Hepatic Glucose Sensing via the CREB Coactivator CRTC2

Renaud Dentin,1 Susan Hedrick,2 Jianxin Xie,2 John Yates III,3 Marc Montminy1*

Chronic hyperglycemia contributes to the development of diabetes-associated complications. Increases in the concentration of circulating glucose activate the hexosamine biosynthetic pathway (HBP) and promote the O-glycosylation of proteins by O-glycosyl transferase (OGT). We show that OGT triggered hepatic gluconeogenesis through the O-glycosylation of the transducer of regulated cyclic adenosine monophosphate response element–binding protein (CREB) 2 (TORC2 or CRTC2). CRTC2 was O-glycosylated at sites that normally sequester CRTC2 in the cytoplasm through a phosphorylation-dependent mechanism. Decreasing amounts of O-glycosylated CRTC2 by expression of the deglycosylating enzyme O-GlcNAcase blocked effects of glucose on gluconeogenesis, demonstrating the importance of the HBP in the development of glucose intolerance.

In fasted animals, increases in the concentration of circulating pancreatic glucagon trigger the gluconeogenic program in part by stimulating the dephosphorylation of CRTC2 at Ser 171 (1, 2). CRTC2 is sequestered in the cytoplasm through an association with 14-3-3 proteins in animals fed ad libitum but translocates to the nucleus after its dephosphorylation, where it mediates transcriptional activation by binding to CREB.

In response to refeeding, increases in circulating concentrations of pancreatic insulin inhibit hepatic glucose production in part through the ubiquitin-dependent degradation of CRTC2 (3). The gluconeogenic program is constitutively induced in diabetes because of insulin resistance and chronic increases in circulating concentrations of glucose, which appear to increase gluconeogenic gene expression independently (4–6). Because a conserved cyclic adenosine 3’-5’ monophosphate (cAMP) response element (CRE) on the glucose-6-phosphatase (G6Pase) promoter is required for the transcription of that gene in response to glucose (7), we explored the role of CRTC2 in this process.

Fig. 1. Activation of CRTC2 in cells exposed to high concentrations of glucose. (A) (Top) Effect of glucose (0, 5, 25 mM: G0, G5, G25) and glucosamine (10 mM GlcN) exposure (6 hours) on amounts of G6Pase mRNA in primary mouse hepatocytes infected with adenovirally encoded unpaired Student’s t test. P < 0.05 for USi compared with CRTC2i cells; n = 6. (Bottom) Western blot showing effect of CRTC2 knockdown on amounts of CRTC2 protein relative to control cells. (For this and subsequent figures, the P value was determined using a two-tailed unpaired Student’s t test; P < 0.05 for USi compared with CRTC2i cells; n = 3). (B) Effect of Glu and GlcN with or without FSK (10 μM, 6 hours) on wild-type and CRE-mutant G6Pase-luciferase reporters (top) and on glucose output (bottom) from primary mouse hepatocytes. Cells were preincubated in glucose for 6 hours, washed, and then assayed for glucose release into glucose-free medium over a 1-hour period. (C) Effect of Glu and GlcN on CRE-luciferase reporter activity in USi- and CRTC2i-expressing mouse hepatocytes, as in (A). (P < 0.05 relative to control cells; n = 3). (Bottom) Western blot of phospho (Ser172) and total CRTC2 in hepatocytes exposed to Glu or GlcN. (D) Immunofluorescence analysis of CRTC2 localization in hepatocytes exposed to Glu, GlcN, or FSK for 6 hours and stained for endogenous CRTC2. DAPI staining shown to visualize nuclei. White arrows point to representative cells with nuclear CRTC2 staining.

1The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA. 2Cell Signaling Technology, 3 Trask Lane, Danvers, MA 01923, USA. 3The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.

*To whom correspondence should be addressed. E-mail: montminy@salk.edu

Clear accumulation of CRTC2, as detected by immunoblotting and immunofluorescence analyses (Fig. 1, C and D, and fig. S1).

Increases in the concentration of extracellular glucose modulate gene expression in part by triggering the O-glycosylation of certain transcriptional activators (8, 9). Thus, we investigated whether CRTC2 is O-glycosylated in hepatocytes. Amounts of O-glycosylated CRTC2 (OG-CRTC2) increased after treatment with Glu or related analogs (mannose, fructose, or galactose), as detected by Western blotting of CRTC2 immunoprecipitated with antisera that recognizes O-glycosylated proteins (10) (Fig. 2A and fig. S2). Glucose analogs and GlcN also stimulated CRE-luc activity in primary hepatocytes, but nonmetabolizable sugars (2-deoxyglucose and L-glucose) and glycolytic intermediates downstream of the HBP did not.

To determine whether the HBP is required for glucose-stimulated activation of CRTC2, we used inhibitors of L-glutamine fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme in this pathway (11). Compared with hepatocytes exposed to high glucose alone, cells incubated with the GFAT inhibitors 6-diazoo-5-oxo-L-norleucine (DON) or azaserine (AZA) had
lower amounts of OG-CRTC2 (Fig. 2B and fig. S3). DON and AZA also inhibited CRTC2 nuclear translocation and CRE-luc activation by glucose, supporting the idea that the HBP mediates effects of glucose on CRTC2 activation in hepatocytes.

O-glycosylation and phosphorylation often exert reciprocal effects on protein activity (12). Indeed, phosphorylated CRTC2 (P-CRTC2), detected by immunoblotting, was confined to the cytoplasm, whereas OG-CRTC2 was detected primarily in nuclear fractions of glucose-stimulated hepatocytes (Fig. 2C). In chromatin immunoprecipitation (ChIP) assays, amounts of OG-CRTC2 associated with the G6Pase promoter increased after exposure of cells to high concentrations of glucose (25 mM) (Fig. 2D and fig. S4). Blocking CRTC2 O-glycosylation with DON disrupted the effects of glucose on CRTC2 recruitment; and co-incubation of DON-treated cells with GlcN rescued both CRTC2 O-glycosylation and occupancy of the G6Pase promoter.

In mass spectrometry studies to characterize O-glycosylation sites in CRTC2, we identified six residues, two of which (Ser70 and Ser171) showed greater amounts of O-glycosylation by mutational analysis (Fig. 3A and fig. S5). A mutant CRTC2 protein containing Ala substitutions at both Ser70 and Ser171 was not detectably O-glycosylated after exposure of hepatocytes to high glucose concentrations. In resting cells, CRTC2 is sequestered in the cytoplasm by 14-3-3 proteins through phosphorylation at Ser\(^{70}\) and Ser\(^{171}\) by members of the AMP-activated protein kinase (AMPK) family of Ser-Thr kinases (13, 2). Consistent with the proposed role of O-glycosylation in blocking protein phosphorylation, alanine mutations at Ser\(^{70}\) and Ser\(^{171}\) disrupted the CRTC2:14-3-3 interaction and promoted localization of these mutant CRTC2 proteins to the nucleus (Fig. 3, B and C, and fig. S6). Phosphorylation-defective Ser\(^{70}\)Ala and Ser\(^{171}\)Ala mutant CRTC2 proteins were also more active than wild-type CRTC2 in stimulating CRE-luc activity under basal conditions (fig. S5). Conversely, substitution of Ser\(^{70}\) and Ser\(^{171}\) with aspartate, to mimic constitutive phosphorylation, rescued recruitment of CRTC2 to the G6Pase promoter in hepatocytes. Effects of GFAT inhibitor (DON) and glucosamine (GlcN) on O-glycosylation at Ser\(^{70}\) and Ser\(^{171}\) increases CRTC2 activity by disrupting 14-3-3 binding. (A) Immunoblot showing relative O-glycosylation of wild-type and mutant CRTC2 proteins containing two Ala substitutions at major O-glycosylation sites (S70,171A). HepG2 hepatocytes were exposed to low (5 mM) or high (25 mM) concentrations of glucose. (B) Relative binding of wild-type and Ser\(^{70}\)Ala mutant CRTC2 to 14-3-3 proteins in primary hepatocytes exposed to G25 or G25+DON (Don). (C) Immunocytochemical analysis showing subcellular localization of wild-type and Ser\(^{70}\)Ala or Ser\(^{171}\)Ala mutant CRTC2 proteins in control or FSK-treated HepG2 cells. DAPI staining shown to visualize nuclei (n = 3). (D) Effect of Glu and FSK exposure on G6Pase luc reporter activity in HepG2 hepatocytes expressing wild-type CRTC2 (WT) and O-glycosylation defective (S70A, S171A; S70,171D) CRTC2 constructs, or empty vector control (CON). Comparable expression of wild-type and mutant CRTC2 proteins was verified by Western blot. (\(^{\ast}\), P < 0.05 compared with 5 mM glucose; n = 3).
Fig. 4. Modulation of O-glycosylation and transcriptional activity of CRTC2 by OGT and GCA. (A) (Top) Coimmunoprecipitation assay of HEK293T cells using epitope-tagged CRTC2 and OGT proteins. Amounts of CRTC2 recovered from IPs of OGT shown. O-glycosylation of CRTC2 (OG-CRTC2) by OGT was detected by immunoblotting. (Bottom) Effect of Ad-OGT or control Ad-GFP on amounts of G6Pase mRNA in primary hepatocytes exposed to various concentrations (0, 5, or 25 mM) of glucose (Glu) or glucosamine (GlcN) (10 mM) for 6 hours. \( P < 0.05 \) for OGT compared with GFP-expressing cells; \( n = 3 \). (B) Effect of OGT small interfering RNA or unspecific control (USi) on amounts of OG-CRTC2 (top) and on gluconeogenic gene expression (G6Pase) (bottom) in hepatocytes exposed to various concentrations of Glu or GlcN. \( *, P < 0.05 \) relative to control; \( n = 3 \). (C) Effect of Ad-O-GlcNAcase (GCA), either alone or in combination with Ad-OGT on CRTC2 O-glycosylation (top) and CRE-luc activity (bottom) in hepatocytes exposed to GlcN as indicated for 6 hours. Wedge shape indicates increasing amounts of Ad-GCA. \( *, P < 0.05 \) relative to control; \( n = 3 \). (D) Effect of GCA inhibitor PUGNAn on amounts of OG-CRTC2 (top) and CRE-luc activity (bottom) in cells exposed to 5 mM or 25 mM Glu \( (*, P < 0.05 \) relative to control; \( n = 3 \)).

Fig. 5. Induction of gluconeogenesis by OG-CRTC2 in response to hyperglycemia. Each panel shows in vivo gluconeogenic profiles, which include hepatic CRE-luc activity (top left), blood glucose concentrations (top right), immunoblot of hepatic OG-CRTC2, P-CRTC2, total CRTC2, P-AKT, AKT, and OGT proteins (bottom left), and mRNA amounts for PEPCK and G6Pase (bottom right). (A) Gluconeogenic profiles of wild-type and db/db mice. \( (*, P < 0.05 \) relative to control; \( n = 4 \) mice per group for all panels). (B) Effect of Ad-GCA or control Ad-GFP on gluconeogenic profiles of wild-type and db/db mice. (C) Gluconeogenic profiles of Ad-GFP and Ad-OGT expressing mice fed ad libitum. (D) Effect of Ad-CRTC2 RNAi or unspecific RNAi (USi) on gluconeogenic profiles in Ad-GFP and Ad-OGT mice.
phosphorylation at these sites, disrupted effects of glucose on CRTC2 activity in hepatocytes, confirming the importance of these sites for transcriptional regulation in response to glucose (Fig. 3D).

O-glycosyl transferase (OGT) catalyzes the O-glycosylation of cellular proteins in response to activation of the HBP (9). In proteomic studies to identify CRTC2-associated proteins, we recovered OGT from IPs of CRTC2 (fig. S7). We confirmed the CRTC2:OGT interaction in IP studies using epitope-tagged OGT and CRTC2 constructs (Fig. 4A). Overexpression of OGT increased amounts of OG-CRTC2 and stimulated CRE-luc activity along with glioglycosylation gene expression (G6Pase) in hepatocytes (Fig. 4A and fig. S8). Conversely, RNAi-mediated knockdown of OGT blocked the effects of Glu or GlcN on CRTC2 glycosylation and on glioglycosylation gene expression (Fig. 4B).

Protein O-glycosylation by OGT is rapidly reversible in vivo through opposing effects of the deglycosylating enzyme O-GlcNAcase (GNA) (12). Expression of adenosinorally encoded GCA (Ad-GCA) in hepatocytes reduced amounts of OG-CRTC2 and disrupted CRE-luc activity in response to GlcN and to Ad-OGT (Fig. 4C). Conversely, treating cells with GCA inhibitors O-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino N-phenyl carbamate (PUGNAc) or streptozotocin (STZ) (14) increased amounts of OG-CRTC2 and stimulated CRE-luc activity (Fig. 4D and fig. S9). These results support the notion that OGT and GCA exert counter-regulatory effects on CRTC2 O-glycosylation and activation in hepatocytes.

We evaluated whether chronic increases in circulating glucose concentrations are sufficient to trigger CRTC2 O-glycosylation and glioglycosylation gene expression in vivo using insulin-resistant db/db diabetic mice and mice fed a high-fat diet (HFD). Db/db and HFD mice had higher glioglycosylation profiles, which include hepatic CRE-luc activity, glioglycosylation gene expression, circulating glucose concentrations, and amounts of hepatic OG-TORC2, than did control animals (Fig. 5A and figs. S10 and S11). Disrupting CRTC2 O-glycosylation in HFD and db/db animals through expression of hepatic Ad-GCA lowered the glioglycosylation profile (Fig. 5B and fig. S12). As a result, HFD and db/db mice expressing Ad-GCA showed increased glucose tolerance and insulin sensitivity. Although GCA could improve glucose homeostasis by deglycosylating components of the insulin signaling pathway, Ad-GCA expression in liver downregulated the glioglycosylation profile comparably to Ad-CRTC2i in streptozotocin-diabetic mice, in which hepatic insulin signaling is absent as a result of the destruction of insulin-producing pancreatic beta cells (fig. S13). Conversely, increasing OG-CRTC2 amounts through expression of Ad-OGT in liver enhanced glioglycosylation profiles in wild-type mice (Fig. 5C and fig. S14). We tested whether CRTC2 was required for Ad-OGT-mediated induction of the glioglycosylation program in RNAi knockdown studies. Relative to control Ad-OGT animals expressing unspecific RNAi (Ad-USi), Ad-OGT mice coinjected with Ad-CRTC2i had lower glioglycosylation profiles (Fig. 5D and fig. S14).

Chronic hyperglycemia is thought to contribute to the development of diabetes-associated complications in part by activating the HBP and increasing protein O-glycosylation at regulatory phosphorylation sites (12, 13). Reducing the O-glycosylation of CRTC2 and other metabolic regulators may improve glucose homeostasis and reduce long-term complications associated with this disease.

### References and Notes

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### Supporting Online Material

www.sciencemag.org/cgi/content/full/319/5868/1402/DC1

**Materials and Methods**

Figs. S1 to S14

**References**

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**Coiled-Coil Irregularities and Instabilities in Group A Streptococcus M1 Are Required for Virulence**

Case McNamara, Annelies S. Zinkernagel, Pauline Macheboeuf, Madeleine W. Cunningham, Victor Nizet, Partho Ghosh

Antigenically variable M proteins are major virulence factors and immunogens of the human pathogen group A Streptococcus (GAS). Here, we report the ~3 angstrom resolution structure of a GAS M1 fragment containing the regions responsible for eliciting type-specific, protective immunity and for binding fibrinogen, which promotes M1 proinflammatory and antiapoptotic functions. The structure revealed substantial irregularities and instabilities throughout the coiled coil of the M1 fragment. Similar structural irregularities occur in myosin and tropomyosin, explaining the patterns of cross-reactivity seen in autoimmune sequelae of GAS infection. Sequence idealization of a large segment of the M1 coiled coil enhanced stability but diminished fibrinogen binding, proinflammatory effects, and antibody cross-reactivity, whereas it left protective immunogenicity undiminished. Idealized M proteins appear to have promise as vaccine immunogens.

M proteins are major virulence factors of group A Streptococcus (GAS), a bacterial pathogen responsible for mild- to-life-threatening diseases against which no vaccines currently exist (1). Fibrils of ~500 Å-long M protein form a dense, covalently attached coat on the streptococcal surface (2, 3). Host proteins, such as fibrinogen (4), bind specifically to M proteins and block deposition of opsonic antibodies and complement, preventing phagocytic elimination of GAS by neutrophils (1, 5). A clone expressing the M1 antigenic variant of M protein emerged nearly three decades ago and has persisted as the leading cause of severe invasive GAS infection (6). Intact M1 and M1 fragments released by neutrophil proteases are sufficient to evoke pulmonary hemorrhage, inflammation, and tissue destruction that is characteristic of severe infection (7). These effects depend on M1 binding to fibrinogen, which triggers release of heparin binding protein (HBP), a mediator of vascular leakage, from neutrophils (7).

M proteins are also prominently associated with autoimmune sequelae of GAS infection, such as rheumatic fever, which is problematic for vaccine development (8) and remains a serious threat in the developing world. In rheumatic fever patients, potently immunogenic M proteins elicit cross-reactive antibodies and T cell receptors directed against host α-helical coiled-coil proteins, such as myosin and tropomyosin (1). Cross-reactivity is probably attributable to molecular mimicry, as M proteins appear to form coiled coils as well (2, 3, 9, 10). As with...