPAS1 mRNA was determined by Northern blot analysis. *C*, expression of EPAS1 mRNA in human venous endothelial cells (Cell Systems) and white adipose cells (ZEN-BIO, Inc.) was determined by Northern blot analysis. *D*, 3T3-L1 cells were induced to differentiate by the standard protocol. Western blot analysis of EPAS1 and actin was performed on whole cell extracts (10 μg for EPAS1 and 5 μg for actin). *E*, expression of EPAS1 mRNA during adipose differentiation in 3T3-L1 cells was determined by Northern blot analysis. *F*, expression plasmids of adipogenesis-related transcription factors were transfected into HEK 293 cells with the reporter plasmid carrying the mouse EPAS1 promoter region. For all constructs, phRL-SV40 vector (Promega) was co-transfected to correct for differences in transfection efficiency, and the normalized luciferase activity was represented as arbitrary units. The averages of three independent experiments are shown.

the expression level of EPAS1 in mice adipose tissue. In white adipose tissue isolated from C57BL/6J mice, EPAS1 was predominantly expressed in fractions containing adipocytes, as compared with stromal-vascular fractions (Fig. 1*A*). Also, white adipose tissue in mice fed a high-fat diet expressed a much higher level of EPAS1 mRNA compared with that in control mice (Fig. 1*B*). These results suggest that the expression of EPAS1 is increased during adipose differentiation *in vivo*. EPAS1 was originally identified as the transcriptional factor abundantly expressed in endothelial cells (22–24). Thus we compared the expression level of EPAS1 in adipocytes with that in endothelial cells. The level of EPAS1 mRNA expression

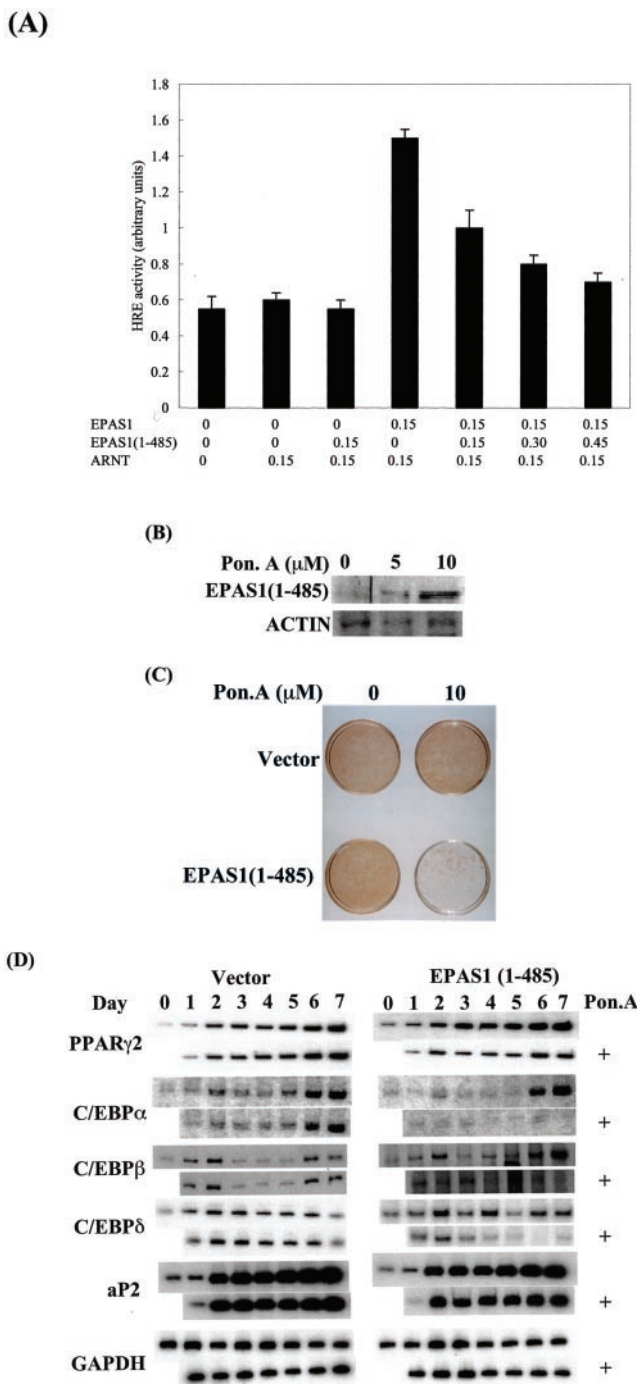

FIG. 2. **Dominant negative effects of EPAS1-(1–485) on adipose differentiation in 3T3-L1 cells.** *A*, dominant negative effects of EPAS1-(1–485) on HRE-driven luciferase activity by full-length EPAS1. The indicated amounts of EPAS1-(1–485) and/or full-length EPAS1 expression plasmid and ARNT expression plasmid were co-transfected into HEK 293 cells with the reporter plasmid pGL3-HRE. For all constructs, pRL-SV40 vector (Promega) was co-transfected to correct for differences in transfection efficiency, and the normalized luciferase activity was represented as arbitrary units. The averages of three independent experiments are shown. *B*, 3T3-L1 preadipocytes stably transfected with pVgRXR and pEGSH-EPAS1-(1–485) were treated with Pon.A for 18 h. Western blot analysis of EPAS1-(1–485) and actin was performed on whole cell extracts (10 μg for EPAS1 and 5 μg for actin). *C*, 3T3-L1 preadipocytes stably transfected with inducible EPAS1-(1–485) or empty vector (*Vector*) were induced to differentiate for 7 days in the presence or absence of Pon.A. Cells were fixed and stained with Oil Red O. Similar results were obtained with three independent clones. *D*, the cell clones were induced to differentiate in the presence or absence of PonA. Total RNA was extracted at the indicated time points, and the expression of adipocyte-related genes was determined by Northern blot analysis.

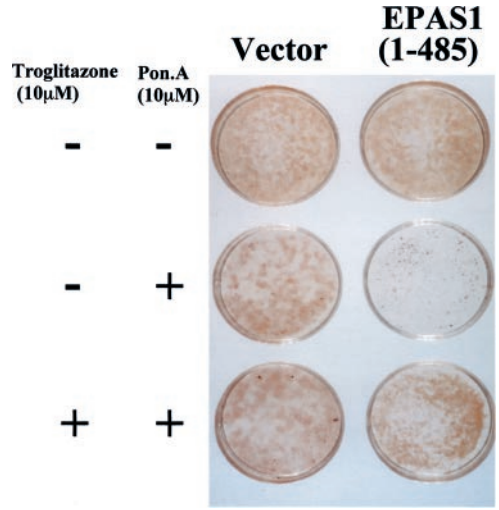

FIG. 3. **Restoration of the differentiation potential of cells expressing EPAS1-(1–485) by treatment with PPARγ2 ligand.** 3T3-L1 preadipocytes stably transfected with inducible EPAS1-(1–485) expression vector (EPAS1-(1–485)) or empty inducible vector (*Vector*) were treated with differentiation medium containing the indicated reagents. On day 7, the cells were fixed and stained with Oil Red O. Similar results were obtained with independent clones.

in adipocytes is as high as that in endothelial cells (Fig. 1*C*). Then, in the next set of experiments, preadipose 3T3-L1 cells were differentiated to adipocytes by the addition of dexamethasone, isobutylmethylxanthine, insulin, and fetal bovine serum, and the expression of EPAS1 during adipogenesis was analyzed by Western blotting (Fig. 1*D*). The adipose differentiation status was confirmed by staining with Oil Red O and by measurement of glyceraldehyde-3-phosphate dehydrogenase activity 8 days after induction (data not shown). The level of EPAS1 protein began to increase 6 days after induction and was markedly expressed in the differentiated cells (8 days after induction). Similar to the protein expression levels, EPAS1 mRNA levels increased during adipose differentiation (Fig. 1*E*). Conversely, expression of HIF-1 α was detected only at the early stage and declined during adipose differentiation (Fig. 1*E*). To characterize the expression of EPAS1 during adipogenesis, the effects of adipocyte-related transcription factors such as C/EBPs and PPARγs on EPAS1 promoter activity were examined (Fig. 1*F*). Luciferase activity driven by the EPAS1 promoter was increased by C/EBPβ or C/EBPδ, whereas C/EBPα and PPARγs had little or no effect on this activity (Fig. 1*F*).

Expression of the Dominant Negative Form of EPAS1 Inhibits Adipose Differentiation in 3T3-L1 Cells—To examine whether EPAS1 exerts an influence on adipogenesis, we first constructed an EPAS1 C-terminal deletion mutant (designated EPAS 1-(1–485)) that lacked a transactivation domain. EPAS1-(1–485) forms a heterodimer with ARNT and binds to the hypoxia-responsive element sequence (32). However, this mutated EPAS1 lacks the ability to transactivate HRE-driven transcription (Fig. 2*A*; Ref. 32). Co-expression of EPAS1-(1–485) suppressed the induction of HRE-driven transcription by full-length EPAS1 in a dose-dependent manner (Fig. 2*A*), indicating that EPAS1-(1–485) functioned as a dominant negative mutant. Although EPAS1-(1–485) also inhibits HIF-1α-dependent transcriptional activity *in vitro* (data not shown), the expression pattern of EPAS1 is distinct from that of HIF-1α during adipogenesis (Fig. 1*E*). Furthermore, HIF-1α protein is unstable due to oxygen-dependent ubiquitination under normoxia conditions, whereas EPAS1 protein is expressed at constant levels regardless of oxygenation (33, 34). Therefore, the effects of EPAS1-(1–485) observed in this study are most likely

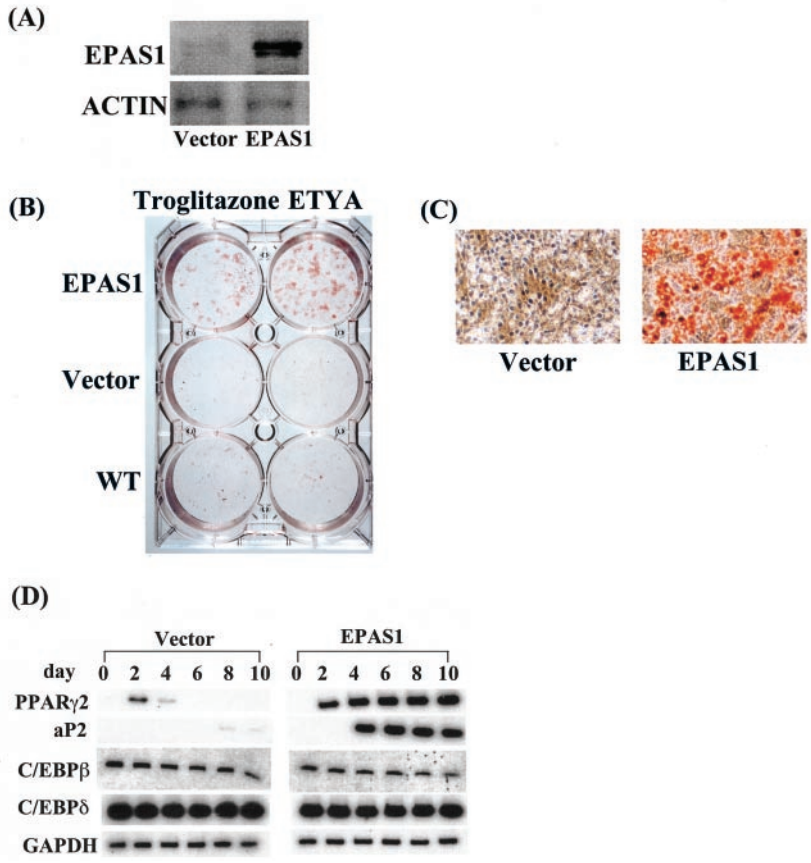

FIG. 4. **Ectopic expression of full-length EPAS1 in NIH 3T3 cells stimulates adipose differentiation.** *A*, NIH 3T3 cells were stably transfected with full-length EPAS1 expression plasmid (*EPAS1*) or pcDNA 3.1 plasmid (*Vector*). Western blot analysis of EPAS1 and actin was performed on whole cell extracts (10 μg for EPAS1 and 5 μg for actin). *B*, cell clones were treated with differentiation medium containing either troglitazone (10 μM) or ETYA (50 μM) for 10 days. Cells were fixed and stained with Oil Red O. *C*, microscopic view of dishes shown in *B* at $\times$100 original magnification. *D*, the cell clones were induced to differentiate with differentiation medium containing ETYA. Total RNA was extracted at the indicated time points, and the expression of adipocyte-related genes was determined by Northern blot analysis. The results shown in *A*–*D* are representative of three to five independent clones with similar results.

on EPAS1 activity, but not HIF-1α activity. We then isolated stable clones from 3T3-L1 cells in which we could induce the expression of EPAS1-(1–485) with insect hormone (Pon.A) (Fig. 2*B*). This system allows us to directly examine the roles of EPAS1 without relying on pharmacological agents or hypoxia conditions that might regulate other signaling pathways and transcriptional activities. Treatment of the control 3T3-L1 cells with Pon.A had no effect on the differentiation states, as judged by Oil Red O staining (Fig. 2*C*). Conversely, the induction of EPAS1-(1–485) allowed cells to accumulate only minimum amounts of intracellular lipid droplets (Fig. 2, *C* and *D*). These results were confirmed by measuring the expression of adipocyte-related genes, such as PPARγ2 and the C/EBP family. As expected, the expression of these genes was strongly induced in the control cells during differentiation (Fig. 2*D*). In contrast, consistent with the morphological observations, minimum amounts of PPARγ2 and C/EBP family were induced in the EPAS1-(1–485)-expressing cells. Consequently, these results indicated that EPAS1 plays a role for execution of the adipose differentiation program in 3T3-L1 cells.

The Combination of the PPARγ Activator and a Conventional Differentiation Mixture Restores the Ability of EPAS1-(1–485)-expressing Cells to Differentiate—Activation of PPARγ with ligands such as thiazolidinediones can promote adipose differentiation. Therefore, we chose to examine whether or not the activation of PPARγ would be able to restore the differentiation potential of EPAS1-(1–485)-expressing cells. Consistent with the results shown in Fig. 2, the induction of EPAS1-(1–485) with PonA resulted in a lower differentiation morphology (Fig. 3, *middle row*). However, treatment with troglitazone allowed the EPAS1-(1–485)-expressing cells to accumulate lipid droplets to the same extent as that of the control cells in the presence of PonA (Fig. 3, *bottom row*). Similarly, co-treatment with the conventional differentiation mixture containing ciglitazone or indomethacin restored the ability of the cells to differentiate (data not shown).

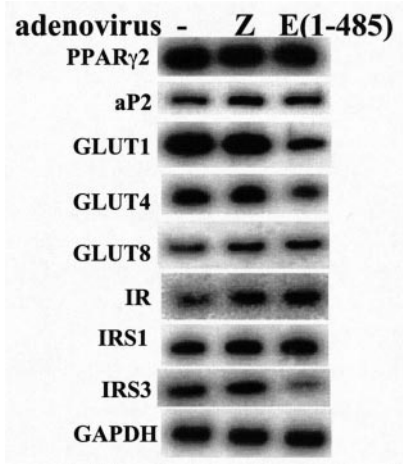

FIG. 5. **Dominant negative effects of EPAS1-(1–485) on the expression of insulin signaling- and glucose transport-related genes in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were infected with the adenovirus carrying EPAS1-(1–485) (*E(1–485)*) or LacZ (*Z*) at a multiplicity of infection of ~50 for 24 h. Total RNA was extracted, and the expression of glucose transport-related genes was determined by Northern blot analysis. The results shown are representative of three to five independent virus preparations with similar results.

Ectopic Expression of EPAS1 in NIH 3T3 Cells Stimulates Adipose Differentiation—The results described above demonstrated that EPAS1 plays a role in adipogenesis in 3T3-L1 cells. Then, we wished to examine whether EPAS1 has the ability to promote adipogenesis in NIH 3T3 cells, a relatively nonadipogenic cell line. NIH 3T3 cells were stably transfected with either a control vector or a vector expressing high levels of full-length EPAS1 mRNA. After the selection and expansion of

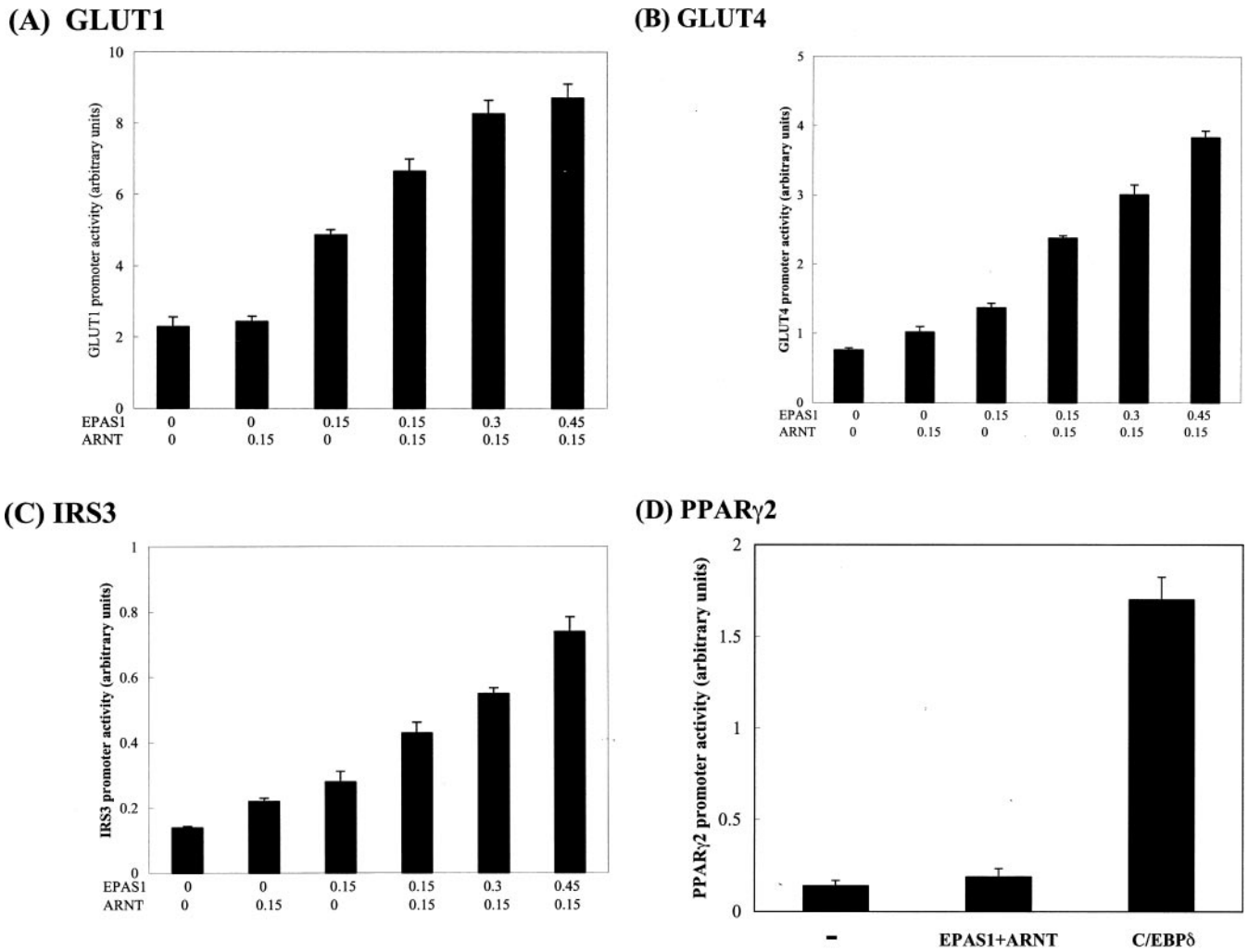

FIG. 6. **EPAS1 activates the promoter activity of GLUT1 (*A*), GLUT4 (*B*), and IRS3 (*C*), but not that of PPARγ2 (*D*).** Full-length EPAS1 and ARNT expression plasmids were transfected into HEK 293 cells with the reporter plasmid (0.15 μg) carrying the promoter region of the indicated genes. In *D*, the PPARγ2 promoter-driven luciferase plasmid (0.15 μg) was transfected with a pair of full-length EPAS1 (0.15 μg) and ARNT expression plasmids (0.15 μg) or C/EBPδ expression plasmid (0.15 μg) as a positive control. For all constructs, pRL-SV40 vector (Promega) was co-transfected to correct for differences in transfection efficiency, and the normalized luciferase activity was represented as arbitrary units. The averages of three independent experiments are shown.

stable clones, the level of the EPAS1 protein was examined by Western blot analysis. As shown in Fig. 4*A*, the cells transfected with the vector expressing EPAS1 mRNA produced more EPAS1 protein than did the control vector-transfected cells. To evaluate the differentiation potency of the clones, the cells were cultured to confluence and then treated with a standard differentiation medium containing PPARγ2 ligand (troglitazone or 5,8,11,14-eicosatetraynoic acid). The extent of differentiation was estimated by adipose staining with Oil Red O. Neither the EPAS1-expressing cell clones nor the control cells showed signs of lipid accumulation when cultured in the absence of differentiation-inducing agents (data not shown). Treatment with adipose differentiation medium containing PPARγ2 ligands enabled the wild-type NIH 3T3 cells and the cells expressing vector mRNA to exhibit only a minimum degree of lipid accumulation (Fig. 4, *B* and *C*). However, the cells overexpressing EPAS1 showed more lipid accumulation than was observed in the control cells 10 days after induction of differentiation (Fig. 4, *B* and *C*). The expression of adipocyte-related genes, such as PPARγ2 and aP2, was greatly induced in the EPAS1-expressing cells, whereas these genes were poorly expressed in the control cells (Fig. 4*D*).

EPAS1 Regulates Glucose Uptake in Adipocytes—In addition to PPARγ2, insulin plays pivotal roles in adipose differentiation. Previous studies have shown that insulin-like growth factor activates EPAS1 in osteoblast-like cells (28). Thus, to understand the molecular basis by which EPAS1 promotes adipose differentiation, we next examined the effects of EPAS1-(1–485) on the expression of genes for insulin signaling and glucose transport. As described in Fig. 2, the induction of EPAS1-(1–485) in preadipocytes resulted in failure of differentiation, and therefore it is not clear whether alterations of gene expression in EPAS1-(1–485)-expressing cells during adipose differentiation reflect direct effects of EPAS1-(1–485) or are an indirect result of the lower level of differentiation of EPAS1-(1–485) cells. Thus, in these experiments, 3T3-L1 cells were induced to differentiate, and mature adipocytes were infected with adenovirus expressing either LacZ or EPAS1-(1–485). As shown in Fig. 5 (−), infection with adenovirus carrying LacZ had no effect on expression of the genes examined. Overexpression of EPAS1-(1–485) in 3T3-L1 adipocytes dramatically suppressed the expression of GLUT1, GLUT4, and IRS3, whereas the expression of other genes such as PPARγ2, aP2, insulin receptor (*IR*), IRS1, and GLUT8 was not significantly altered (Fig. 5). We then examined the ability of EPAS1 to directly regulate the transcriptional activity of these genes. The luciferase reporter gene driven by the promoter/enhancer of GLUT1, GLUT4, IRS3, and PPARγ2 was constructed and

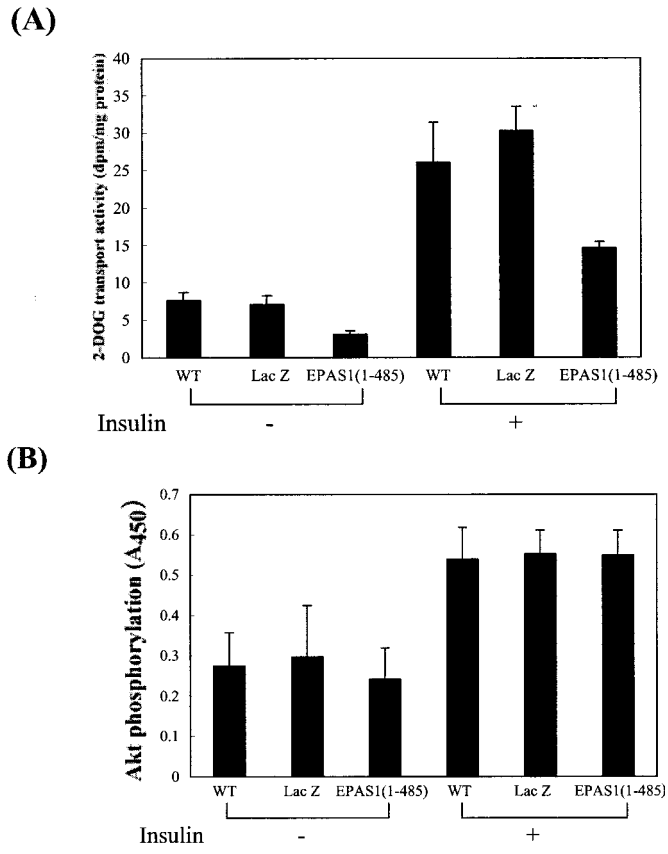

FIG. 7. **Dominant negative effects of EPAS1-(1–485) on glucose transport in 3T3-L1 adipocytes.** 3T3-L1 adipocytes were infected with adenovirus carrying EPAS1-(1–485) or LacZ at a multiplicity of infection of ~50 for 24 h. The glucose transport activity (*A*) and Akt phosphorylation status (*B*) of the cells were determined as described under "Experimental Procedures." The results shown are averages of three independent experiments.

transfected into 293 cells with different amount of EPAS1 expression vector. As shown in Fig. 6*A*–*C*, EPAS1 enhanced the promoter/enhancer activity of GLUT1, GLUT4, and IRS3 genes in a dose-dependent manner. Also, consistent with the mRNA expression results (Fig. 5), EPAS1/ARNT was found to have no effect on PPARγ2 promoter activity (Fig. 6*D*). To evaluate whether these changes in gene expression had an effect on the biological activity of adipocytes, glucose transport activity in EPAS1-(1–485)-expressing adipocytes was determined. In the absence of insulin, the amount of glucose transported into EPAS1-(1–485)-expressing cells was <50% of that in the control cells (Fig. 7*A*). Although the addition of insulin increased the amount of glucose transported into the cells, the glucose transport activity of the EPAS1-(1–485)-expressing cells was still significantly lower than that of the control cells (Fig. 7*A*). In contrast to glucose transport activity, the degree of Akt phosphorylation was the same in EPAS1-(1–485)-expressing cells and control cells (Fig. 7*B*).

DISCUSSION

EPAS1 was originally identified as a transcription factor response to hypoxia conditions (21–23). Although EPAS1 and HIF-1α share high amino acid homology, they may have distinct functions because of differences in tissue distribution and in the developmental expression profiles of these proteins (21–23, 35, 36). A recent study has revealed that the target genes of EPAS1 are, at least in part, distinct from those of HIF-1α (37). More interestingly, oxygen-dependent protein degradation is restricted to HIF-1α (33). In many cell lines such as mouse embryo fibroblasts, EPAS1 escapes oxygen-dependent protein degradation and is no longer a hypoxia-inducible factor (34). Together with the results from knockout mice studies, these results strongly suggest that EPAS1 plays a critical role in embryonic development and homeostasis under normoxia conditions (25–27). In this study, we demonstrated that EPAS1 is highly induced during adipose differentiation *in vivo* and *in vitro* (Fig. 1). Also, the C/EBP family can activate EPAS1 promoter activity *in vitro* (Fig. 1*D*). Although little has been known about the role of this factor in adipose differentiation, the present observations suggest that EPAS1 may play a role in adipogenesis. The expression of a dominant negative form of EPAS1 suppressed the morphological differentiation of 3T3-L1 cells, as well as induction of adipocyte-related genes (Fig. 2). Conversely, overexpression of full-length EPAS1 in nonadipogenic NIH 3T3 cells facilitated lipid droplet accumulation in the cells (Fig. 4), although the presence of the PPARγ ligand is required for adipose differentiation of EPAS1-expressing NIH 3T3 cells (data not shown). The morphological differentiation in EPAS1-expressing NIH 3T3 cells was accompanied by the induction of adipogenic markers such as PPARγ2 and aP2 (Fig. 4). Finally, DEXA scan analysis revealed that adipose tissues in EPAS1 knockout mice are smaller than those in control mice.[2] Consequently, EPAS1 appears to play a role in promotion of adipose differentiation.

In this study, the results obtained using the luciferase reporter assay indicate that the EPAS1 promoter is differentially regulated by different C/EBPs (Fig. 1*F*). A number of other studies show differential action of C/EBPα and C/EBPβ in activating various promoters including PPARγ2 (38–42). Although precise analysis on the EPAS1 promoter has not yet been performed, computer analysis revealed that the putative binding sites of C/EBPs on the EPAS1 promoter region are not identical to those found in PPARγ2 (data not shown). These results, taken together, support idea that the action of C/EBPs involves context-specific effects and depends on promoter composition as reported previously (43).

Adipose differentiation is a complex process accompanied by the alteration of expression of several hundred genes (1). The coordination of this complex process is driven mainly by PPARγ2 (4). Although the induction of a dominant negative form of EPAS1 in 3T3-L1 preadipocytes reduced the expression level of PPARγ2 (Fig. 2*D*), this may be an indirect result of the lower level of differentiation of EPAS1-(1–485) cells because of the following reasons. First, in mature adipocytes, dominant negative EPAS1 had no effect on PPARγ2 expression (Fig. 5). Second, the early expression level of PPARγ2 in EPAS1-expressing NIH 3T3 cells was not substantially different from that in the control cells (Fig. 4*D*). Moreover, EPAS1 had no effect on PPARγ2 promoter activity (Fig. 6*D*). Therefore, it is unlikely that PPARγ2 is a direct target of EPAS1.

We demonstrated in this study that EPAS1 regulates both basal and insulin-dependent glucose transport into cells (Fig. 7*A*). EPAS1-(1–485) had no effects on phosphorylation of Akt (Fig. 7*B*), suggesting that EPAS1 is not likely to be involved in regulation of the Akt signaling pathway. Therefore, the mechanism by which EPAS1 regulates glucose uptake may be the direct transcriptional regulation of several factors including GLUT1 and GLUT4 (Figs. 5 and 6), although we cannot exclude the possibility that EPAS1-(1–485) suppressed the translocation activity of the glucose transporter. Several lines of evidence have suggested that these factors play pivotal roles in the promotion of adipose differentiation. A previous study using transgenic mice revealed that GLUT4 regulates the num-

[2] J. A. Garcia, personal communication.

ber of fat cells as well as insulin sensitivity (44). GLUT4-null mice exhibit decreased longevity associated with cardiac hypertrophy and severely reduced adipose tissue deposits (45). GLUT1 expression and GLUT4 translocation can be regulated by PPAR (46), suggesting that restoration of the ability of EPAS1-(1–485) cells to differentiate (Fig. 3) by troglitazone is likely due to the recovery of these factors. If this is indeed the case, the present results support the notion that the reduced expression of GLUT1 and GLUT4 is partly responsible for the reduced differentiation potency of EPAS1-(1–485) cells. The promotion of glucose flux and subsequent glycolysis by these factors are known to contribute to an increase in lipid synthesis in adipocytes (47, 48). In addition to GLUT1 and GLUT4, IRS3 could be the target gene of EPAS1 (Figs. 5 and 6). White adipose tissue of $Irs1^{-/-}/Irs3^{-/-}$ double knockout mice was reduced by ~80% as compared with $Irs1^{-/-}$ mice, suggesting that IRS3 may be at least partly involved in the formation of white adipose tissue (49). Taken together, the results suggest that the transcriptional regulation of these factors, followed by enhanced glucose uptake, may account in part for a putative mechanism by which EPAS1 promotes adipose differentiation.

Scortegagna *et al.* (27) reported an alteration of serum acylcarnitin profiles in EPAS1-null mice. The markedly high C16:C2 ratio and the spectrum of intermediate acyl-fatty acids species in EPAS1-null mice suggest that EPAS1 participates in the regulation of lipid metabolism (27). Consequently, we are led to conclude that EPAS1 plays several supporting roles in maintaining specific aspects of adipogenesis and adipocyte function, such as regulation of glucose uptake followed by lipid synthesis, despite not being directly responsible for the induction of adipogenesis *per se*.

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