Inhibition of Gluconeogenesis through Transcriptional Activation of EGR1 and DUSP4 by AMP-activated Kinase*

Stephen B. Berasi, Christine Huard, Dongmei Li, Heather H. Shih, Ying Sun, Wenyan Zhong, Janet E. Paulsen, Eugene L. Brown, Ruth E. Gimeno, and Robert V. Martinez

From the Departments of Biological Technologies and Cardiovascular and Metabolic Diseases, Wyeth Research, Cambridge, Massachusetts 02140

Increased hepatic gluconeogenesis is an important contributor to the fasting hyperglycemia found in Type 2 diabetic patients. Low energy states activate the intracellular energy sensor AMP-activated kinase (AMPK). AMPK activation by the AMP mimetic AICAR (5-aminoimidazole-4-carboxamide riboside) has been shown to inhibit hepatic gluconeogenesis. We used transcriptional profiling to search for AICAR-regulated genes in hepatocyte cell lines. We report that a dual specificity phosphatase, Dusp4, is induced by AMPK in AML12, H4IIE, and Fao cells at both mRNA and protein levels. AMPK also induces the immediate early transcription factor Egr1 (early growth response 1), a known transcriptional activator of Dusp4, and it directly binds the Dusp4 promoter at its known binding site.

Both reporter gene assays and real time PCR demonstrate that exogenous DUSP4 inhibits the promoter activity and expression of both glucose-6-phosphatase (Glc-6-P) and phosphoenolpyruvate carboxykinase (PEPCK) to an extent similar to both AICAR and constitutively active AMPK. Conversely, depletion of EGR1 or DUSP4 using siRNA not only partially abrogates the inhibition of Pepck expression by AICAR, but also importantly affects glucose production by Fao cells. In Fao cells, small interfering RNA targeted EGR1 also depletes DUSP4 expression following treatment with AICAR, further supporting a direct link between EGR1 and DUSP4 activation. Expression of a constitutively active form of p38, a known effector of cAMP-mediated gluconeogenesis, rescues the DUSP4-mediated repression of PEPCK. These results suggest that the inhibition of hepatic gluconeogenesis by AMPK may, in part, be mediated by an immediate early gene response involving EGR1 and its target, DUSP4.

Understanding the pathways that maintain proper glucose homeostasis is a central focus of discovery efforts for treatments of Type 2 diabetes that now affects over 20 million Americans, with levels increasing at 6% a year (1, 2). Proper glucose homeostasis requires a balance between glucose uptake by skeletal muscle and adipose tissue, and production by the liver. Type 2 diabetic patients lose this balance due to a reduction of glucose uptake during the fed state, as well as improper fasting gluconeogenesis by the liver. During the fasted state, glucagon secretion by the pancreas and resulting cAMP-mediated signaling through CREB, as well as glucocorticoid release, results in gluconeogenic gene transcription. In the fed state this transcriptional program is suppressed by insulin signaling due to repression of the nuclear hormone receptor co-activator peroxisome proliferator-activated receptor-γ coactivator-1α, which is necessary for CREB-mediated transcription. Phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (Glc-6-P) are two rate-limiting enzymes for gluconeogenesis that are transcriptionally regulated by glucagon and insulin, and are widely used as markers for gluconeogenesis (3).

Due to the important role of dysregulated gluconeogenesis in the pathology of Type 2 diabetes, further insight into the mechanisms of repression of these genes by insulin-independent mechanisms could lead to treatments of insulin-resistant individuals (4). Activation of AMPK (AMP-activated kinase) is one insulin independent means of gluconeogenesis repression. AMPK has been termed a “master switch” of cellular energy status, being highly conserved from simple eukaryotes to humans. In mammalian systems it is activated in multiple organs during conditions that cause a low ATP/AMP ratio. These include exercise and starvation on the whole body level as well as many cellular stresses such as glucose deprivation, oxidative stress, ischemia, and exposure to metabolic poisons that inhibit ATP synthesis (5, 6). When activated, AMPK switches on ATP generating processes and switches off those that consume ATP. Both in vivo and in vitro studies support that AMPK activation inhibits gluconeogenesis (7, 8). Treatment of rat hepatoma cells or primary hepatocytes with the AMPK activator AICAR inhibits transcription of the Glc-6-P and Pepck genes (8). In vivo, activation of AMPK in the livers of fasted mice has been shown to reduce glucose production (9) and gluconeogenic gene expression (10). Additionally, AMPK has been suggested to mediate the beneficial and detrimental effects of adiponectin and resistin, respectively, on hepatic glucose output (11, 12). Recently this has been supported by the finding that genetic deletion of the AMPK α2 isoform in the

2 The abbreviations used are: CREB, cAMP response element-binding protein; AMPK, AMP-activated kinase; DUSP, dual specificity phosphatase; EGR1, early growth response 1; AICAR, 5-aminoimidazole-4-carboxamide riboside; PEPCK, phosphoenolpyruvate carboxykinase; Glc-6-P, glucose-6-phosphatase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; HNF, hepatic nuclear factor; FBS, fetal bovine serum; ACC, acetyl-CoA carboxylase; CA-AMPK, constitutively active AMP-activated kinase; HA, influenza hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; GFP, green fluorescent protein; bis-tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; siRNA, small interfering RNA.
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AMPK achieves its downstream effects by immediate direct phosphorylation of enzyme substrates as well as long-term effects on gene expression. For example, AMPK phosphorylates and inactivates acetyl-CoA carboxylase, resulting in a suppression of the conversion of acetyl-CoA to malonyl-CoA.

The lower levels of malonyl-CoA allows entry of fatty acids into the mitochondria and their subsequent oxidation (15, 16). Other direct targets that can be phosphorylated by AMPK include glycogen synthase, IRS-1, and hydroxymethylglutaryl-CoA reductase (17–19). The effects of AMPK on gene expression, and the role of the target genes in mediating the physiological effects of AMPK activation are much less well understood, although it has been shown that AMPK activation decreases HNF-4 expression levels leading to repression of its logical effects of AMPK activation.

We identified the immediate early response transcription factor early growth response 1 (Egr1) and the dual specificity phosphatase 4 (Dusp4) as transcriptional targets of AMPK that are necessary for its ability to fully repress glucose production. Additionally, our data suggests a link between the p38 MAP kinase and DUSP4 in regulating gluconeogenesis.

EXPERIMENTAL PROCEDURES

Gene Expression Profiling—Double-stranded cDNA was synthesized starting with 5 μg of total RNA using the SuperScript System (Invitrogen). The cDNA was purified by filtration through Multiscreen filter plate (Millipore), and transcribed in vitro using T7 RNA polymerase (Epicenter, Madison, WI) and biotinylated nucleotides (PerkinElmer Life Sciences). Hybridization buffer containing the spike pool reagent was added to each of the fragmented cRNA mixtures and each sample was hybridized to the Mouse Genome 430 2.0 array or Rat Genome 230 2.0 array (Affymetrix, Santa Clara, CA) at 45 °C for 18 h as recommended by the manufacturer. The hybridized chips were scanned using an Affymetrix Genechip Scanner 3000 (Affymetrix, Santa Clara, CA), and then streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR), followed by biotinylated antibody against streptavidin (Vector Laboratories, Burlingame, CA), and then streptavidin-phycoerythrin. The chips were scanned using an Affymetrix Genechip Scanner 3000 and .cel files were generated with Affymetrix Microarray Suite 5.0 (MAS 5.0) software.

Cell Culture—The rat hepatoma cell lines H4IE and Fao were cultured in DMEM containing 5% fetal calf serum and RPMI1640 with 10% fetal calf serum, respectively. AML12 cells were maintained in DMEM/F-12 medium containing 10% fetal calf serum, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 40 ng/ml dexamethasone.

Chromatin Immunoprecipitation—To cross-link protein and DNA, formaldehyde was added at a 1% (v/v) concentration to H4IE cells in a 100-mm dish. Fixation proceeded for 10 min at room temperature with gentle shaking and was stopped with the addition of glycine to a final concentration of 0.125 M, and then the mixture was incubated at room temperature for 5 min. The cells were then washed twice with cold phosphate-buffered saline, harvested, and washed once with phosphate-buffered saline containing phenylmethylsulfonyl fluoride. The cell pellet was lysed with immunoprecipitation assay lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 5 mM EDTA, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 10 μg of leupeptin/ml)) and incubated on ice for 10 min. The resulting lysate was sonicated to give chromatin of an average length of 600 to 1,000 bp. The lysate was then microcentrifuged at 14,000 × g for 10 min at 4 °C. The lysate was then preclarified with 50 μl of protein A beads (IPA 400; Repligen) for 15 min. Immunoprecipitations were performed overnight with 2 μg of antibody in a total volume of 500 μl at 4 °C. 50 μl of protein A beads were then added to the lysates and incubated for 30 min at 4 °C. The beads were then washed twice with immunoprecipitation dilution buffer (2 mM EDTA, 50 mM Tris-Cl (pH 8.0), and 4 times with immunoprecipitation wash buffer (100 mM Tris-Cl (pH 8.0), 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid). The immunocomplexes were eluted twice in 200 μl of elution buffer (50 mM NaHCO3, 1% SDS) for 15 min. A 1-μl aliquot of high concentration RNase was added, and the NaCl concentration was adjusted to 0.3 M. Samples were then incubated at 67 °C for 5 h to reverse cross-links. Two volumes of ethanol were added for overnight precipitation at −20 °C. The resulting pellets were collected, air dried, and resuspended in 100 μl of TE. Twenty-five microliters of 5× PK buffer (50 mM Tris-Cl (pH 7.5), 25 mM EDTA, 1.25% SDS) was added with 1 μl of proteinase K. The samples were digested at 45 °C for 2 h. 175 μl of TE was then added. The DNA was purified by standard phenol-chloroform and chloroform extractions and precipitated with 0.3 M NaCl, 5 μg of glycogen carrier, and 2 volumes of ethanol for overnight precipitation at −20 °C. The purified DNA was resuspended in 20 μl of water. A 5-μl aliquot was analyzed by PCR with Vent polymerase (New England Biolabs). For the rat Dusp4 promoter, the sequence to be amplified resides at positions −72 to −264 for which the PCR primer sequences were: 5′-GGCGGCTCATTGGAGCGCAG-3′ and 5′-CCGACTCTTGAATGGAGCGCCG-3′ and positions −1310 to −1528 for which the primers were: 5′-AAGAAACG-CAGTGGCATTGGGACC-3′ and 5′-CGCTTAAACCTCT-TCTCACATGTT-3′.

Western Blots—Lysates were prepared with a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium vanadate, and 1 μg of leupeptin/ml. 20 μl of lysate for each sample was run on NuPAGE 10% polyacrylamide bis-tris gels (Invitrogen) and transferred to polyvinylidene difluoride membrane. Following blocking with 5% milk in Tris-buffered saline, anti-DUSP4 (SC-1200, Santa Cruz) was blotted in Tris-buffered saline with 0.1% Tween at 1:300, anti-EGR1 (SC-110, Santa Cruz) at 1:1000, p38 and phos-
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Phospho-p38 (Cell Signaling Technology), acetyl-CoA carboxylase (ACC) (Cell Signaling Technology), phospho-ACC (Cell Signaling Technology), and anti-α-tubulin (Abcam) at 1:2000. Goat anti-mouse and rabbit horseradish peroxidase (Jackson ImmunoResearch) were used at 1:5000 and blots were visualized by using enhanced chemiluminescence (PerkinElmer Life Sciences).

**TaqMan Real Time Quantitative PCR**—RNA was isolated and purified from H4IIE cells using RNeasy kit (Qiagen) according to the manufacturer’s instructions. Dusp4, Egr1, Pepck, and Gls-6-P mRNA levels were measured by TaqMan real time quantitative PCR using Assay-on-Demand TaqMan reagents (Applied Biosystems). TaqMan was performed in an ABI PRISM 7000 Sequence Detection System (PE Applied Biosystems). Data were analyzed according to the manufacturer’s instructions.

**Generation of Adenoviruses**—cDNA expressing human CA-AMPK (a2312f(172f) (21)), GFP, and human DUSP4 were subcloned into pAdori1–3 (22). Each pAdori1–3 cDNA-expressing plasmid was digested with Ascl and BstZ17I and the fragment containing cDNA inserts and adenoviral genomic sequence was isolated. Recombinant adenoviral genomic DNA was linearized with PacI. The pAdori1–3 DNA fragment was co-transfected with adenoviral genomic DNA into early passage 293 cells using Lipofectamine 2000 (Invitrogen). Ten to 14 days post-transfection, the recombinant adenovirus was harvested and subjected to another two rounds of amplification in 293 cells. The resulting virus was used to infect 293 cells at a large scale followed by purification by CsCl gradient ultracentrifugation (Viraquest, North Liberty, IA).

**Reporter Assays**—The rat Pepck promoter (−548 to +73) was amplified from rat genomic DNA using primers 5’-GCTCTGAGCTCGAGTTCCACGAGCCCTGTTG-3’ and 5’-GCAAGCTTAAGCTTCCTCGGGATTTCCCTGTTTGC-3’ (23). The human Glc-6-P promoter (−1227 to +57) was amplified from human genomic DNA using primers: 5’-GCTCTGAGCTCGAGTTCCACGAGCCCTGTTG-3’ and 5’-GCAAGCTTAAGCTTCCTCGGGATTTCCCTGTTTGC-3’. The promoters were subsequently cloned into Xhol and HindIII sites of pSEAP-BASIC vector (Clontech).

pSEAP2-control, for which SEAP expression is regulated by the SV40 early promoter, was used as a negative control. H4IIE cells were seeded in 24-well plates and transfected at 90% confluence using Tfx-50 Reagent (Promega). 0.9 μg of each SEAP reporter and 0.2 μg of pCMV-β-galactosidase (BD Biosciences) were included. 0.4 μg of pCMV-XL5-human-DUSP4 (NM_001394, Origene), pCMV-Sport6-human EGR1 (BC073983, Open Biosystems), pMEV2HA-P38α-WT and P38α-EE (Biomyx), or empty pCMV-Sport6 were included. SiGenome SMARTpool reagents were included at 25 nM. 24 h post-transfection cells were starved for 5 h in DMEM with 0.5% FBS followed by treatment with 200 μl of DMEM with 0.1% FBS with 100 μM cAMP (Calbiochem) and dexamethasone (Sigma) and/or 500 μM AICAR for 3 h. 60 μl of the media was utilized for the SEAP assay (Great EscAPE SEAP Kit, BD Biosciences) and cell lysate was used for the β-galactosidase assay (Luminescent β-galactosidase detection kit, BD Biosciences) and cell lysate was used for the β-galactosidase activity as a transfection control.

**Glucose Output**—1 million Fao cells were transfected with siGenome SMARTpool reagents for EGR1 (number M-100247-00), DUSP4 (number M-098966-00), or siCONTROL Non-targeting siRNA (Dharmacon) using OPTI-fect (Invitrogen) at 25 nM final concentration. 24 h post-transfection cells were washed once with RPMI1640 and incubated in RPMI1640 containing 0.5% bovine serum albumin, 1 μM dexamethasone (Sigma catalog D1881), and 1 mM cAMP (Calbiochem catalog 28745) for 16 h with and without 500 μM AICAR. Cells were then incubated in 0.35 ml (per well) of phenol red-free, glucose-free DMEM containing 2 mM pyruvate and 20 mM lactate containing dexamethasone and cAMP with or without AICAR as indicated in the figure legends. Media were collected 5 h later and subjected to glucose measurement using the Amplex® Red Glucose/Glucose Oxidase Assay Kit (Molecular Probes). Cells were lysed and protein concentration was determined. The glucose output rate was normalized by cellular protein concentr-
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Expression and expressed as micrograms of glucose/mg of protein/5 h. Untreated output of samples was set to 1 and the data were presented as glucose output relative to the untreated samples.

**RESULTS**

Identification of Transcripts Significantly Regulated by AICAR in Hepatocytes—To identify transcriptional targets of activated AMPK in hepatocytes, with a goal of further understanding its role in negatively regulating gluconeogenesis, the mouse hepatoma cell line AML12 was treated with 500 μM AICAR for 2, 6, or 24 h in triplicate. Processed RNA was hybridized on the Affymetrix MOE430 2.0 array, as described under “Experimental Procedures.” Using normalized data, after removing lowly expressed genes, we performed an analysis of variance. Qualifiers that were induced or repressed in a statistically significant (p ≤ 0.05) way at all three time points between AICAR and vehicle treated samples were identified. The expression patterns of these 43 qualifiers are represented in Fig. 1. Among the qualifiers that increased with greatest significance upon AICAR treatment were Dusp4 and the zinc finger transcription factor Egr1. The normalized frequency of each of these qualifiers at each time point with and without AICAR treatment is also shown in Fig. 1. Dusp4 message increased 3-, 6-, and 5-fold over the time course relative to untreated controls with the highest individual expression occurring at 6 h of treatment. Egr1 increased 3-fold at 2 and 6 h of treatment relative to control and 2-fold at 24 h.

AICAR treatment was also performed over the time course period on the H4IIE rat hepatoma line. Total RNA was isolated from these cells and hybridized on the RAE430 2.0 array and analyzed in the same manner as the AML12 cells. The number of qualifiers changing was much more robust with treatment of these cells. Using similar filtering conditions as described above, the expression of 54 qualifiers were induced or repressed in a statistically significant (p ≤ 1 × 10−4 versus p ≤ 0.05) manner at every time point when comparing AICAR versus vehicle treated samples. The normalized frequency of Egr1 over the time course of this experiment is shown in Fig. 2A. Expression increased 32-, 7.5-, and 3-fold relative to time matched controls at 2, 6, and 24 h of treatment. To further investigate the time course of EGR1 induction with AICAR treatment an anti-EGR1 Western blot was performed at time points ranging from 10 min of treatment up to 24 h. As shown in Fig. 2B, EGR1 expression increases as early as 20 min of treatment, increasing up to 6 h, and slightly decreasing by 24 h. DUSP4 expression also increased with AICAR treatment in H4IIE cells. Fig. 2C shows the results of a TaqMan real time PCR analysis of Dusp4 mRNA levels following 2, 6, and 24 h of treatment of H4IIE cells. TaqMan was utilized because the Dusp4 probe set on the RAE430 2.0 array was not as sensitive as the Dusp4 probe set on the MOE430 2.0 array. DUSP4 message was increased 20-fold at 2 h of treatment, and 4-fold at both 6 and 24 h of treatment. Western blot analysis (Fig. 2D) showed an increase of DUSP4 protein at 2 and 6 h and at 12-h levels were similar to no treatment. The 500 μM AICAR treatment did not activate AMPK as ACC was phosphorylated at 2, 6, and 24 h of treatment (Fig. 2E).

To confirm that AMPK activation resulted in Dusp4 and Egr1 transcription an adenovirus expressing constitutively active AMPKα2 (CA-AMPK), which is truncated to 312 residues and contains an activating mutation (T172D), was created (21). Fig. 3A shows that 24 and 48 h of infection of H4IIE cells with CA-AMPK adenovirus at multiplicity of infection of 100, resulted in a 10- and 8-fold increase, respectively, in Egr1 mRNA, as determined by TaqMan real time PCR with the Assay on Demand primer set (Applied Biosystems). E, Western blot of DUSP4 following AICAR treatment for the indicated times. The anti-tubulin blot is a loading control. E, Western blot of total and phosphorylated ACC at the indicated times of AICAR treatment.
results of Figs. 2 and 3 indicate that activation of AMPK either through 500 μM AICAR treatment or expression of a constitutively active mutant both increase the expression of EGR1 and DUSP4.

Dusp4 was previously identified as a transcriptional target of the immediate early transcription factor EGR1 in response to gonadotropin releasing hormone in pituitary cells (24). Upon treatment with gonadotropin-releasing hormone, EGR1 bound and activated the Dusp4 promoter. To test whether EGR1 bound the Dusp4 promoter in AICAR-treated H4IIE cells, chromatin immunoprecipitation experiments were performed. Fig. 4A shows a schematic of the DUSP4 promoter including the reported EGR1 site and putative binding sites of other transcription factors. The EGR1 site identified as a response element during gonadotropin-releasing hormone signaling is located 119 base pairs upstream of the transcriptional start site. Primers were designed to amplify this site and PCR was performed on DNA following immunoprecipitation of EGR1 protein-DNA complexes after AICAR treatment for either 2 or 6 h. As shown in Fig. 4B, only upon AICAR treatment and immunoprecipitation with anti-EGR1 antibodies (not anti-VP16 as a nonspecific antibody) was the EGR1 site amplified, as identified by the presence of a 189-bp PCR product. The results shown in Fig. 4C show that the region of the Dusp4 promoter containing the site identified in Fig. 4B is specific as an upstream sequence of the promoter, including −1528 to −1310 that does not contain an EGR1 site, was not immunoprecipitated by anti-EGR1 antibodies following AICAR treatment. These results show that both Egr1 and Dusp4 are induced within 2 h of AICAR treatment (Egr1 within 20 min), and that EGR1 specifically occupies an element of the Dusp4 promoter previously shown to be necessary for its activation by gonadotropin-releasing hormone.

EGR1 and DUSP4 Inhibit Gluconeogenic Gene Transcription—Activation of AMPK via AICAR inhibits hepatic gluconeogenesis through inhibition of the transcription of the rate-limiting enzymes Pepck and Glc-6-P (8). To determine whether EGR1 and DUSP4 are effectors of AICAR-mediated inhibition of Pepck and Glc-6-P, reporter assays were utilized. The rat PEPCK promoter (−548 to +73) was cloned into a secreted alkaline phosphatase reporter (pSEAP-Basic, Promega) and the effects of altering DUSP4 or EGR1 expression levels on this reporter were determined in transient transfection assays. As has been previously reported, we found that treatment of H4IIE cells with 500 μM AICAR inhibits the cAMP and dexametha-

FIGURE 3. Constitutively active AMPK activates EGR1 and DUSP4 transcription. Relative expression of EGR1 mRNA (A) and DUSP4 (B) compared with uninfected cells, as determined by TaqMan real time PCR with Assay on Demand primer set (Applied Biosystems) following infection of H4IIE hepatoma cells with the specified adenoviral vectors for the indicated times. C, Western blot showing expression of CA-AMPK and ACC phosphorylation after infection with the specified virus for the indicated time.

FIGURE 4. EGR1 binds to the rat DUSP4 promoter following treatment with AICAR. A, schematic of the rat DUSP4 promoter including putative binding sites for additional transcription factors is shown (EGR1, HNF1, NFκB, and CCAAT displacement protein). Arrows indicate sites of primers used for chromatin immunoprecipitation assay (−72 to −261 bp (specific for EGR1) and −1310 to −1528 bp (nonspecific control) from the transcriptional start site). B and C, ethidium bromide-stained agarose gel showing results of PCR with the indicated primers in the DUSP4 promoter primers following chromatin immunoprecipitation with either anti-EGR1 or anti-VP16 as a negative control after H4IIE treatment with AICAR for the indicated time.
FIGURE 5. DUSP4 expression levels modulate PEPCK promoter activity and expression of both PEPCK and GLC-6-P mRNA. A, H4IIE hepatoma cells were transfected with the PEPCK-SEAP reporter plasmid and either a DUSP4 expression plasmid or a DUSP4 siRNA smart pool. 24 h post-transfection the media was changed to 0.01% FBS/DMEM for 5 h. The media was then changed to 0.1% FBS/DMEM with cAMP and dexamethasone (Dex) with or without 500 μM AICAR for 16 h and SEAP activity in the media was determined. B, relative expression of PEPCK mRNA and GLC-6-P (C) as determined by TaqMan real time PCR with the Assay on Demand primer set (Applied Biosystems) following infection of H4IIE hepatoma cells with the specified adenoviral vectors and the indicated treatments (same treatment conditions as in A). D, Western blot showing expression of HA-DUSP4, CA-AMPK, and ACC phosphorylation after infection with the indicated adenovirus and treatment. E, H4IIE cells were transfected as in A while replacing the PEPCK-SEAP with pSEAP2-control regulated by the SV40 early promoter.
sone-induced activation of the Pepck promoter (Fig. 5A). Interestingly, overexpression of DUSP4 in H4IIE cells inhibited the cAMP and dexamethasone activation of the Pepck promoter almost as potently as AICAR (from ~36-fold inhibition with AICAR to 6-fold with DUSP4 ($p = 0.01$)), suggesting that up-regulation of DUSP4 alone could mediate the effects of AICAR on Pepck transcription. Additionally, depletion of DUSP4 via RNA interference using a siRNA smart pool (a mix of 4 individual DUSP4 targeting siRNA molecules) impaired the ability of AICAR to completely inhibit Pepck transcription (Fig. 5A). A control smart pool of non-targeting siRNA had an insignificant effect on the ability of AICAR to repress the activation by cAMP and dexamethasone. Similarly, addition of siRNA had an insignificant effect on the ability of AICAR to repress the activation by cAMP and dexamethasone without AICAR. To further confirm the ability of DUSP4 to inhibit gluconeogenic gene transcription, an adenovirus expressing DUSP4 was utilized. Fig. 5, A–C, shows that DUSP4 expression not only inhibits Pepck transcription but also inhibits endogenous Pepck and Glc-6-P mRNA expression induced by cAMP and dexamethasone. As shown in Fig. 5B, cAMP and dexamethasone increased endogenous Pepck mRNA expression ~10-fold without adenovirus, and 14-fold with infection by aden-GFP. This induction was repressed strongly by both 500 $\mu$M AICAR and adenoviral expression of CA-AMPK. Adenoviral expression of DUSP4 also repressed Pepck nearly as strongly as CA-AMPK. Fig. 5C shows a similar effect on endogenous Glc-6-P mRNA expression. Dexamethasone plus cAMP induced Glc-6-P expression by ~25-fold with and without adenoviral expression of GFP. Treatment with 500 $\mu$M AICAR and either CA-AMPK or DUSP4 adenoviral expression strongly inhibited the Glc-6-P induction. The Western blots of Fig. 5D show that activated AMPK either through AICAR treatment or constitutively active kinase expression both induced ACC phosphorylation, whereas DUSP4 expression did not. The reporter assay of Fig. 5E shows that co-transfection of DUSP4 as well as AICAR treatment does not repress transcription under the control of the SV40 early promoter (pSEAP2-control), indicating that the DUSP4 effect is not global. The results of Fig. 5 strongly suggest that DUSP4 mediates part of the repressive effect of AMPK on gluconeogenic gene transcription.

The effect on Pepck promoter activity following modulation of EGR1 levels is shown in Figs. 6, A and B. Unlike DUSP4, transfection of exogenous EGR1 had no effect on Pepck promoter activity. However, when EGR1 was overexpressed in the presence of a relatively low AICAR concentration (100 $\mu$M), which by itself was not sufficient to inhibit Pepck activation (Fig. 6B), Pepck transcription was significantly reduced from 12- to
3-fold. Thus, EGR1 can potentiate the effects of AICAR on PEPCK transcription. The Western blots of Fig. 6C show that 100 μM AICAR treatment does result in a marginal increase in EGR1 protein expression at 6 h of treatment, but to a much lesser extent compared with 500 μM AICAR treatment. This further supports that exogenous EGR1 can potentiate the lower AICAR dose. It is possible that, in addition to inducing EGR1 transcription, AMPK activation also results in a modification to EGR1, such as phosphorylation, which is required for EGR1 activity. Alternatively, AMPK could also activate a co-factor necessary for EGR1-mediated transcription, although the expression of the repressors of EGR1 transcription NAB-1 or -2 did not change in our experiments (data not shown). It is interesting to note that EGR1 overexpression alone is also unable to modulate other cellular effects (e.g. apoptosis) known to be dependent on EGR1, and co-factor requirements for EGR1 function have been suggested previously (25).

Depletion of EGR1 via siRNA smart pool partially abrogated the ability of AICAR to inhibit the Pepck promoter (Fig. 6A). Whereas in this set of experiments cAMP and dexamethasone activated the Pepck reporter 13-fold, siRNA-mediated knockdown of EGR1 resulted in a 6-fold activation by cAMP and dexamethasone in the presence of AICAR, whereas AICAR fully repressed the activation when control siRNA was included. EGR1 transfection did not affect SV40 promoter activity, also suggesting that EGR1 is not affecting global transcription (Fig. 6D). These reporter assays and analyses of endogenous Pepck and Glc-6-P expression show that the induction of EGR1 and DUSP4 by AMPK is necessary for the ability of AMPK to fully inhibit the induction of these gluconeogenic genes. Similar experiments, overexpressing and depleting DUSP4 and EGR1, were performed using a Glc-6-P promoter, and yielded similar results (data not shown), suggesting that modulation by DUSP4 and EGR1 is not specific to the Pepck promoter.

Depletion of DUSP4 and EGR1 Abrogates the Ability of AICAR to Inhibit Glucose Production—Fig. 7 shows the results of glucose production assays upon reduction of EGR1 and DUSP4 by siRNA smart pool transfection. Transfection of Fao hepatoma cells with DUSP4 and EGR1 siRNA smart pools resulted in a 60 and 70% reduction in their relative basal expres-
These results are statistically significant with a Student’s t test value of p = 0.01. Consistently, chromatin immunoprecipitation experiments show that EGR1 binds to the Dusp4 promoter at the same promoter element. Modulating the expression levels of either EGR1 or DUSP4 affected the transcriptional activity of this gene in FaO rat hepatoma cells.

Constitutively Active p38 Rescues DUSP4 Repression of Pepck Transcription—Members of the DUSP family show differing specificities for the MAPK family of kinases. DUSP4 has been previously shown to dephosphorylate and inactivate the MAPK family members extracellular signal-regulated kinase (ERK) and JNK, but not p38 (26, 27, 29). Interestingly both JNK and p38 have been shown to be positive regulators of gluconeogenesis, with p38 being downstream of protein kinase A/cAMP (30, 31). Because JNK and p38 have an inverse relationship to gluconeogenesis relative to DUSP4, it was tested whether the activity of either of these kinases is regulated by AICAR or DUSP4 in H4IIE cells. As shown in Fig. 8A, addition of cAMP to serum-starved H4IIE cells for 2 h leads to an increase in the phosphorylation of p38 and that it is inhibited with the addition of either AICAR or a specific p38 chemical inhibitor SB205830. In contrast, JNK phosphorylation was not inhibited by AICAR (data not shown). As indicated in Fig. 3B, DUSP4 was induced following 2 h of AICAR treatment. Because the amount of active p38 present in the cAMP-treated H4IIE cells decreases upon 2 h of AICAR treatment, which corresponds to the time point of DUSP4 induction (Fig. 3B), a possible link between DUSP4 and p38 was examined. The Western blots of Fig. 8B show that as with AICAR treatment, expression of either CA-AMPK or DUSP4 via adenoviral infection for 48 h also inhibited the phosphorylation of p38 following 2 h of cAMP treatment of serum-starved H4IIEC cells. This result suggests that DUSP4 represses gluconeogenesis through dephosphorylation of p38. In further support of this hypothesis, Fig. 8C shows that, whereas DUSP4 overexpression represses AMPK activation of the Pepck promoter almost as efficiently as AICAR, inclusion of the constitutively active p38 (p38EE) mutant with the activation phosphorylation sites Thr-180 and Tyr-182 replaced with glutamate, but not wild type p38, abrogates the ability of DUSP4 to repress the Pepck promoter (32). Importantly, p38EE, which is refractory to DUSP4 regulation, is unable to activate the Pepck promoter on its own, indicating that active p38 is necessary but not sufficient for mediation of the cAMP effect on Pepck. In summary, based on our results, we propose a model, illustrated in Fig. 9, where AMPK activation in hepatocytes leads to activation and induction of EGR1 and DUSP4, which in turn dephosphorylates p38 leading to inhibition of gluconeogenesis. It is important to note that the transcriptional pathway uncovered in this study likely mediates only a portion of the effects of AMPK on gluconeogenic gene expression, as evidenced by the finding that neither the addition of exogenous DUSP4 nor the depletion of EGR1 can completely mimic the effects of AICAR or CA-AMPK.

Discussion

In this study we show that transcriptional targets of activated AMPK are necessary for its ability to repress hepatocyte glucose production. Using transcriptional profiling of AML12 and H4IE hepatocyte cell lines treated with AICAR, we identified two new transcriptional targets of AMPK, EGR1 and Dusp4. Our data establish that EGR1 and DUSP4 act sequentially to mediate the effect of AMPK on inhibiting hepatic gluconeogenesis. This is supported by the following data: first, EGR1 protein expression is present 20 min after AICAR treatment followed by a DUSP4 increase at 2 h. Second, chromatin immunoprecipitation experiments show that EGR1 binds to the Dusp4 promoter at 2 and 6 h of treatment. Third, siRNA-mediated knockdown of EGR1 also knocks down expression of DUSP4. A previous study has shown that EGR1 can transcriptionally regulate Dusp4 through a specific binding site in the Dusp4 promoter (24). Similarly, in our system we found that the increase in EGR1 protein is accompanied by increased binding of EGR1 to the Dusp4 promoter at the same promoter element. Modulating the expression levels of either EGR1 or DUSP4 affected the ability of AICAR to repress both Pepck and Glc-6-P activated transcription. Furthermore, DUSP4 expression repressed transcription of these two gluconeogenic genes to a similar extent as AICAR and CA-AMPK expression. In addition,
siRNA-mediated knockdown of DUSP4 and EGR1 in Fao cells shows that these genes mediate at least part of the inhibition of glucose production by AICAR in these cells. Additionally, DUSP4 overexpression, but not EGR1, causes repression of gluconeogenesis, suggesting that additional factors or modifications to EGR1 are necessary for EGR1 to induce Dusp4 and repress gluconeogenesis. This is further suggested by the result that EGR1 can potentiate the AICAR signal, as EGR1 overexpression can repress Pepck transcription with the addition of AICAR at a concentration that is ineffective by itself. EGR1 has been shown to activate its own transcription by directly binding its promoter, so an initial activation of the resting pool of EGR1 could result in a quick and immediate increase of its expression (33). The inability of exogenous EGR1 to affect Pepck transcription in the absence of AICAR reported here is similar to its role in apoptosis, as its overexpression does not cause apoptosis on its own (25). The fact that overexpression of DUSP4 can mimic the effect of AICAR suggests that it is a primary protein mediating the effect of EGR1 up-regulation. We cannot, however, exclude that other EGR1 target genes make major contributions.

A recent study has shown that p38 is necessary for the cAMP-dependent activation of gluconeogenic genes (30). Blockade of p38 prevents transcription of peroxisome proliferator-activated receptor-γ coactivator-1α and phosphorylation of CREB (30). Transcriptional up-regulation of DUSP4 following AICAR treatment could result in blockage of the necessary p38-mediated downstream events. We have demonstrated that inhibition of cAMP driven p38 phosphorylation by both exogenous DUSP4 expression and by AICAR treatment at 2 h (the time of DUSP4 induction) occurs. Furthermore, the rescue of DUSP4 repression of PEPCk by a constitutively activated form of the MAPK p38, which is refractory to regulation by DUSPs, suggests DUSP4 may act by dephosphorylating p38. Two other members of the DUSP family have previously been implicated in gluconeogenesis. Surprisingly, DUSP9 (MKP-4) and DUSP6 (MKP-3) activate PEPCk transcription (23, 34). DUSP9 inhibits insulin signaling and reverses insulin-induced repression of PEPCk, whereas DUSP6 activates PEPCk in the presence of cAMP and dexamethasone and activates glucose production. Both are more highly expressed in insulin-resistant tissues when compared with normal tissues. The ability of DUSP4 to repress PEPCk and Glc-6-P expression is opposite to that reported for other members of this family. Whether this is due to substrate or tissue specificity, or is an AMPK-specific effect of DUSP4 is unknown. This complexity among DUSP family members indicates that any treatment to activate DUSP4 to inhibit gluconeogenesis in Type 2 diabetic livers would potentially have to be selective for DUSP4 and not DUSP6 or -9.

A recent study by Koo et al. (35) demonstrated that TORC2 regulation by salt inducible kinase 1 (SIK1) and AMPK is key to proper hepatic regulation of glucose homeostasis (35). Phosphorylation of TORC2 by either SIK1 or AMPK on serine 171 sequesters it in the cytoplasm, where it is unable to co-activate transcription by CREB. Interestingly, TORC2 only mediates part of the response of AICAR, as shown by partial inhibition of the AICAR effect with AICAR-insensitive TORC2. Here we show that similar to expression of dominant negative TORC2, abrogation of EGR1 and DUSP4 induction partially restores Pepck promoter activity in the presence of AICAR. Whereas phosphorylation of TORC2 is a direct effect of AMPK activation, Egr1 and Dusp4 represent a new class of transcriptional targets of AMPK that are necessary for its function. As of now it appears that Dusp4 activation could be a second and distinct but complementary mechanism of Pepck repression by AMPK.

To date, very few genes regulated by AICAR have been reported and the direct transcriptional mechanism in this case is unclear. Many of the genes are down-regulated. Interestingly, we find preponderance of up-regulated genes in our profiling experiment. Additionally, AMPK has generally been found to exert effects through inhibition of transcription by factors such as carbohydrate-responsive element-binding protein, HNF-4α, and p300, as well as inhibit translation through TSC2 and mTOR, yet here we show an acute and immediate induction of Egr1 and Dusp4 that appears to be necessary for downstream effects (20, 36–38). Many of the known genes are regulated later (e.g. HNF-4 protein levels decrease after 6 h of treatment) as well. It is possible that the induction of EGR1 is so strong as to overcome other inhibitory effects of AMPK activation, and it has been shown that AMPK activation down-regulates nuclear hormone receptor-driven transcription while preserving p300-mediated activation of non-nuclear receptor transcription factors, of which EGR1 is one (28). Future work will lead to understanding the mechanism of EGR1 activation by AMPK.

Thus far, our data are in vitro. Studies are underway to address the in vivo importance of EGR1 and DUSP4 in gluconeogenesis. Comparing the in vivo results of alteration of EGR1 and DUSP4 on gluconeogenesis to the known p38 data could further establish the functional p38/DUSP4 link. It is possible that the EGR1-DUSP4-p38 pathway may allow a novel approach to modulate dysfunction in hepatic glucose output in Type 2 diabetes.

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