Carbohydrate response element-binding protein (ChREBP) is a glucose-responsive transcription factor that plays a critical role in the glucose-mediated induction of genes involved in hepatic glycosylation and lipogenesis. Circulating blood glucose levels affect ChREBP activity in hepatocytes largely by posttranslational mechanisms that include phosphorylation-dependent subcellular localization. Previously, we showed that ChREBP is retained in the cytosol by phosphorylation-dependent binding to 14-3-3 protein dimers and identified the α2 helix (residues 125–135) phospho-Ser140 domain as the primary 14-3-3 binding site (Sakiyama, H., Wynn, R. M., Lee, W. R., Fukasawa, M., Mizuguchi, H., Gardner, K. H., Repa, J. J., and Uyeda, K. (2008) J. Biol. Chem. 283, 24899–24908). To enter the nucleus in response to high glucose, ChREBP must bind importin-α; this heterodimer then forms a complex with importin-β to interact with the nuclear pore complex. In this work, we recharacterized the importin-α binding nuclear localization signal (NLS) of rat ChREBP, identifying it as an extended classical bipartite NLS encompassing minimally residues 158–190. Replacing Lys159/Lys190 residues of ChREBP with alanine resulted in loss of importin-α binding, glucose-stimulated transcriptional activity and nuclear localization. A secondary 14-3-3 protein binding site also was identified, the α3 helix (residues 170–190) phospho-Ser196 domain. Importin-α and 14-3-3 were found to bind competitively to this secondary site. These results suggest an important mechanism by which importin-α and 14-3-3 control movement of ChREBP in and out of the nucleus in response to changes in glucose levels in liver and thus further suggest that the extended NLS of ChREBP is a critical glucose-sensing, glucose-responsive site.

Carbohydrate response element binding protein (ChREBP) is a transcription factor that, in response to elevated blood glucose levels, increases transcription of multiple genes whose promoters contain carbohydrate-responsive elements. ChREBP is expressed at particularly high levels in liver. In liver, ChREBP increases expression of the liver pyruvate kinase (LPK) gene and all lipogenic enzyme genes resulting in the conversion of excess dietary carbohydrate into triglycerides that can be stored as fat (1, 2).

When circulating glucose levels are low, inactive ChREBP is mainly localized to the cytoplasm in liver hepatocytes (3). Glucose stimulation of ChREBP activity occurs at two levels, translocation of ChREBP from the cytoplast into the nucleus and transcriptional control (3). Previously, we showed that phosphorylation and dephosphorylation of residue Ser196 of ChREBP plays an important role in regulating the subcellular localization of ChREBP (3, 4). The underlying mechanism, however, has been unclear.

The shuttling of proteins such as ChREBP between the nucleus and cytosol is controlled by various soluble nuclear transport factors that bind the “cargo” proteins to be transported to mediate their passage through the nuclear pore complex. The nuclear transport factors bind to nuclear localization signals (NLS) and nuclear export signals (NES) within the sequence of the cargo proteins to effect their nuclear import and export, respectively (4). In addition to NLS- and NES-mediated transport, binding of 14-3-3 proteins to other, less well characterized sequences is an important additional mechanism that has been shown to facilitate both nuclear export and cytosolic retention of several cargo proteins (5, 6). It has been suggested that the latter may result in part from inhibition of nuclear reimport.

Previously, we determined that the N-terminal region of rat ChREBP (residues 1–251) controls both basal cytosolic and glucose-stimulated nuclear localization of ChREBP (7). Consistent with this observation, both an NES (residues 85–95, now “NES2”) and a classical NLS (residues 158–175) were initially identified within this region by computer analysis (1, 8, 9). Subsequently, we predicted three α helices within this region (7) and, more recently (10), a second leucine-rich NES site (residues 5–15, “NES1”). The originally identified NES2 (85–95) was noted to be nearly identical with the α1 helix (residues 84–95), and both NES1 and NES2 sites were determined to be essential for binding of CRM1 (exportin) for ChREBP export to the cytoplasm. The α2 helix we identified as a 14-3-3 binding site and found that 14-3-3 binding was facilitated by phosphorylation of both the nearby Ser140 as well as Ser196 residues. Phosphorylation-dependent interaction of 14-3-3 proteins with ChREBP was initially reported by Merla et al. (11), but they were unable to identify the involved site(s). The previously assigned classical NLS (residues 158–175) also was investigated in our previous report, and we determined that replacing all five
Extended NLS of ChREBP

basic residues within this region with alanine indeed disrupted importin-α binding. Importin-α binding to the N-terminal ChREBP region also was found to be decreased by phosphorylation of the Ser140 and Ser196 residues, and this effect was less pronounced in the absence of 14-3-3 (7). The mechanism for the apparent interference of 14-3-3 with importin-α binding, however, was not clear.

Prompted by recent structural and biochemical data that have shown classical NLSs of some proteins are much longer and more complex than thought previously (12, 13), we now have more extensively characterized importin-α binding to ChREBP. We report here that the NLS of ChREBP is an importin-α binding, extended bipartite classical NLS that extends minimally from Arg158 to Lys190. Of note, this extended NLS overlaps with the previously identified α3 helix (residues 170–190). We also report that the α3 helix and nearby phosphorylated Ser196 residue comprise a secondary 14-3-3 binding site. The competitive binding of 14-3-3 and importin-α to the α3 helix and extended NLS of ChREBP provides, at last, a mechanism for glucose sensing and for the observed role of cAMP-activated protein kinase-mediated phosphorylation of Ser196 in the glucose-dependent regulation of the nuclear/cytoplasmic localization of ChREBP as originally proposed in 2001 (3).

EXPERIMENTAL PROCEDURES

Chemicals and Vectors—All chemicals were purchased from Sigma unless otherwise indicated. Bacterial expression vectors for GST-tagged importin-α and mouse 14-3-3β were gifts from Dr. Y. Yoneda (Osaka University, Osaka, Japan) and that for human 14-3-3β was a gift from Dr. Steven L. McKnight (University of Texas Southwestern Medical Center, Dallas, TX). Mammalian expression vectors for FLAG-tagged or GFP-tagged rat ChREBP and FLAG-ChREBP N-terminal peptide (1–250) were those described previously (7). Site-specific mutants were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions.

Proteins and Peptides—GST fusion proteins were purified using glutathione-Sepharose (GE Healthcare) according to the manufacturer’s instructions. His-tagged mouse and human 14-3-3β were transformed into Escherichia coli strain BL21. Expression of the 14-3-3 proteins was facilitated by the addition of isopropyl 1-thio-β-D-galactopyranoside (0.1 mM) followed by incubation at 20 °C with shaking at 120 rpm for 16 h. His-tagged proteins were purified by affinity chromatography using nickel-nitrilotriacetic acid (GE Healthcare). Peptides used for isothermal titration calorimetry (Fig. 2 and Table 1) were synthesized by the Peptide Synthesis Group at the University of Texas Southwestern.

In Vitro Binding Assays of ChREBP and ChREBP Mutants with Importin-α and/or 14-3-3 Proteins—FLAG-tagged ChREBP, ChREBP N-terminal peptide (1–250), or indicated ChREBP mutants were expressed in HEK293T cells and purified from cell lysates by incubating with beads (Sigma Aldrich) bearing anti-FLAG antibodies (Sigma Aldrich) in buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 10 mM sodium fluoride, and 1% Nonidet P-40 for 1 h at 4 °C with gentle rocking. Beads with bound FLAG-ChREBP and/or ChREBP mutants were incubated with indicated concentrations of homogeneous preparations of bacterially expressed GST-tagged importin-α and/or 14-3-3β in 500 μl of the above reaction mixture containing 0.5% BSA for 1.5 h at 4 °C with gentle rocking. Under these conditions, we found that Mlx had little effect on the ChREBP-importin interaction. The beads were washed three times with reaction buffer without BSA, eluted with SDS-PAGE sample buffer, and then subjected to PAGE on 8 or 10% SDS-PAGE gels and transferred to Trans-Blot nitrocellulose membranes (Whatman) for Western blotting. The following primary antibodies were used to detect importin-α (anti-GST, Upstate Biologicals), endogenous 14-3-3 and exogenous His-tagged 14-3-3β (pan 14-3-3, Santa Cruz Biotechnology, sc-629), and FLAG-ChREBP (anti-FLAG, Sigma Aldrich). Horseradish peroxidase conjugated anti-rabbit or mouse IgG (Zymed Laboratories Inc.) was used as the secondary antibody, and bound proteins were visualized with an ECL Western blotting detection system.

Primary Hepatocyte Culture and Transfection with Luciferase Reporters—Male Sprague-Dawley rats were obtained from Jackson Laboratories. All animals used for this study was approved by the University of Texas Southwestern Medical Center and the Veterans Affairs Medical Center Institutional Animal Care and Use Research Advisory Committees. Isolation of primary hepatocytes from rat liver and subsequent transfection of the cells with luciferase reporter was performed as described previously (1). Briefly, 5 h after isolation, the culture medium was replaced with DMEM (5.5 mM glucose) with 1 mM insulin and 100 nM dexamethasone but without antibiotics. Attached cells (5 × 10⁵/well) were transfected by adding 0.2 μg of the pRL-TK reporter plasmid containing Renilla luciferase as an internal control, 2.0 μg of the experimental reporter pGL3-LPK plasmid, which expresses firefly luciferase, and 1.0 μg of FLAG-ChREBP or FLAG-ChREBP mutant expression plasmids using Lipofectamine 2000 (Invitrogen) diluted with Opti-MEM (Invitrogen). Four hours later, the medium was replaced with DMEM containing either 5.5 or 27.5 mM glucose supplemented with 1 mM insulin, 100 mM dexamethasone, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% dialyzed fetal bovine serum. Cells were incubated for an additional 20 h period, and the luciferase reporter activity was determined using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

Subcellular Localization of ChREBP—To determine the subcellular localization of ChREBP, hepatocytes were plated onto 35-mm glass-bottomed dishes (MatTek) coated with type I collagen at a density of 5.0 × 10⁵ cells per dish, and 3.2 μg of expression plasmid encoding wild-type or mutated versions of GFP-ChREBP were transfected into the attached hepatocytes by Lipofectamine 2000 as described for luciferase reporter assays. Four hrs later the cultures received fresh DMEM containing either a low or high glucose concentration and the cells were incubated for an additional 20 h. The cells were washed once with phosphate-buffered saline, and fixed with phosphate-buffered saline-buffered 4% formaldehyde for 15 min at room temperature followed by replacement with phosphate-buffered saline containing 50 mM glycine. The subcellular localization of GFP-ChREBP was determined using confocal laser
microscopy. Typically for each experimental point, three sets of ~100 fluorescent cells were counted and scored for subcellular localization in each of three independent experiments.

Isothermal Titration Calorimetry (ITC) Measurement—Isolated human 14-3-3β protein was dialyzed exhaustively against the ITC buffer containing 50 mM Tris-HCl (pH 7.35) and 0.1 mM EDTA. For measuring binding of ChREBP peptides, a solution of 200 μM of the ChREBP peptide in a syringe was injected in 8 μl increments into the reaction cell containing 1.4 ml of 25 μM of 14-3-3β protein (based on the homodimer for each 14-3-3 protein was dialyzed exhaustively against the ITC buffer) (MicroCal, Northampton, MA). Binding constants (Kₐ) and enthalpy (ΔHₒ) were calculated with ORIGIN v7.0 software (MicroCal). The concentration of 14-3-3 protein was determined by absorbance at 280 nm using a calculated extinction coefficient (mg⁻¹ ml⁻¹ cm⁻¹) of 25.7. Synthetic peptides were weighed as solids and dissolved into a ITC buffer solutions.

Data Analysis—Statistical analysis was performed using GraphPad Prism5 software (GraphPad Software, San Diego CA). Data were analyzed by one-way analysis of variance, followed by a Newman-Keuls post hoc test, and groups designated with asterisks are statistically different (*, p < 0.05; **, p < 0.01) from control.

RESULTS

NLS of ChREBP—The initially assigned classical NLS was proposed to extend from amino acid residue Arg¹⁵⁸ to Glu¹⁷² of mouse ChREBP and the human orthologue, WBSCR14 (9), the sequence is indicated in Fig. 1A. Reinspection of the surrounding sequence however suggested that the ChREBP NLS might belong to a more recently recognized extended bipartite form. To investigate this possibility, we first systematically mutated the lysine residues between Arg¹⁵⁸ and Lys¹⁹⁴ in pairs by substitution with alanine and determined the effects on importin-α binding in in vitro pulldown assays (Fig. 1B). Among the putative extended bipartite NLS lysine mutants of FLAG-ChREBP, K159A/K190A and K171A/K190A exhibited the greatest decrease in importin-α binding, 75% p = 0.004 and 65% p =

Extended NLS of ChREBP

FIGURE 1. Modulation of ChREBP importin-α binding, transcriptional activity, and nuclear localization by mutation of putative bipartite NLS lysine residues. A, residues 101–200 of ChREBP indicating locations of the "classical" NLS site, α2 and α3 helices, Ser¹⁴⁰ and Ser¹⁹⁶ phosphorylation sites, and lysine residues of the putative bipartite NLS. B, interaction of importin-α with ChREBP. WT FLG-FLAG-ChREBP and the indicated FLAG-ChREBP lysine mutants expressed in HEK293T cells were immunoprecipitated from cell lysates with anti-FLAG beads; GST-importin-α antibodies. These experiments were repeated three times, and a representative Western blot is shown. The bar diagrams were generated by densitometric scanning to quantitate importin-α binding to ChREBP. *, p < 0.01; **, p < 0.05. C, transcriptional activity of FLAG-ChREBP lysine, Ser¹⁴⁰, and Ser¹⁹⁶ mutants. DNA constructs expressing the indicated FLAG-ChREBP proteins, firefly luciferase under control of LPK promoter, and Renilla luciferase (an internal control) were co-transfected into primary cultured rat hepatocytes during a 4-h incubation in DMEM containing 5.5 mM glucose (Glc). Fresh medium containing either 5.5 (open bars) or 27.5 mM (filled bars) glucose was added, and the cells were incubated for an additional 20 h. One set of cultures received cAMP (0.1 mM) in addition. Luciferase activities were measured and expressed as firefly luciferase activity relative to Renilla luciferase activity. The values presented are the mean ± S.D. of the results of all five independent experiments. *, p < 0.05; **, p < 0.01. D, nuclear localization of ChREBP in primary hepatocytes. Primary cultured rat hepatocytes were transfected with DNA constructs expressing WT GFP-ChREBP or indicated GFP-ChREBP lysine mutants during a 4-h incubation in DMEM with 5.5 mM glucose. Fresh medium containing either 5.5 (open bars) or 27.5 mM (filled bars) glucose was added, and the cells were incubated an additional 20 h. The values presented are the mean ± S.D. of three sets of ~100 fluorescent cells. *, p < 0.05.
0.004, respectively. Importin-α binding to the double lysine substitution mutant K159A/K171A of the previously assigned NLS signal also was decreased but to a lesser extent (40%).

To investigate the effect of the lysine mutations on transcriptional activity of ChREBP, primary rat hepatocytes were co-transfected with a luciferase reporter under control of the ChREBP-dependent LPK promoter and the same FLAG-ChREBP lysine to alanine NLS mutants (Fig. 1C). The cells then were incubated an additional 20 h in medium containing either a low (5.5 mM) or high (27.5 mM) glucose concentration. For comparison, replicate cultures also were co-transfected with S140A/S196A and S140D/S196D FLAG-ChREBP mutants that mimic the fully dephosphorylated or phosphorylated forms of these phosphorylation sites. The loss of glucose-stimulated transcriptional activity observed with the S140D/S196D mutant was similar in magnitude to that obtained by cAMP treatment (Fig. 1C). No apparent effect on glucose-stimulated nuclear localization was observed with the shorter classical NLS K159A/K171A mutation or with the K159A/K189A ChREBP mutant compared with WT. Glucose-stimulated transcriptional activity also was nearly eliminated by the K171A/K189A bipartite mutation. In contrast, transcriptional activity of the K159A/K171A ChREBP mutation within the shorter classical NLS was only partially inactivated (39%) compared with wild-type (Fig. 1C). This is consistent with the less robust effect of this mutation on disruption of importin-α binding.

Effects of the same bipartite lysine mutations on basal and glucose-stimulated nuclear localization of WT GFP-ChREBP and GFP-ChREBP mutants in primary hepatocytes were examined using confocal microscopy (Fig. 1D). No apparent effect on glucose-stimulated nuclear localization was observed with the shorter classical NLS K159A/K171A mutation or with the K159A/K189A or K171A/K189A GFP-ChREBP mutants compared with WT. In contrast, nuclear migration of the K159A/K190A, K159A/K189A, K171A/K190A, and K171A/K189A GFP-ChREBP mutants in response to glucose was significantly lower (33–50%).

Isothermal titration calorimetry was used to determine the dissociation constant ($K_d$) for importin-α binding to ChREBP peptides containing the originally identified classical NLS (Fig. 2A) or the putative extended bipartite NLS (Fig. 2B). There was no measurable interaction between importin-α and the shorter NLS peptide, but importin-α bound to the peptide containing the extended NLS with a dissociation constant of $\sim 3.03 \mu M$ with a stoichiometry of $\sim 1.0$ NLS-peptide per importin-α monomer. Taken together, the results suggest that the NLS of ChREBP is a signature long classical NLS encompassing the key residues Lys$^{159}$ to Lys$^{190}$.

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14-3-3 Protein Dimers Bind to Two Sites on ChREBP in Vitro—Previously, we reported that endogenous 14-3-3 proteins in HEK293 cells bind to a region of ChREBP containing the α2 helix (residues 125–135, see Fig. 1A) and that this binding is facilitated by phosphorylation of the Ser$^{140}$ and Ser$^{196}$ residues (7). Moreover, in pulldown assays, the S140D/S196D ChREBP mutant bound significantly less importin-α than the S140A/S196A ChREBP mutant.
S196A mutant in the presence of exogenous 14-3-3β but not in the absence of 14-3-3β. This suggested that 14-3-3 and importin-α proteins may bind to the same, or over-lapping, regions of ChREBP.

To further characterize the binding of 14-3-3 to ChREBP, in vitro pulldown assays were used to examine 14-3-3 binding to full-length FLAG-ChREBP (WT) and the α2 helix mutants A129P/Y131P-ChREBP and Δ126–134 in which residues 126–134 are deleted (Fig. 3, left panel). Substituting proline for the Ala129 and Tyr131 residues leads to complete loss of the α2 helical structure as determined by circular dichroism (7). We noted that endogenous 14-3-3 proteins (14-3-3(end)) that remained bound to WT FLAG-CHREBP during its initial purification from HEK293 cell lysates and the exogenously added 14-3-3-His (14-3-3(exo)) were distinguishable owing to differences in electrophoretic mobility due to His tags in the latter. Endogenous 14-3-3 proteins were observed bound only to WT-ChREBP and not to either α2 helix mutant. In contrast, exogenous 14-3-3β added to the in vitro incubations bound both to WT and α2 helix ChREBP mutants, indicating that there might be an additional binding site. To localize the additional site, 14-3-3 binding to an N-terminal (1–250) FLAG-ChREBP peptide and α2 helix mutants of the N-terminal peptide (Fig. 3, right panel) was examined. The results were the same as those obtained with the full-length FLAG-ChREBP, indicating that the second binding site also was located in the N-terminal region of ChREBP.

When the concentration of exogenous 14-3-3β in reaction mixtures was varied, increasing amounts of 14-3-3β were bound to ChREBP in a sigmoidal fashion and reached a maximum saturation value at ~0.8 μM and with a K_s of ~0.4 μM (Fig. 4). The amount of bound endogenous 14-3-3 remained constant with the addition of increasing amounts of exogenous 14-3-3β from 0.07 to 1.71 μM, which suggests that the initially bound 14-3-3(endo) did not readily exchange with the added 14-3-3(exo) because of tight binding. At much higher 14-3-3(exo) concentrations (>10 μM), the initially bound endogenous 14-3-3 was displaced by 14-3-3(exo) suggesting that the off-rate is kinetically slow. Exogenously added 14-3-3 appears to bind to ChREBP with a single K_s value. This suggests that K_s values for the two sites are very similar or, possibly, that 14-3-3β(exo) is binding almost exclusively to the second site in the in vitro assays because the primary site is nearly saturated with the tightly bound, co-purified 14-3-3(endo). ITC measurements using peptides corresponding to the primary and secondary sites indicate that the K_s values are very similar, 0.43 μM (7) and 0.62 μM (Table 1), respectively, providing additional support for the former possibility. The lack of detectable endogenous 14-3-3 protein remaining bound to the secondary site of the WT and mutant FLAG-ChREBPs during the initial purification is likely to result because 14-3-3(endo) is not expressed at a concentration sufficient to saturate even the primary 14-3-3 binding site when the WT and mutant FLAG-ChREBP proteins are expressed in HEK293 cells at very high levels needed for purification. Because of toxicity problems, we have not found it possible to simultaneously overexpress 14-3-3β to a similar level in mammalian cells. Differences in endogenous and exogenous 14-3-3 binding to the secondary site are not likely to result from differing affinities of the various 14-3-3 isoforms. We previously determined that 14-3-3β is the predominant endogenous 14-3-3 isoform bound to ChREBP in vivo in liver.3

3 W. R. Lee and K. Uyeda, unpublished observations.
Although the structural requirements for 14-3-3 binding are not completely understood, we suspected that the secondary binding site for 14-3-3 might include the H923 helix (residues 170–190) and/or the Ser196 phosphorylation site. To evaluate the role of these motifs in 14-3-3 binding to the putative secondary site, we prepared a V177P/Y187P mutant of FLAG-ChREBP that partially disrupts the H923 helix and a combined H922/H923 helix mutant, A129P/Y131P-V177P/Y187P. The partial loss of the H923 helix resulted in a significant decrease in 14-3-3 binding in pulldown assays with more than saturating concentrations of exogenous 14-3-3, whereas the combined H922/H923 helix mutant lost the ability to bind 14-3-3 nearly completely (Fig. 5A). These results suggest the H922 and H923 helices of ChREBP are important determinants of the 14-3-3 primary and secondary binding sites, respectively. Both the H923 helix and combined H922/H923 helix mutations resulted in loss of glucose-stimulated ChREBP-dependent transcriptional activity and a modest but significant decrease in basal levels of reporter gene transcription in cells cultured with low glucose medium (Fig. 5B). Nuclear localization of the α2 helix and combined α2/α3 helix mutant was similarly decreased in response to high glucose and under basal low glucose conditions (Fig. 5C).

Isothermal titration calorimetry also was used to evaluate binding of 14-3-3β to a ChREBP peptide, residues 170–202, containing the α3 helix and either phosphorylated or dephosphorylated Ser196 (Table 1). The $K_d$ for 14-3-3 binding to the phospho-α3-Ser196 peptide was 0.62 μM, which is in good agreement with the $K_d$ (0.4 μM) of full-length WT ChREBP determined by pulldown assay (Fig. 4). However, the $K_d$ for the dephospho-α3-Ser196 peptide was 10-fold higher than that for binding to the phospho-α3-Ser196 peptide, indicating a significant decrease in affinity of 14-3-3 binding to the dephosphorylated form. The $K_d$ for 14-3-3 binding to phospho-α3-Ser196p 187–202, a ChREBP peptide containing phosphorylated Ser196 but in which most of the α3 helix region was deleted, was even higher than that for dephospho-α3-Ser196 170–202 peptide (Table 1). Taken together, these results suggest that the secondary binding site of 14-3-3 is comprised of the α3 helix-Ser196p region of ChREBP. Of note, this region overlaps extensively with the extended importin-α binding bipartite NLS site (see Fig. 7). Also of note, both the extended NLS and the α3 helix/

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**TABLE 1**

Dissociation constants ($K_d$) for interactions of human 14-3-3β protein with human ChREBP peptides as determined by isothermal titration calorimetry.

| Proteins                                      | $K_d$ (μM) |
|-----------------------------------------------|------------|
| 14-3-3β + α3-Ser196 (phospho-Ser196, including amino acid residues 170–198) | 0.62 ± 0.03 |
| 14-3-3β + α3-Ser196 (including amino acid residues 170–198) | 6.3 ± 0.41 |
| 14-3-3β + α3-Ser196 (phospho-Ser196, including amino acid residues 187–201) | 21 ± 1.1 |

Although the structural requirements for 14-3-3 binding are not completely understood, we suspected that the secondary binding site for 14-3-3 might include the α3 helix (residues 170–190) and/or the Ser196 phosphorylation site. To evaluate the role of these motifs in 14-3-3 binding to the putative secondary site, we prepared a V177P/Y187P mutant of FLAG-ChREBP that partially disrupts the α3 helix and a combined α2/α3 helix mutant, A129P/Y131P-V177P/Y187P. The partial loss of the α3 helix resulted in a significant decrease in 14-3-3 binding in pulldown assays with more than saturating concentrations of exogenous 14-3-3β, whereas the combined α2/α3 helix mutant lost the ability to bind 14-3-3 nearly completely (Fig. 5A). These results suggest the α2 and α3 helices of ChREBP are important determinants of the 14-3-3 primary and secondary binding sites, respectively. Both the α3 helix and combined α2/α3 helix mutations resulted in loss of glucose-stimulated ChREBP-dependent transcriptional activity and a modest but significant decrease in basal levels of reporter gene transcription in cells cultured with low glucose medium (Fig. 5B). Nuclear localization of the α2 helix and combined α2/α3 helix mutant was similarly decreased in response to high glucose and under basal low glucose conditions (Fig. 5C).
Ser<sup>196</sup> motifs are nearly identical (>98%) in mouse, rat, and human.

**Importin-α and 14-3-3 Compete for Binding to 14-3-3 Secondary Binding Site**—To investigate whether 14-3-3 and importin-α compete for binding to ChREBP, saturating levels of 14-3-3β and increasing amounts of importin-α were added to reaction mixtures, and binding of both proteins to WT FLAG-ChREBP was evaluated in pulldown assays (Fig. 6, left). At high concentrations, importin-α inhibited binding of exogenous 14-3-3β but not endogenous 14-3-3 proteins to ChREBP, suggesting that importin-α competes for 14-3-3 binding to the secondary rather than primary 14-3-3 site. Consistent with this suggestion, increasing amounts of importin-α similarly inhibited 14-3-3β binding to the A129P/Y131P helix mutant (Fig. 6, right) in which the primary 14-3-3 binding site is disrupted, as indicated by the lack of 14-3-3(end) binding to the α3 helix-Ser<sup>196</sup> mutant site.

**DISCUSSION**

To elucidate the mechanism(s) by which changes in glucose concentration regulate the subcellular localization of ChREBP, we have focused recently on the mechanisms of ChREBP nuclear export and import and, in particular, the function of three α helices identified previously in the N-terminal region of ChREBP (7). Based on data from the current study, we conclude that the actual NLS of ChREBP extends beyond the originally assigned NLS (residues 158–173) to include the predicted highly conserved α3 helix (see Figs. 1 and 7). The originally assigned classical NLS contains an N-terminal basic cluster linked to a C-terminal basic cluster by an 11-amino acid linker (in bold) (158RKPEAVVLEGNYKRR173), whereas the newly described NLS extends the C-terminal cluster of basic residues (LysLys) with an additional 16 amino acid linker to Lys<sup>190</sup> (in bold) (158RKPEAVVLEGNYKRRIEVVMREYHKRR1YYK<sup>190</sup>).

From a comparison with three-dimensional structures of other cargo proteins that interact with importins and CRM1 for import and export trafficking (12, 13), we can make a number of predictions concerning the molecular structures of the NLS and previously identified NES sites (10) that are localized within the N-terminal region (residues 1–250) of ChREBP. An important requirement for the location of NLS and NES is accessibility. The extended ChREBP bipartite classical NLS contains a high proportion of basic residues and is likely exposed for importin-α binding. Similarly, the leucine-rich NES1 (residues 5–15) (10) located at the N terminus consists of a disordered region that is likely to be accessible for CRM1 binding for export. The NES2 site of ChREBP is instead an α helix (7, 10). The region between the NES1 and NES2 sites however contains highly polar amino acids and a mixture of acidic and basic residues (eight Asp and eight Arg within the residues 15–84) that suggest its location on the surface of the ChREBP protein, consistent with the observed CRM1 binding to both NES1 and NES2 sites.

The current findings that the extended NLS overlaps with the α3 helix and our identification of the α3 helix-phosphorylated Ser<sup>196</sup> (α3 helix-Ser<sup>196</sup>(p)) as a secondary 14-3-3 binding site helps to explain our previous finding that 14-3-3 inhibits the nuclear import of ChREBP (7). The data suggest that importin-α and 14-3-3 directly compete for binding to the overlapping NLS and 14-3-3 secondary binding sites. Because importin-α is known to bind to extended peptide structures (4, 14, 15), whereas 14-3-3 proteins bind to phosphorylated sites and α helices (6, 16–18), a question may be raised as to how ChREBP can bind two proteins that recognize such different structures. One explanation is that this region of ChREBP adopts different conformations when bound to importin-α or 14-3-3. At one extreme, the α3 helix may completely unravel, and the ChREBP NLS may adopt a completely extended conformation when bound to importin-α. Previous structural studies have shown that a classical NLS peptide is in extended conformation while bound to importin-α but adopts an α-helical conformation when bound to a different partner (19, 20). It is also possible that the N- and C-terminal basic clusters of

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**Extended NLS of ChREBP**

**FIGURE 6.** Importin-α and 14-3-3 proteins compete for binding to wild-type ChREBP and the α2 helix mutant A129P/Y131P. Anti-FLAG beads were used to isolate WT FLAG-ChREBP or the A129P/Y131P mutant with bound endogenous (end) 14-3-3 from lysates of transfected HEK293 cells. Varying concentrations of purified importin-α were added to reaction mixtures in the presence of purified 14-3-3β (0.18 μM). Following incubation and washing as described in the legend to Fig. 1, bound proteins were analyzed by SDS-PAGE and Western blotting with FLAG, GST, and pan-14-3-3 antibodies. These experiments were repeated twice with similar results.

**FIGURE 7.** Residues 1–200 of ChREBP show locations of motifs and phosphorylation sites key for glucose-regulated nuclear import and export of ChREBP. The α1, α2, and α3 helices, the bipartite lysine importin-α binding NLS site, NES1 and -2, CRM1 (exportin) binding sites, and Ser<sup>146</sup> and Ser<sup>196</sup> phosphorylation sites are indicated. ChREBP of rat (24), mouse (3), and human (9) are compared.
ChREBP NLS are in extended conformation, whereas the linker includes the helical α3 helix. A partially helical linker is formally possible even though it has not yet been observed in the few structures of importin-α bound to bipartite NLS. Although it is clear that both the α3 helix and Ser196 phosphorylation site contribute positively to 14-3-3 binding, it is possible that 14-3-3 also can bind the phosphorylated site alone when the helix is not exposed because ITC measurements indicated that 14-3-3β also can bind to a short phosphorylated Ser196 peptide (residues 187–200), albeit with significantly increased $K_d$. The results suggest that a monomer of the 14-3-3 dimer binds to the primary site (α2 helix-140(p)) on ChREBP, and the other monomer could easily cover up the NLS site by binding to the phosphorylated Ser196.

The apparent differences in the binding sites of ChREBP between the endogenous 14-3-3 proteins and exogenously added 14-3-3β can be explained as follows. The difference is not due the fact that endogenous 14-3-3 was a mixture of three major 14-3-3 isoforms, including 14-3-3β, whereas the exogenously added 14-3-3 is homogeneous 14-3-3β. A reasonable explanation for the differences may be that the 14-3-3(end) bound tightly to the primary site (α2–140(p)) on ChREBP and does not easily exchange with 14-3-3(exo) in the in pulldown assay. This explains why only a single apparent $K_d$ value for the exogenous 14-3-3 binding was observed. The reason why the 14-3-3(end) bound to ChREBP only to the primary site and not to the secondary site is as follows: 1) either the amounts of 14-3-3 in vivo is limited and insufficient to saturate even the primary site or 2) the affinity of 14-3-3 to the primary site are significantly tighter than that for the second. However, the latter explanation is less likely because ITC measurements using the peptides corresponding to those sites produced similar binding affinities. It is clear that the interaction between ChREBP and 14-3-3 proteins is complex, and additional structural studies are required to understand exactly how 14-3-3 binds to the two sites and competes with importin-α on ChREBP. We recently have initiated an investigation of the three-dimensional structures of ChREBP-14-3-3 complexes by x-ray crystallography.

In Fig. 8, we present our proposed pathway for nuclear cytoplasmic shuttling of phosphorylated/dephosphorylated ChREBP. With a rise in glucose levels, the pentose shunt intermediate, xylulose5P (Xu5P), increases. This activates a specific protein phosphatase PP2AB8C, which leads to dephosphorylation of ChREBP, including Ser196 specifically (21) (data not shown in the figure). Xu5P is thus a glucose signaling compound for ChREBP activation. The resulting loss in 14-3-3 binding to the secondary site facilitates importin-α binding directly NLS site of dephospho-ChREBP, whereas the 14-3-3 dimer may either remain bound to the primary site or may dissociate completely. The ChREBP-importin-α complex then binds importin-β to enter the nucleus and turn on transcription of ChREBP-dependent genes under high glucose conditions. In response to a drop in glucose levels ChREBP is phosphorylated by protein kinase A in the nucleus on Ser196; ChREBP(p) then binds the 14-3-3 dimer and CRM1 to localize in the cytosol (Fig. 8). ChREBP complexed with 14-3-3 dimer remains in the cytosol under low glucose. Under the starved conditions, 14-3-3 proteins bind to α3 helix-S196(p) and masks a part of NLS to prevent importin-α binding and nuclear localization of ChREBP. Thus, in response to changes in circulating glucose levels, protein kinase A-mediated phosphorylation stimulated by glucagon/cAMP and dephosphorylation mediated by Xu5P-PP2A initiate nuclear export and import of ChREBP, respectively, and regulate the subcellular distribution in liver. It is interesting to note that ChREBP NLS variants investigated in these studies, which failed to show glucose-stimulated increases in ChREBP-dependent transcription and nuclear
translocation did not exhibit marked increases in either transcrip-
tion or nuclear localization under basal low glucose con-
ditions. Recently, Davies et al. (22) analyzed the effects of
mutating individual amino acids within all five “Mondo” con-
served regions (MCRs) of ChREBP, MCR1–4 are contained
within the N-terminal residues 1–250 of ChREBP but MCR5,
comprising residues 277–294 is outside of this region. Davies et al.
(22) found that most amino acid mutations within the
MCR1–4 domains were inactivating and distinct from their
previously reported effects of MCR1–4 deletion. However, they
concluded that the MCR5 region may be the target of repres-
sion under basal, low glucose conditions since the majority of
mutations within this domain increased ChREBP activity in
both low and high glucose conditions. Li et al. (23) also have
proposed that MCRV(MCR5) is responsible for repression of
ChREBP activity under low glucose conditions. These latter
suggestions are not consistent with data from our labora-
tory that find the N-terminal region of ChREBP consisting of resi-
dues 1–251 is both necessary and sufficient for regulation of
ChREBP subcellular regulation under basal and glucose-stimu-
lated conditions. These differences could be due to the differ-
ences in cell types because our investigations have used primary
hepatocytes, whereas other laboratories have examined cul-
tured cell lines of various types that do not respond to changes
in circulating glucose concentrations in the same manner as
liver. Davies et al. (22) also failed to identify the longer NLS in
their mutation studies because they mutated only the C-termi-
nal basic cluster. Their negative results also lend additional sup-
port for the conclusion that the NLS of ChREBP is an extended
bipartite classical NLS.

The regulation of ChREBP is complex. Results from the cur-
rent study, however, suggest that phosphorylation-regulated
interactions between ChREBP, importin-α, and 14-3-3 proteins
represent critical mechanisms of glucose-sensing and glu-
cose-responsive activation/inactivation of ChREBP. Although
additional regulatory mechanisms and functional domains are
well known to be important in regulating ChREBP DNA bind-
ing and transcriptional activities by the C-terminal region (resi-
dues 252–865 (7)), nuclear localization of ChREBP is critical
for the effects of these additional mechanisms to be realized. In
support of this conclusion, mutations of the NLS, α3 helix, and
Ser196 phosphorylation site were found consistently in this
study to coordinately affect ChREBP binding to importin-α and
14-3-3, nuclear localization, and transcription. In summary,
identification of the extended bipartite classical NLS and over-
lapping 14-3-3 site has elucidated a physiologically significant
mechanism regulating the glucose-dependent nuclear localiza-
tion and hence activity of ChREBP.

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