Original Article

Glucose-Dependent Transcriptional Regulation by an Evolutionarily Conserved Glucose-Sensing Module

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We report here a novel mechanism for glucose-mediated activation of carbohydrate response element binding protein (ChREBP), a basic helix-loop-helix/leucine zipper (bHLH/ZIP) transcription factor of Mondo family that binds to carbohydrate response element in the promoter of some glucose-regulated genes and activates their expression upon glucose stimulation. Structure-function analysis of ChREBP in a highly glucose-sensitive system using GAL4-ChREBP fusion constructs revealed a glucose-sensing module (GSM) that mediates glucose responsiveness of ChREBP. GSM is conserved among Mondo family members; MondoA, a mammalian paralog of unknown function, and the GSM region of a *Drosophila* homolog were also found to be glucose responsive. GSM is composed of a low-glucose inhibitory domain (LID) and a glucose-response activation conserved element (GRACE). We have identified a new mechanism accounting for glucose responsiveness of ChREBP that involves specific inhibition of the transactivation activity of GRACE by LID under low glucose concentration and reversal of this inhibition by glucose in an orientation-sensitive manner. The intramolecular inhibition and its release by glucose is a regulatory mechanism that is independent of changes of subcellular localization or DNA binding activity, events that also appear to be involved in glucose responsiveness. This evolutionally conserved mechanism may play an essential role in glucose-responsive gene regulation. *Diabetes* 55:1179–1189, 2006

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Received for publication 28 June 2005 and accepted in revised form 24 January 2006.

Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org.

bHLH/ZIP, basic helix-loop-helix/leucine zipper; ChREBP, carbohydrate response element binding protein; DBD, DNA binding domain; GFP, green fluorescent protein; GRACE, glucose-response activation conserved element; GSM, glucose-sensing module; LID, low-glucose inhibitory domain; L-PK, liver pyruvate kinase; MCR, Mondo conserved region; Mlx, max-like protein x; PADRE, protein NH_2-terminal domain of repression; PKA, cAMP-dependent protein kinase; PP2A, protein phosphatase 2A; TAD, transactivation domain; UAS, upstream activating sequence; WPRE, woodchuck hepatitis posttranscriptional regulatory element.

DOI: 10.2337/db05-0822

To satisfy their energy needs, all organisms have developed a variety of methods to adapt to fluctuations in food supply. Unicellular organisms can switch on and off different sets of genes in the presence of different carbon sources. Higher multicellular organisms, however, maintain a homeostatic internal environment in which only a few carbon sources, most importantly glucose, are available for utilization. Glucose homeostasis is primarily achieved with hormonal and neural regulation. Glucose itself can also regulate some genes involved in glycolysis, lipogenesis, and other metabolic processes (1–3). A carbohydrate response element was found in promoter regions of some glucose-responsive genes, e.g., liver pyruvate kinase (L-PK) and acetyl-CoA carboxylase (4–7). Carbohydrate response element binding protein (ChREBP), a basic helix-loop-helix/leucine zipper (bHLH/ZIP) transcription factor, was lately found to bind carbohydrate response element and regulate glucose-inducible expression of these genes (8–10). ChREBP knockout mice display reduced glycolysis and lipogenesis and severe simple sugar intolerance, underscoring the importance of ChREBP in glucose and lipid homeostasis (11).

ChREBP has been identified previously under the names WBSCR14 and MondoB (12,13). Homologs of ChREBP, collectively named Mondo family, exist in species from nematodes to mammals (13,14). Among this family, two paralogs, i.e., ChREBP and MondoA, can be found in vertebrates (13). WBSCR14/ChREBP and MondoA are mainly cytoplasmic because of two highly conserved regions in both ends of the protein. The NH_2-terminal conserved region was called protein NH_2-terminal domain of repression (PADRE) (14) or Mondo conserved region (MCR) (13); it harbors a nuclear export signal and a binding site for 14-3-3 proteins, which also facilitates nuclear export (15). The COOH-terminal region, called WBSCR14 max-like protein x [Mlx] C-tail (14) or dimerization and cytoplasmic localization domain (15), dimerizes with Mlx, which is essential for DNA binding and contributes to cytoplasmic retention of MondoA (9,14,15). Despite the striking similarity among Mondo proteins, two cAMP-dependent protein kinase (PKA) phosphorylation sites considered essential for glucose responsiveness of ChREBP are not conserved in other Mondo proteins (16,17), whose physiological function remains unknown.

In hepatocytes, Kawaguchi et al. (16) attributed glucose responsiveness of rat ChREBP to phosphorylation status of two key residues S196 and T666. Under low glucose concentrations, S196 and T666 are phosphorylated by PKA, which inhibits nuclear transport and target binding of

ChREBP. Glucose, via its metabolite xylulose-5-phosphate, activates protein phosphatase 2A (PP2A), which dephosphorylates ChREBP at these two residues, allowing its nuclear entry and DNA binding (16,18). However, as pointed out by Stoeckman et al. (9), a S196A/T666A mutant of ChREBP remained glucose responsive (16), implying alternative mechanisms for its glucose responsiveness.

In this study, we established a sensitive system, based on the GAL4 DNA binding domain (DBD) fusion proteins and upstream activating sequence $(UAS)_{GAL}$-driven luciferase reporter in insulinoma cell line 832/13, to examine the role of Mondo family in glucose-regulated gene transcription. Structure-function analysis of ChREBP using this system revealed a glucose-sensing module (GSM) encompassing a low-glucose inhibitory domain (LID) and a glucose-response activation conserved element (GRACE). Glucose responsiveness of ChREBP is mediated by inhibition of the transactivation activity of GRACE by LID and relief of the inhibition by glucose. GSM is evolutionally conserved and functionally interchangeable among Mondo proteins. Our investigation has thus uncovered a novel mechanism for glucose-regulated gene transcription mediated by an evolutionarily conserved GSM.

RESEARCH DESIGN AND METHODS

Plasmid construction. pCMX-GAL4 (19) was used for c-*myc*–tagged GAL4 fusion constructs. pGAMPAC was modified from pCMX-GAL4 with a protein-A tag (gift of Dr. R. Schwartz, Texas A&M University, Houston, TX) (20) to the 3′ of multiple cloning site. Retroviral vector pBpCWGFP was modified from pBabe-puro (21) with cytomegalovirus promoter driving expression of green fluorescent protein (GFP) fusion proteins and a woodchuck hepatitis post-transcriptional regulatory element (gift of Dr. K. Oka, Baylor College of Medicine) for mRNA stability. VP16 transactivation domain (TAD) fusion constructs were based on pCMX-VP16 (19). UAS_{GAL}-driven luciferase reporter pG5-luc was from Promega (Madison, WI); L-PK promoter–driven luciferase reporter PLPK-luc was cloned by inserting L-PK promoter (−196 to +32) (GenBank accession no. X05684) into pGL3-Basic (Promega). UAS_{GAL}-driven Rapid-Response luciferase reporter pG5-R2.2luc was cloned by replacing the firefly luciferase gene in pG5-luc with the Rapid-Response luciferase gene in pGL3(R2.2)-Basic (Promega). Leucine zipper regions of c-Fos and c-Jun were from pHybLex/Zeo-Fos2 and pYesTRP-Jun (Invitrogen, Carlsbad, CA), respectively. Mouse ChREBP (GenBank accession no. AF245475), MondoA (AY968204), and *Drosophila* Mio (Mlx interactor) (AF264754) were PCR cloned from mouse liver, skeletal muscle, and *Drosophila* embryo cDNA, respectively. S196A/T665A mutant of ChREBP was made by PCR-based site-specific mutagenesis. Cloning procedures are available upon request.

Cell culture and transfection. 832/13 cells (gift of Dr. C. Newgard, Duke University, Durham, NC) were cultured as described previously (22). Lipofectamine 2000 (Invitrogen) was used for transfection according to the manufacturer's instructions.

Luciferase assay. Specified expression plasmids and firefly luciferase reporters with internal control pRL-TK (Promega) were transfected into 832/13 cells, which were then treated with indicated concentration of glucose and/or cantharidic acid (Sigma, St. Louis, MO) for a specified period of time. A dual-luciferase kit (Promega) was used for luciferase assay. Results were shown as fold activation over reporter activity with empty vector or GAL4 only under low glucose concentration if not otherwise suggested.

Fluorescence imaging. cDNAs for GFP fusion proteins were cloned into pBpCWGFP. 832/13 cells transfected with these constructs were treated with low- or high-glucose medium for 6 h and fixed in 10% buffered formalin phosphate before fluorescence imaging analysis.

Nuclear and cytosolic extracts. Retroviruses expressing c-*myc*–tagged ChREBP and its derivatives were infected into 832/13 cells, which were treated with low or high glucose for 6 h. Nuclear and cytosolic extracts were prepared as described (23). The quality of fractionation was monitored with Western blot for glyceraldehyde-3-phosphate dehydrogenase and histones with corresponding antibodies (Chemicon, Temecula, CA). The levels of ChREBP and its derivatives were examined by Western blot with anti–c-*myc* antibody (Research Diagnostics, Flanders, NJ).

RESULTS

A sensitive system for glucose-dependent transcriptional regulation. It was reported that L-PK promoter activity can be upregulated by ChREBP in high-glucose medium in INS-1, an insulinoma cell line (24,25). In this study, we used a particularly glucose-sensitive cell line, INS-1–derived 832/13 (22). L-PK promoter–driven luciferase reporter in 832/13 cells showed mild (approximately threefold) stimulation by glucose, and further overexpression of ChREBP did not significantly change its glucose responsiveness (Fig. 1*A*), probably because of some rate-limiting cofactor(s). To improve the glucose sensitivity, we constructed a fusion protein of GAL4 DBD and mouse ChREBP (GAL4-ChREBP) and tested its ability to activate UAS_{GAL}-driven luciferase reporter in response to glucose. We reasoned that multiple GAL4 binding sites and low background activity of the promoter would significantly improve sensitivity; meanwhile, the reporter activity would be solely induced by exogenous GAL4-ChREBP, a fact that greatly simplifies data interpretation. Furthermore, because the DNA binding activity of GAL4-ChREBP is provided by GAL4 DBD instead of the bHLH/ZIP domain of ChREBP, it allows us to study the glucose responsiveness of ChREBP independent of the DNA binding activity of bHLH/ZIP domain. We found that GAL4-ChREBP displayed ~42-fold higher transactivation activity than GAL4 DBD in high-glucose medium, while having little activity in low-glucose medium; in contrast, a fusion protein of GAL4 DBD with VP16 TAD (GAL4-VP16) strongly activated reporter expression in both low- and high-glucose medium (Fig. 1*B*). To our knowledge, this is the most sensitive system to date for glucose-responsive gene regulation, which enabled us to study the dynamics and kinetics of glucose response of ChREBP.

To determine the time frame of glucose signaling events that lead to activation of ChREBP, we used Rapid-Response firefly luciferase reporter, which harbors protein and mRNA degradation signals and consequently has a very short half-life, enabling us to examine transcription activity approximating real-time. The results showed that GAL4-ChREBP transcription activity was turned on within 2 h after switching to high-glucose medium, peaked at 3 h, and stayed elevated up to 12 h (Fig. 1*C*). This suggests that glucose signaling has a short response time but is not subject to the quick desensitization observed with many other signaling pathways.

We next examined the dose response of GAL4-ChREBP transactivation activity to changes in glucose concentration and found that the reporter activity started to increase at ~7.5 mmol/l glucose and plateaued at ~25 mmol/l (Fig. 1*D*). The linear range largely overlapped physiological inter-meal glucose concentrations in portal venous blood (5–20 mmol/l) (26). Taken together, these data suggest that ChREBP is tightly regulated by glucose in a dose-dependent manner, and the glucose responsiveness seems most sensitive to physiological excursions of intra-hepatic glucose concentrations, underscoring the importance of ChREBP in nutrient physiology.

Structure-function analysis of ChREBP. In the quest for the mechanism underlying glucose responsiveness of ChREBP, we started with defining the functional domain mediating glucose response. We created GAL4 fusion proteins with serial deletions of ChREBP (Fig. 2*A*). All constructs expressed desired proteins with predicted molecular weights in Western blots (data not shown). Among

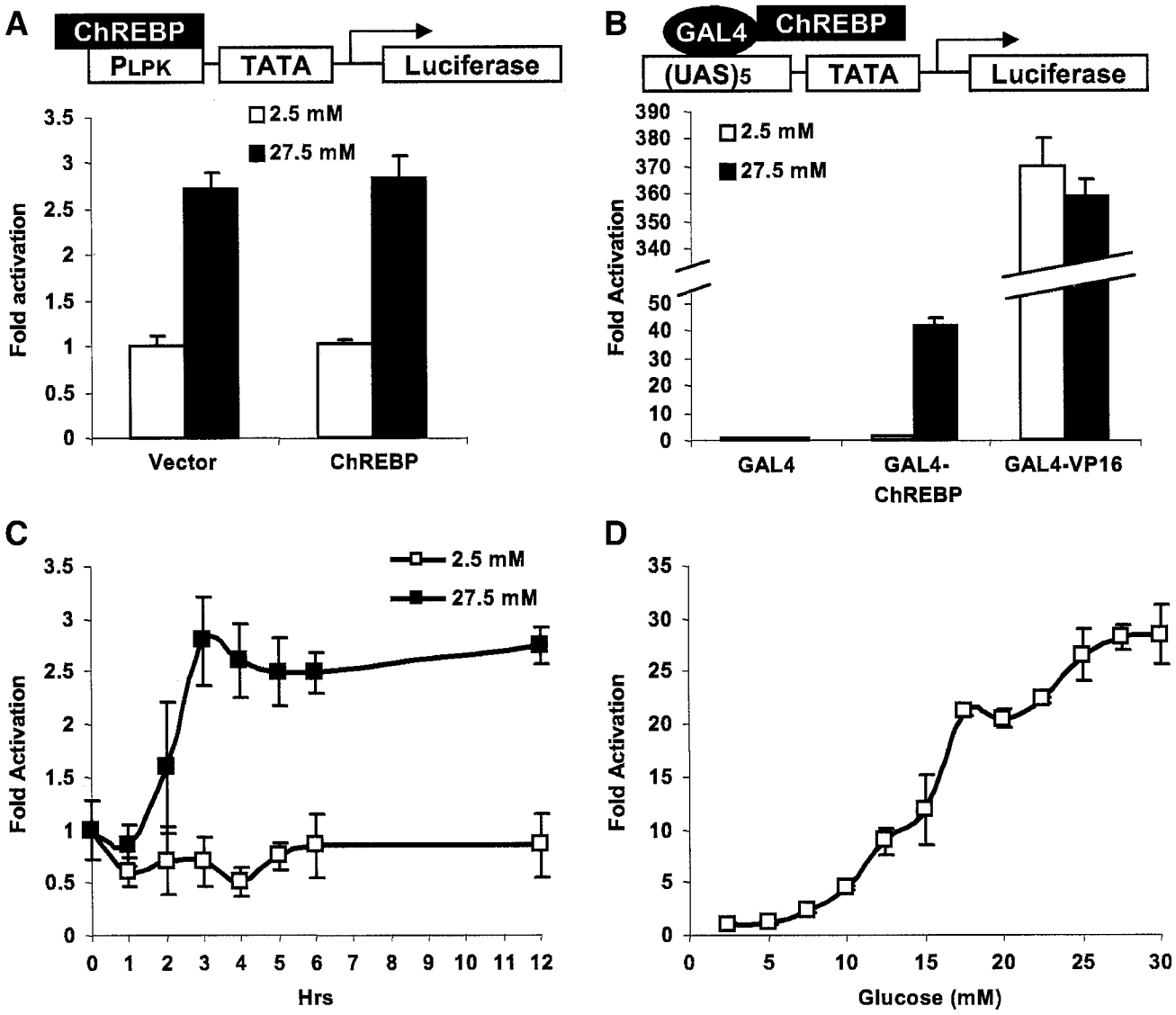

FIG. 1. A sensitive glucose-responsive system. Luciferase assay results were obtained under the following conditions. *A*: PLPK-luc and pRL-TK were cotransfected with empty vector or vector harboring ChREBP into 832/13 cells; *B*: pG5-luc and pRL-TK were cotransfected with plasmids encoding GAL4, GAL4-ChREBP, or GAL4-VP16 into 832/13 cells. Transfected cells (*A* and *B*) were treated with 2.5 or 27.5 mmol/l glucose for 24 h. *C*: pG5-R2.2luc and pRL-TK were cotransfected with plasmid encoding GAL4-ChREBP into 832/13 cells, which were primed with 2.5 mmol/l glucose for 24 h and then treated with 2.5 or 27.5 mmol/l glucose for a specified period of time. Results are shown as fold activation over reporter activity before treatment. *D*: pG5-luc and pRL-TK were cotransfected with plasmid encoding GAL4-ChREBP into 832/13 cells that were treated with indicated concentration of glucose for 24 h.

NH_2-terminal deletion mutants, N1 (Δ1–36) remained glucose responsive, whereas N2 (Δ1–196) became constitutively active in both low- and high-glucose medium. As to the COOH-terminal deletions, the shortest mutant that remained responsive to glucose is C4 (Δ299–864) (Fig. 2*B*). Taken together, the minimal requirement for glucose response seems to be aa.37–298. Notably, this region is also the most conserved region among Mondo proteins (13,14), suggesting that glucose responsiveness might be evolutionally conserved. We named this region (aa.37–298) the GSM.

Because transactivation activity was highest in N2 (Δ1–196) among NH_2-terminal truncation mutants and reduced to a negligible level in N3 (Δ1–298) (Fig. 2*B*), the TAD for ChREBP is most likely located within aa.197–298. Consistently, transactivation activity of M5 (aa.197–298) was significantly higher than that of M1 (aa.299–658) (Fig. 2*C*), which covers the middle activation domain as in RelB (MADRE) region previously proposed as the TAD of WBSCR14 (14). aa.197–298 is located within the highly conserved GSM and was therefore designated as GRACE. MADRE, although dispensable for glucose response, may contribute to transactivation of ChREBP in cooperation with GRACE, as could be inferred from the much stronger activity of M4 (aa.197–482) than that of M1 and M5 combined (Fig. 2*C*).

Because N1 (Δ1–36) showed the strongest glucose response and N2 (Δ1–196) the transactivation activity, it seems that aa.37–196 inhibits the transactivation activity of GRACE under low glucose concentrations, and this inhibition can be relieved in high-glucose medium. To substantiate the boundary between this inhibitory region and GRACE, we inserted GFP between aa.192–197 and found this insertion construct (GFPins) to be glucose responsive (Fig. 2*D*). The data prompted us to designate aa.37–192 LID. The fact that GFP insertion did not abolish

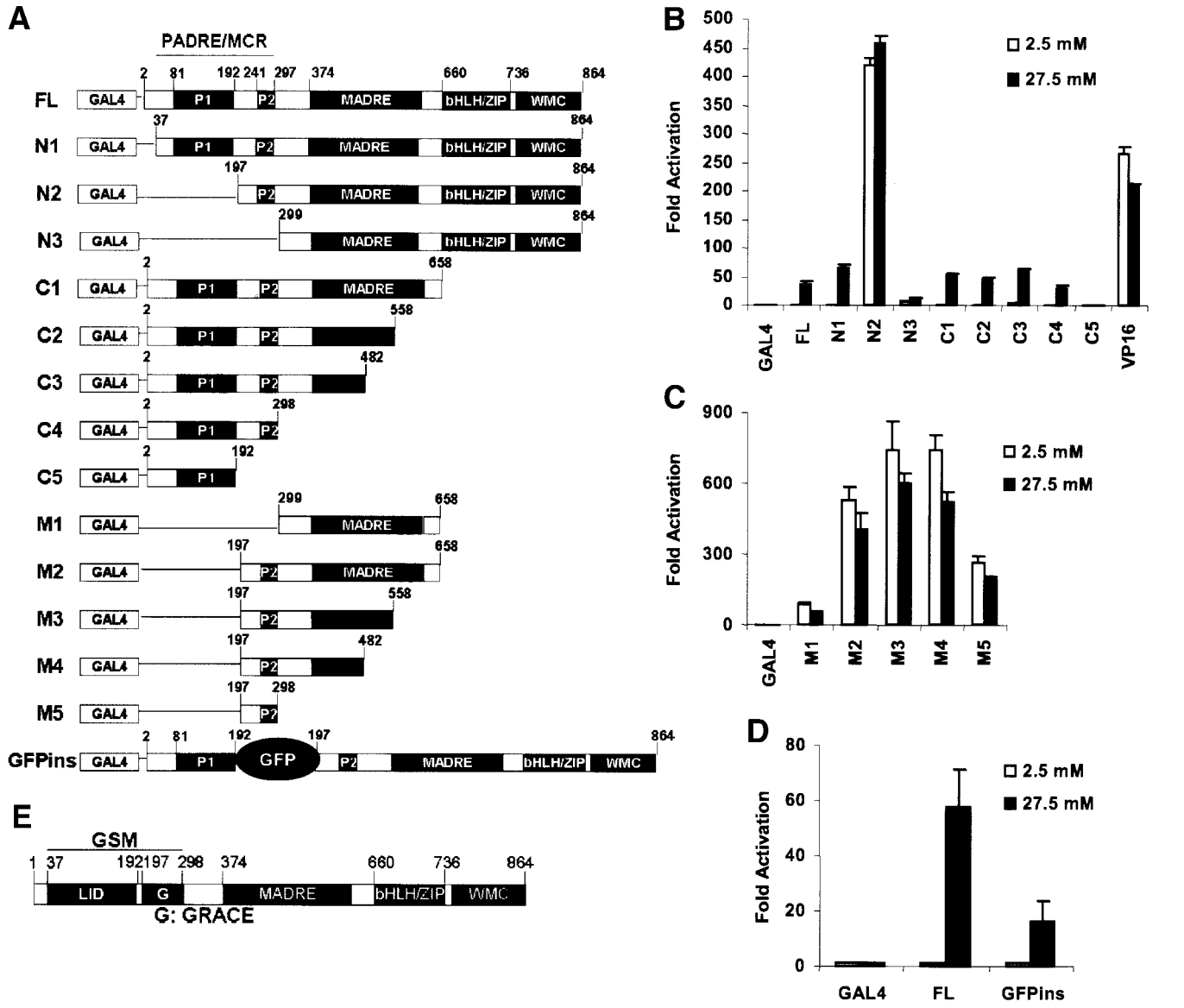

FIG. 2. Structure-function relationship of ChREBP. *A*: **Schematic representation (not to scale) of the deletion constructs in pGAMPAC in reference to current domain definition of WBSCR14.** *B–D*: **pG5-luc and pRL-TK were cotransfected with indicated plasmids into 832/13 cells that were treated with 2.5 or 27.5 mmol/l glucose for 24 h and assayed for luciferase activity.** *E*: **Schematic representation of GSM.**

glucose responsiveness of ChREBP also indicates that LID and GRACE are functionally distinct submodules within GSM. Consistent with this interpretation, LID and GRACE each encompass a highly conserved block within GSM, as revealed by sequence alignment of the GSM region (supplemental Fig. 1, which is detailed in the online appendix [available at http://diabetes.diabetesjournals.org]): LID roughly corresponds to PADRE1 in WBSCR14 or MCRI-IV in MondoA, whereas GRACE covers PADRE2 or MCRV in WBSCR14 and MondoA, respectively (14,15). In conclusion, our results suggest that glucose response of ChREBP results from reversal of LID-mediated inhibition of GRACE by glucose within the evolutionally conserved GSM (Fig. 2*E*)

GSM is evolutionally conserved among Mondo proteins. The high degree of sequence similarity in GSM led us to hypothesize that other Mondo proteins may also mediate glucose-responsive gene regulation. We constructed GAL4 fusion proteins with mouse MondoA (GAL4-MondoA) or the GSM region (aa.3–388) of a *Drosophila* homolog dMio (GAL4-dMio). Glucose activated both constructs at a level comparable with GAL4-ChREBP (Fig. 3*A*). We next engineered domain swap constructs in which we replaced aa.2–196 (LID region) of ChREBP in GAL4-ChREBP with the corresponding region of MondoA (aa.2–236) or dMio (aa.3–265). Both constructs displayed even stronger glucose responses than GAL4-ChREBP (Fig. 3*B*), suggesting that LID and GRACE from different homologs are interchangeable without compromising glucose responsiveness. These experiments indicate that Mondo proteins are capable of glucose responsiveness.

The potential involvement of other Mondo proteins in glucose-mediated gene regulation prompted us to analyze the evolutionary relationship within Mondo family. From available databases, the simplest organism in which ChREBP homolog can be identified is *Caenorhabditis elegans*, an early bilaterian species. Two copies of Mondo genes, namely ChREBP and MondoA, were found in species from fish to mammals, suggesting that the duplication event producing these paralogs occurred no later

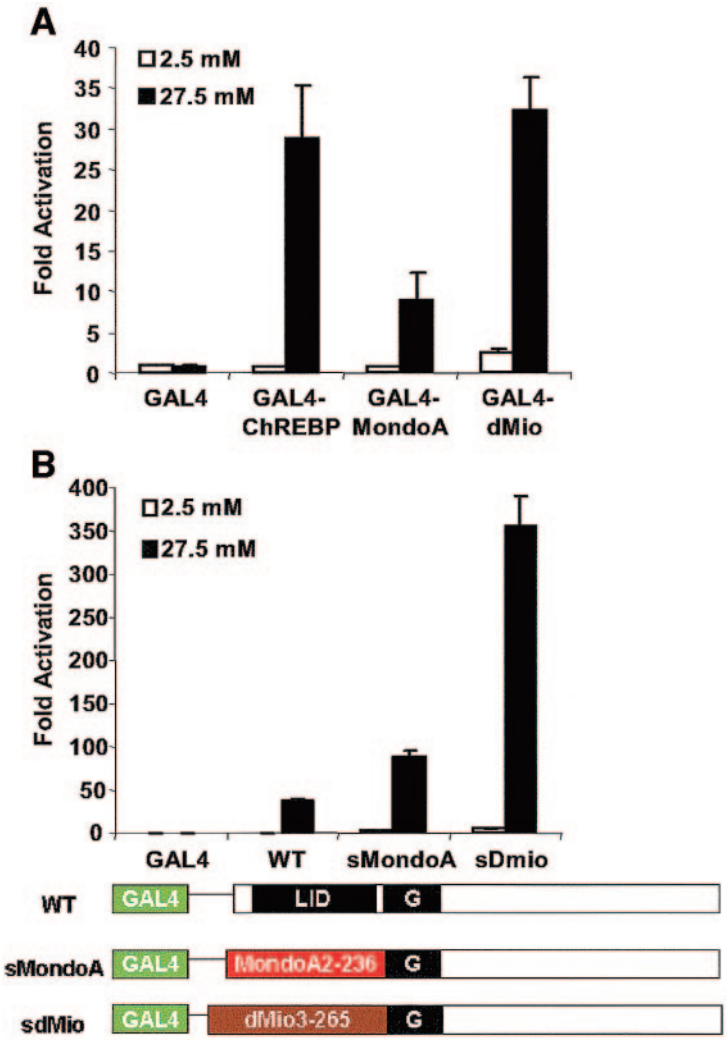

FIG. 3. Mondo proteins respond to glucose. Luciferase assay results were obtained under the following conditions. *A*: MondoA and aa.3–388 region of dMio were cloned into pGAMPAC (GAL4-MondoA and GAL4-dMio). pG5-luc and pRL-TK were cotransfected with specified plasmids into 832/13 cells that were treated with 2.5 or 27.5 mmol/l glucose for 24 h. *B*: In pGAMPAC-ChREBP, aa.2–196 of ChREBP was replaced with aa.2–236 of MondoA (sMondoA) or aa.3–265 of dMio (sdMio). pG5-luc and pRL-TK were cotransfected with specified plasmids into 832/13 cells that were treated with 2.5 or 27.5 mmol/l glucose for 24 h.

than the emergence of vertebrates. We generated a phylogenetic tree based on sequences of three MondoAs (human, mouse, and fish), four ChREBPs (human, mouse, rat, and fish), and two homologs from *Drosophila melanogaster* and *C. elegans*. MondoAs and ChREBPs form separate clusters that are together joined with the *Drosophila* and *C. elegans* homolog. The topology within each cluster fits well with the known phylogenetic relationship (species tree). ChREBP appears to have evolved faster than MondoA, as revealed by the longer branch within the ChREBP cluster (supplemental Fig. 2*A*). According to the phylogenetic tree, homologs from *Drosophila* and *C. elegans* seem to represent the ancestral genes that diverged into MondoA and ChREBP before vertebrates split. This scenario is supported by the phylogenetic tree based on the sequences of GSM (supplemental Fig. 2*B*), which are substantially better conserved (supplemental Fig. 1) and thus more reliable for evolutionary reconstruction. In conclusion, the Mondo family most likely originated in early bilaterians; it diverged into two subgroups before vertebrates split, which may mediate glucose-induced changes where they are preferentially expressed.

Glucose responsiveness of ChREBP is independent of S196/T665 phosphorylation and dephosphorylation. GSM is most likely the structural basis of glucose responsiveness, given its self-sufficiency for glucose response (Figs. 2*B* and 3*A*) and high degree of sequence similarity (supplemental Fig. 1). In contrast, the lack of conservation of the S196/T666 equivalents in MondoA and dMio suggests that these two residues and their phosphorylation status may not be required for glucose responsiveness of ChREBP. We tested this hypothesis with specific ChREBP mutants and a dephosphorylation inhibitor.

First, to confirm the reversible inhibitory effect of LID in physiological situation, we produced an NH_2-terminal deletion mutant of ChREBP (ChREBPΔ1–196) in which LID (together with S196) was removed, and we tested its effect on an L-PK promoter–driven luciferase reporter. Consistent with other experiments with LID deletion (Fig. 2), ChREBPΔ1–196 was constitutively active without response to glucose (Fig. 4*A*). To determine whether the expression of endogenous glucose/ChREBP target genes also responds to ChREBPΔ1–196, we infected 832/13 cells with retrovirus overexpressing ChREBPΔ1–196, taking advantage of the high integration rate and antibiotics selectability of retrovirus to minimize glucose response from noninfected cells. Consistently, mRNA levels of L-PK and acetyl-CoA carboxylase were elevated significantly at both low and high glucose concentrations (supplemental Fig. 3). These data indicate that T665 (equivalent to T666 in rat ChREBP), which is intact in this mutant, is not directly involved in glucose responsiveness of ChREBP in 832/13 cells, because ChREBPΔ1–196 is active under low glucose concentration when it is not supposed to bind

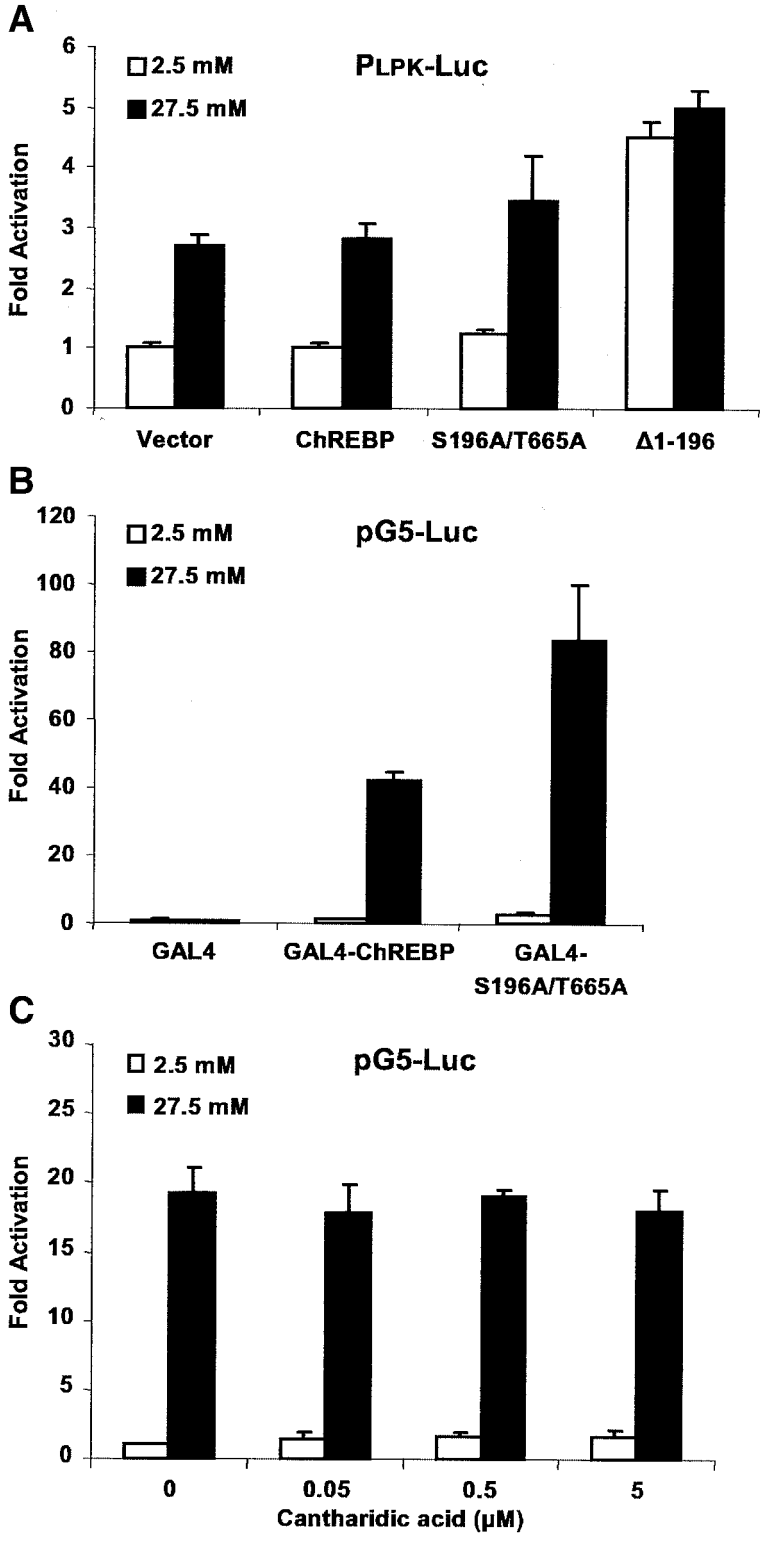

FIG. 4. Glucose responsiveness of ChREBP is independent of dephosphorylation of S196/T665. Luciferase assay results were obtained under the following conditions. *A*: PLPK-luc and pRL-TK were cotransfected with empty vector, vector expressing wild-type, S196A/T665A, or Δ1–196 mutant ChREBP into 832/13 cells; *B*: pG5-luc and pRL-TK were cotransfected with plasmids encoding GAL4, GAL4-ChREBP, or GAL4-S196A/T665A mutant into 832/13 cells. Transfected cells (*A* and *B*) were treated with 2.5 or 27.5 mmol/l glucose for 24 h. *C*: Plasmid encoding GAL4-ChREBP was cotransfected with pG5-luc and pRL-TK into 832/13 cells that were treated with indicated concentrations of cantharidic acid and glucose for 24 h. Results are shown as fold activation over reporter activity treated with 2.5 mmol/l glucose.

DNA because of T665 phosphorylation (16). Likewise, neither is the nuclear localization signal (aa.155–174), which was previously proposed to be activated by S196 dephosphorylation (16) and deleted in this construct, required for activation of ChREBP in 832/13 cells.

In support of the nonessential role of S196/T665, a S196A/T665A mutant of ChREBP was shown to be glucose responsive to activate L-PK promoter in 832/13 cells (Fig. 4*A*); however, the presence of endogenous ChREBP made it difficult to reach a definitive conclusion. We therefore generated a GAL4 fusion protein with the S196A/T665A mutant (GAL4-S196A/T665A), taking advantage of the fact that endogenous ChREBP cannot activate UAS_{GAL}-driven promoter. We found that this mutant displayed even stronger glucose response than wild-type ChREBP (Fig. 4*B*), further confirming that these two residues are dispensable for glucose responsiveness.

To test whether dephosphorylation of other residues in ChREBP by PP2A is involved in its glucose response, we treated 832/13 cells transfected with GAL4-ChREBP con-

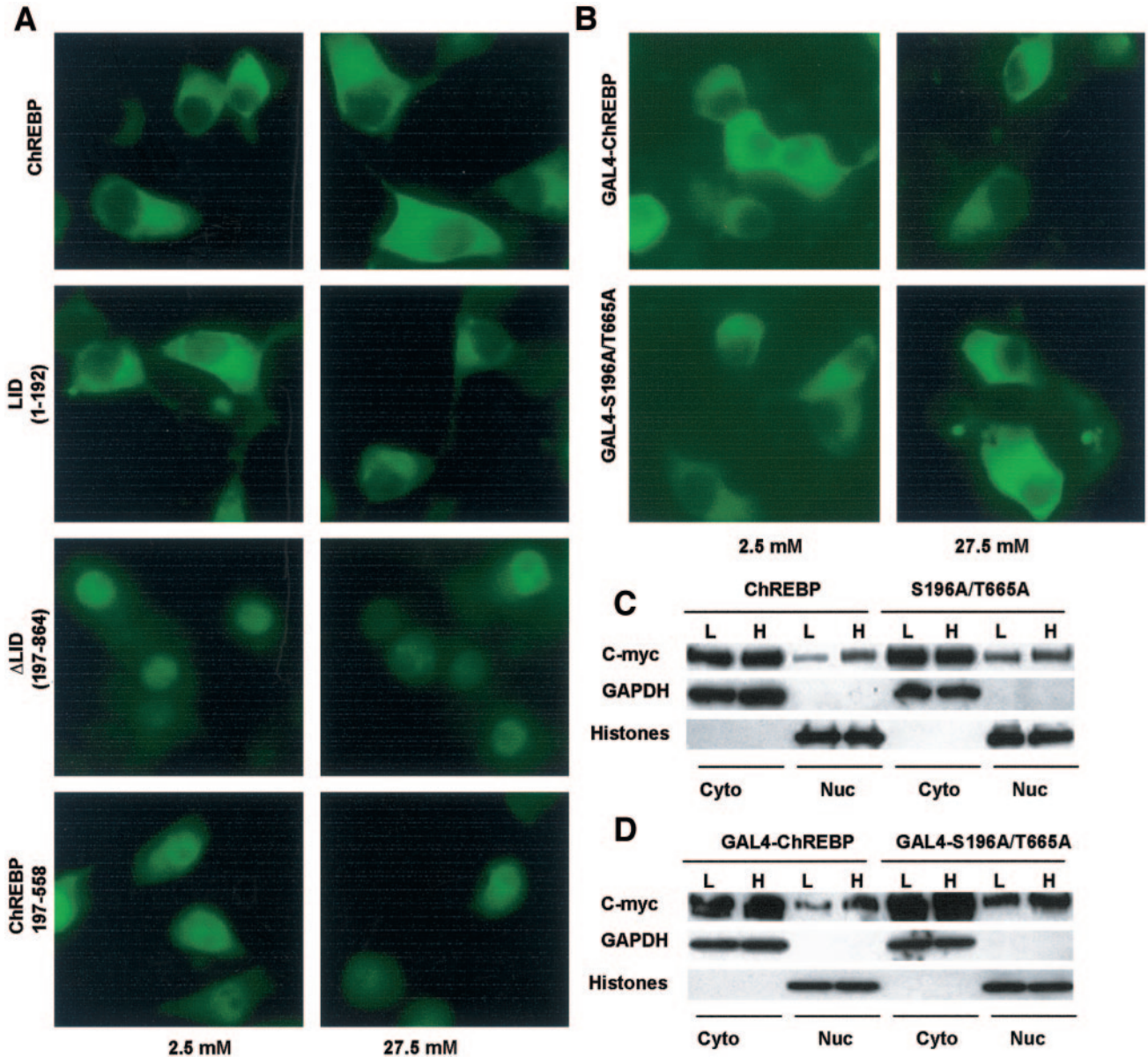

FIG. 5. Glucose responsiveness of ChREBP is independent of subcellular translocation of ChREBP. *A* and *B*: Specified proteins were cloned into pBpCWGFP. 832/13 cells transfected with these constructs were treated with 2.5 or 27.5 mmol/l glucose for 6 h and examined for fluorescence microscopy. *C* and *D*: Western blots of c-*myc*–tagged ChREBP, S196A/T665A mutant (*C*), GAL4-ChREBP, and GAL4-S196A/T665A (*D*) in cytosolic (Cyto) and nuclear (Nuc) fraction with low- (2.5 mmol/l, L) or high- (27.5 mmol/l, H) glucose treatment. These proteins were expressed with stably infected retrovirus. Infected cells were exposed to the appropriate glucose medium for 6 h before subcellular fractionation.

struct with different concentrations of cantharidic acid, a potent inhibitor of PP2A, in low- or high-glucose medium. We found that cantharidic acid up to 5 μmol/l did not significantly affect glucose responsiveness of GAL4-ChREBP (Fig. 4*C*); at the same doses, the inhibitor significantly stimulated the activity of a cAMP response element–driven luciferase reporter (data not shown). These experiments further support that dephosphorylation of ChREBP by PP2A may not be required for its glucose response, although the phosphorylation state of these two residues may independently contribute to glucose response by modulating DNA binding activity and nuclear transport. Nonetheless, the data demonstrate that mechanism(s) other than S196/T665 phosphorylation or dephosphorylation must exist and play a fundamental role in glucose responsiveness of ChREBP.

Mechanism(s) independent of change in subcellular distribution of ChREBP contributes to glucose responsiveness. It was reported previously that the GSM region harbors a strong cytoplasmic localization signal that retains WBSCR14/ChREBP and MondoA in the cytoplasm (15,27). To determine whether glucose can drive ChREBP into the nucleus in 832/13 cells, we generated GFP fusion proteins with wild type and select mutants of ChREBP and examined their subcellular localization under different glucose concentrations. We confirmed that both the LID region (aa.1–192), which harbors the nuclear export signal and 14-3-3 binding site, and full-length ChREBP were mainly cytoplasmic (Fig. 5*A*), although faint fluorescence is visible in the nucleus. In contrast, mutants with deletion of LID (ΔLID and ChREBP197–558) were mainly nuclear (Fig. 5*A*). Within the limits of its sensitivity

(see below), fluorescence microscopy failed to reveal any significant change in subcellular distribution of full-length ChREBP or LID upon glucose stimulation (Fig. 5*A*). Consistent with a previous report (15), GAL4-ChREBP and GAL4-S196A/T665A were also mainly cytoplasmic under either low or high glucose concentration (Fig. 5*B*). However, given the robust glucose-stimulated reporter expression, the small amount of ChREBP in nucleus must be sufficient for reporter activation in high-glucose medium.

The strong cytoplasmic fluorescence in these experiments made it difficult to detect small changes in the nuclear abundance of ChREBP. We therefore performed subcellular fractionation experiments to further examine whether glucose affects the relative distribution of ChREBP in the cytosol versus the nucleus in 832/13 cells. The results confirmed that ChREBP occurs predominantly in cytoplasm. Although wild-type ChREBP showed a moderate increase in its nuclear abundance with glucose stimulation, the S196A/T665A double mutant showed higher level in nuclei under low glucose concentration compared with that of wild-type ChREBP but only a minor change in nuclear abundance responding to high glucose stimulation (Fig. 5*C*), an observation reminiscent of that of a recent study conducted in primary hepatocytes (28). Fractionation of cells stably expressing fusion proteins of GAL4-ChREBP and GAL4-S196A/T665A double mutant also revealed a similar pattern of distribution, i.e., higher basal level in nuclei under low glucose and minimal increase in nuclear abundance with glucose stimulation (Fig. 5*D*).

The above data suggest that there must be other mechanism(s) at work accounting for the glucose responsiveness of ChREBP besides glucose-induced nuclear translocation, because the strong glucose response of GAL4-ChREBP (Fig. 1*B*) and GAL4-S196A/T665A (Fig. 4*B*) cannot be explained by the moderate increase in nuclear abundance of GAL4-ChREBP and the minor increase for GAL4-S196A/T665A (Fig. 5*D*) in response to glucose. In addition, the higher basal level of the double mutant in nuclei under low glucose did not translate into stronger transactivation. Furthermore, ChREBP turnover also seems not to be involved in its glucose response, because glucose did not change the protein level of ChREBP by fluorescent microscopy and Western blotting (Fig. 5).

Glucose response depends on modulation of specific intramolecular inhibition of GRACE transactivation activity by LID. The fact that an LID-dependent mechanism mediates glucose responsiveness that is largely independent of changes in subcelluar localization or DNA binding activity suggests that the transactivation activity of GRACE is the most likely target for the reversible inhibition by LID. To determine whether LID alone is sufficient for this effect, we inserted LID between GAL4 DBD and VP16 TAD and tested whether LID would confer glucose responsiveness to GAL4-LID-VP16 fusion protein. We found that GAL4-LID-VP16 was constitutively active under different glucose concentrations (Fig. 6*A*), suggesting that this intramolecular inhibition by LID is specific for GRACE, which cannot be replaced by VP16 TAD. We next repositioned LID to the COOH-terminus of GRACE and found that this reshuffled construct (Rev) became constitutively active (Fig. 6*B*). These data suggest that glucose responsiveness of ChREBP is dependent on specific interplay between LID and GRACE, which is sensitive to their relative orientation. To determine whether this interplay involves direct interaction between LID and GRACE, we performed a mammalian two-hybrid assay but found no detectable interaction between them (Fig. 6*C*). PolyHis pulldown assay also failed to reveal binding between these domains in vitro (data not shown). However, glucose responsiveness could be restored when LID and GRACE were brought together via interaction between the leucine zipper regions of c-Jun and c-Fos (Fig. 6*C*). In conclusion, glucose responsiveness of ChREBP depends on specific, orientation-sensitive, and reversible inhibition of GRACE transactivation activity by LID.

DISCUSSION

The discovery of ChREBP was a milestone in the understanding of glucose-mediated metabolic regulation. However, the existing model fails to fully explain the glucose responsiveness of ChREBP. In this study, we developed a sensitive system for glucose-responsive transcriptional regulation mediated by ChREBP. Structure-function analysis of ChREBP with this system revealed an evolutionally conserved GSM that mediates glucose responsiveness of ChREBP. We found that glucose responsiveness is mediated by a reversible intramolecular inhibition of GRACE transactivation activity by LID. This mechanism is also observed in ChREBP homologs not previously known for glucose-regulated function, suggesting their involvement in glucose-responsive gene regulation.

The evolution of glucose-responsive gene regulation. ChREBP most likely originated in early bilaterians, according to analysis of available databases (supplemental Fig. 2). With the emergence of mesoderm, bilaterians were able to build a separate internal environment, which makes humoral regulation of homeostasis possible. It is thus not surprising that ChREBP and some other factors important for homeostatic regulation such as nuclear receptors (29), SREBP, and C/EBP (M.V.L., L.C., personal observation) seem to have emerged at this time of evolution.

According to our evolutionary analysis, a major event during the evolution of Mondo family was the diversification of the ancestral gene into two subgroups, i.e., ChREBP and MondoA, in vertebrates. Although ChREBP is highly expressed in the liver (8), MondoA is predominantly expressed in skeletal muscle (13), an expression pattern that may contribute to the difference in glucose-regulated metabolism in these two tissues. Although ChREBP regulates mainly lipogenic and glycolytic genes (10,11), we have no knowledge of MondoA target genes. Considering the importance of skeletal muscle in glucose disposal, it would be interesting to find out what these genes are and whether and how MondoA affects their expression in response to glucose.

The molecular nature of the glucose response of ChREBP. Although changes of subcellular localization, DNA binding activity, or protein turnover are common mechanisms to regulate the transactivation activity of many transcription factors, they do not seem necessary for the glucose response of ChREBP, at least in our system (Fig. 5). This indicates that the transactivation activity of ChREBP itself is the most likely target of glucose regulation. Although it was reported previously that the LID region has a documented repression activity (14), this activity was later attributed to its cytoplasmic retention effect, rather than corepressor recruitment (27). Consistently, we found that LID cannot confer glucose responsiveness to GAL4-LID-VP16 as we would expect if glucose responsiveness is due to reversible recruitment of core-

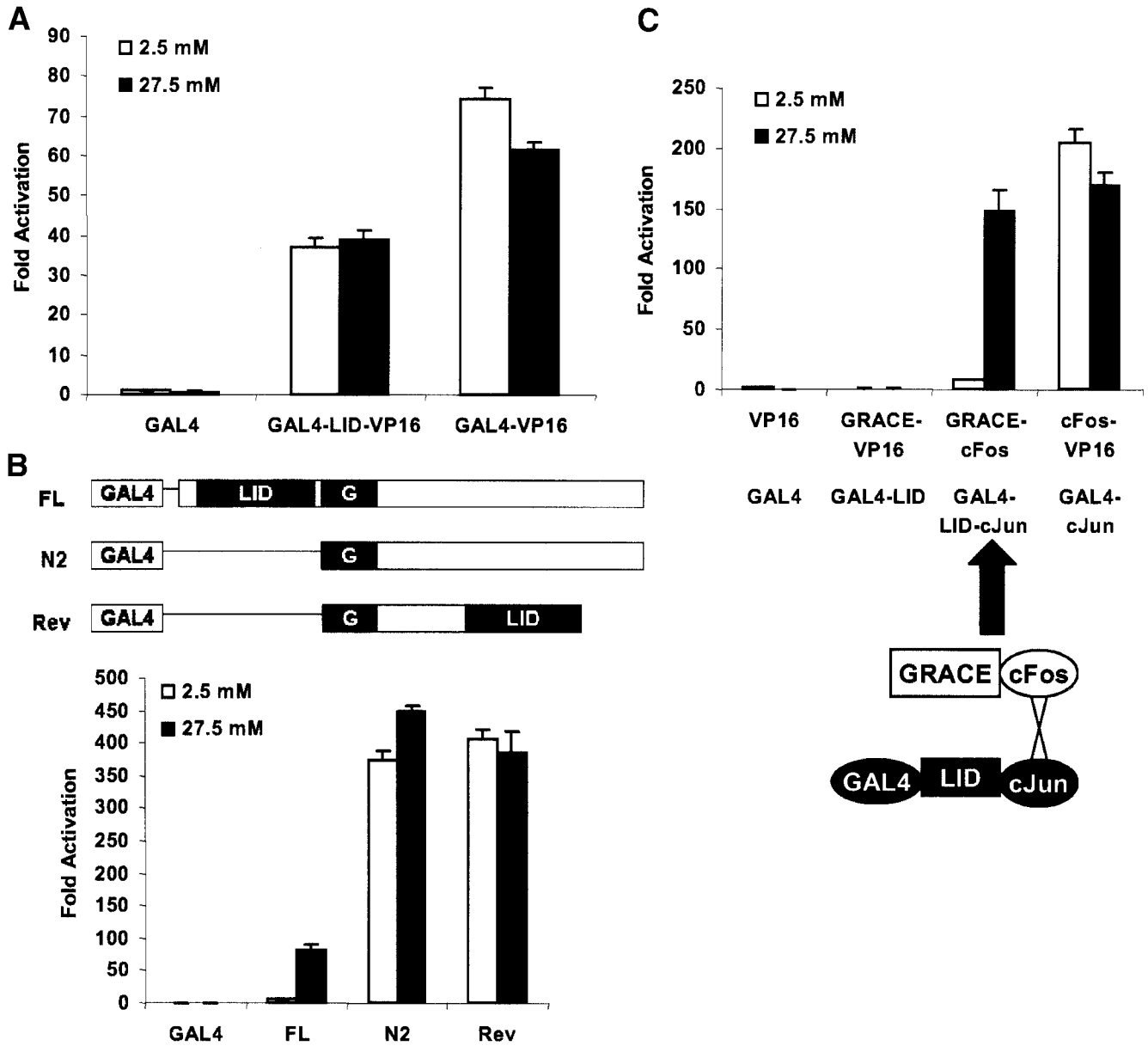

FIG. 6. Glucose responsiveness of ChREBP is mediated by reversible and specific intramolecular inhibition of GRACE by LID. Luciferase assay results were obtained under the following conditions. *A*: pG5-luc and pRL-TK reporters were cotransfected with plasmids encoding GAL4, GAL4-LID-VP16, or GAL4-VP16 into 832/13 cells. *B*: Schematic representation of Rev (LID/GRACE reversion) in reference to wild type (FL) and constitutively active (N2) controls. Indicated plasmids were cotransfected with pG5-luc and pRL-TK into 832/13 cells. *C*: For two-hybrid between LID and GRACE, aa.2–192 of ChREBP was fused with GAL4 (GAL4-LID) and aa.197–558 with VP16 (GRACE-VP16). GAL4-cJun and cFos-VP16 were positive control, and GAL4-LID-cJun and GRACE–c-Fos were used for reconstitution of glucose responsiveness. Indicated plasmids were cotransfected with pG5-luc and pRL-TK into 832/13 cells. Transfected cells (*A–C*) were treated with 2.5 or 27.5 mmol/l glucose for 24 h.

pressors by LID (Fig. 6*A*). We further showed that the inhibitory effect of LID is sensitive to the relative orientation of LID and GRACE, because repositioning of LID COOH-terminal to GRACE abolished the glucose responsiveness of ChREBP (Fig. 6*B*). These data indicate that glucose responsiveness is dependent on specific and orientation-sensitive interplay between LID and GRACE, although this interplay does not seem to involve direct interaction, as indicated by mammalian two-hybrid (Fig. 6*C*) and polyHis pulldown assay (data not shown). However, the molecular mechanism of this intramolecular interplay remains unknown. We can envision two possibilities. One is that LID directly blocks the recruitment of coactivators by GRACE through physical interference, which is circumvented by a conformation change after glucose stimulation; alternatively, LID and GRACE together can recruit some corepressor(s) that is released upon glucose stimulation. Elucidation of the exact mechanism requires additional investigation, especially in pursuit of binding proteins of ChREBP, the effect of glucose on their interactions, and their three-dimensional structures.

Cytoplasmic localization of ChREBP. Our results corroborated the finding of Merla et al. (27), i.e., the predominant cytoplasmic localization of ChREBP (Fig. 5). As if one were not enough, evolution imposes two separate cytoplasmic localization signals along with a 14-3-3 binding site to keep Mondo proteins away from nucleus (15,27). Although it is counterintuitive that a functional transcription factor should be mainly cytoplasmic, this distribution pattern may be functionally important. Most

likely, ChREBP needs to be activated by some cytoplasmic signal turned on by glucose. It is difficult, however, to definitively prove this hypothesis before the putative signal is identified, because we were not able to drive ChREBP into nucleus even with multiple nuclear localization signal sequences engineered into ChREBP (M.V.L., L.C., unpublished data). If this cytoplasmic activation process exists, it possibly involves at least another protein in addition to diffusible signaling compounds such as glucose metabolites, because the latter are less likely confined to the cytoplasm.

Phosphorylation-dependent and -independent regulation of ChREBP. Kawaguchi et al. (16) reported convincing evidence that phosphorylation status of S196/T666 in rat ChREBP directly regulates its nuclear transport and DNA binding activity in hepatocytes. These data have been confirmed by several other groups. Stoeckman et al. (9) showed that S196A/T666A double mutation significantly increased DNA binding activity of ChREBP; using subcellular fractionation, Dentin et al. (28) and our own work corroborated the increase in nuclear abundance of ChREBP after glucose treatment, an effect that is attenuated by introduction of mutations at S196 and T666 (T665 for mouse). Nonetheless, the double mutant was highly glucose responsive, suggesting that in addition to phosphorylation-induced changes in DNA binding activity and subcellular localization, there must be other mechanism(s) that mediate glucose responsiveness. One such mechanism is the intramolecular inhibition within GSM discussed earlier. The high degree of evolutionary conservation of GSM suggests that this mechanism must have arisen earlier in evolution and play a fundamental role in glucose responsiveness of Mondo proteins. In contrast, the phosphorylation-dependent regulation of ChREBP must have emerged more recently, because the two phosphorylation sites are not conserved across species. The acquisition of this latter mechanism presumably allows fine tuning of ChREBP activity by changing nuclear abundance and DNA binding activity and integrating glucose signaling with other pathways, such as that of glucagon, which activates PKA. It is not surprising that the liver, a major site for ChREBP expression and a central organ in nutrient metabolism, has acquired a multifaceted and sophisticated regulatory mechanism for glucose homeostasis.

In conclusion, we have identified a new glucose-sensing mechanism for activation of Mondo transcription factors by glucose. This mechanism is mediated by a reversible intramolecular inhibition within an evolutionally conserved GSM. This study sheds light on a novel glucose signaling pathway leading to glucose-responsive gene regulation.

ACKNOWLEDGMENTS

L.C. has received National Institutes of Health Grants DK-68037 and HL-51586 and was supported by the Rutherford Chair for Diabetes Research from St. Luke's Episcopal Hospital and the T.T. & W.F. Chao Foundation.

We thank Dr. Christopher Newgard for 832/13 cells, Dr. Kazuhiro Oka for woodchuck hepatitis posttranscriptional regulatory element DNA, Dr. Robert Schwartz for protein-A cDNA, and Drs. David Moore, Jiemin Wong, and Vijay Yechoor for critical reading of the manuscript.

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