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Articles

Glucose as a Protein-Condensing Cellular Solute

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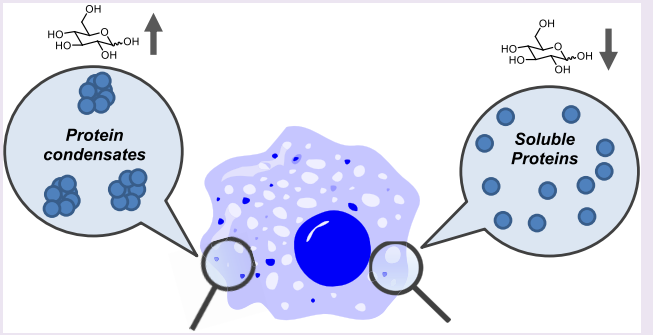
ABSTRACT: The present study reports a surprising protein-condensing effect of glucose, prompted by our accidental observation during chemical library screening under a high-glucose condition. We noticed "glucosing-out" of certain compounds, in which physiological concentrations of glucose induced compound aggregation. Adapting the "glucosing-out" concept to proteins, our proteomic analysis identified three cellular proteins (calmodulin, rho guanine nucleotide exchange factor 40, and polyubiquitin-C) that displayed robust glucose-dependent precipitation. One of these proteins, calmodulin, formed glucose-dependent condensates that control cellular glycogenolysis in hepatic cells. Our findings suggest that glucose is a heretofore underappreciated driver of protein phase separation that may have profound effects on cellular homeostasis.



INTRODUCTION

Phase separation has been shown to bolster the formation and disassembly of biological condensates that are capable of containing, protecting, and concentrating proteins without an encapsulating membrane.[1,2] The biological roles of such mesoscale protein condensates are particularly prominent in the regulation of enzymes: enzymes can be enriched with substrates to increase the turnover rate, or conversely, can be sequestered away from substrates to limit their activity.[3−9]

The formation and disassembly of these so-called membrane-less organelles are influenced by intracellular solutes.[10,11] Ions, nucleic acids, osmolytes, and surrounding proteins have been identified as abundant solutes that affect protein phase separation. Recent studies[12,13] have also shown that cellular adenosine triphosphate (ATP) serves not only as an energy source but also as a hydrotrope that solubilizes the biological condensates around its physiological concentration range of 2−8 mM.

Glucose is another abundant solute in the body and the main player in carbohydrate metabolism. Its hydroxyl group-rich structure resembles that of water clusters,[14] making it an excellent solute for body fluids. The physiological glucose concentration is highly dynamic. The blood glucose levels are regulated to around 5 mM in healthy fasting individuals. In diabetics, on the other hand, it increases to 11.1 mM, and postprandial blood glucose levels can reach as high as 20 mM.[15,16] However, whether alterations of cellular glucose levels affect the formation and disassembly of particular protein condensates remains unknown. The present study reports a surprising protein-condensing effect of glucose, prompted by our serendipitous observation during chemical library screening under a high-glucose condition.

RESULTS

Chemical Screening under a High-Glucose Condition.

The original goal of our project was completely different: the search for compounds that form coassemblies with glucose. However, as described below, the unexpected results of the chemical library screening under a high-glucose condition allowed us to propose that physiological concentrations of glucose have the intrinsic capability of aggregating, rather than coassembling, particular small molecules.

When a small molecule coassembles with glucose in water, it would be expected to form insoluble particles or increase the sizes of its preformed particles. Therefore, we screened an in-house chemical library of 8000 molecules by monitoring the particle size using a high-throughput dynamic light scattering (DLS) instrument (DynaPro Plate Reader II). This instrument permits quick estimation of dynamic light scattering in a 384-well format. The 8000−compound library contains relatively large-sized compounds (molecular weights > 500) that are enriched in hydrophobic and/or aromatic functional groups,

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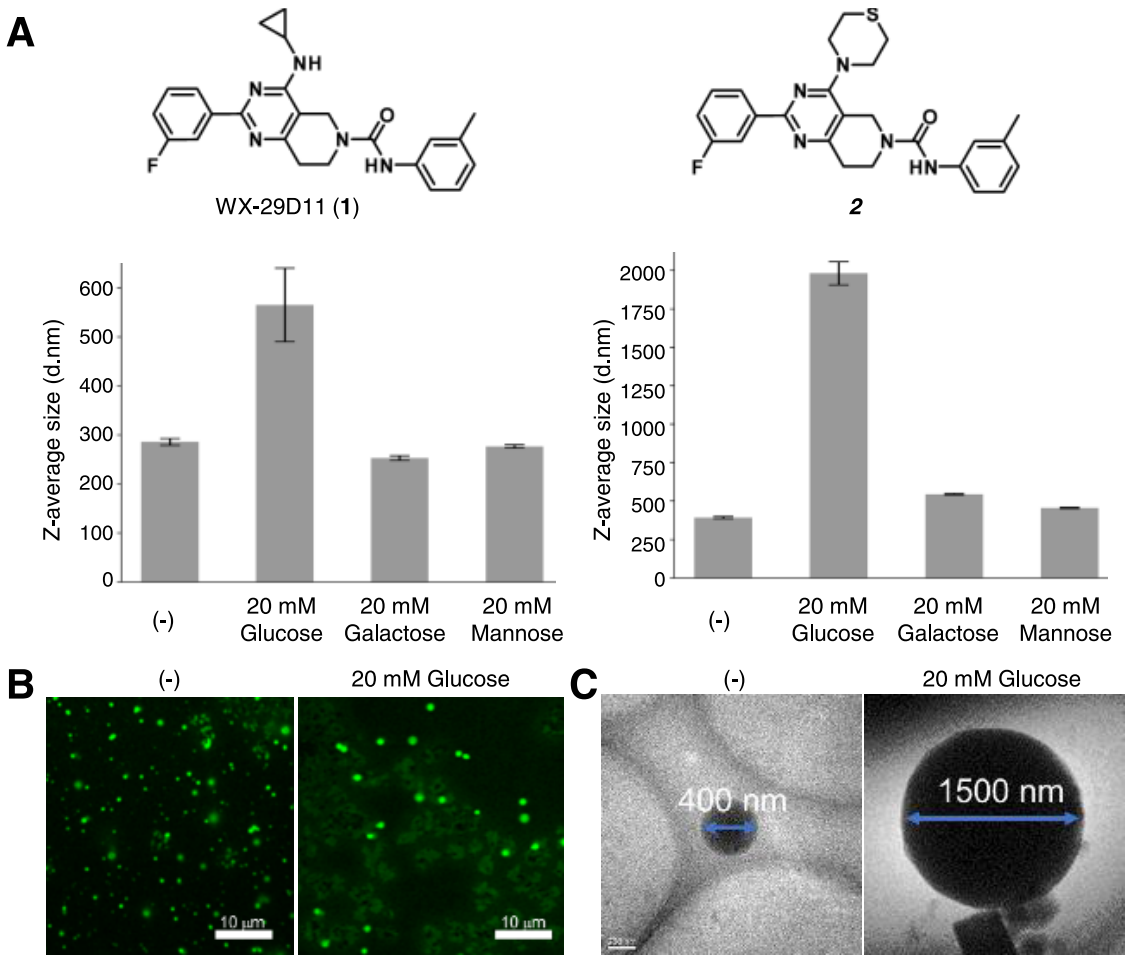

Figure 1. Discovery of WX-29D11 (**1**) and molecule **2**. (A) Z-average sizes of molecules **1** and **2** as measured by DLS (Zetasizer Nano S). Their chemical structures are also shown. The concentrations used for DLS were 50 μM. Data represent mean ± S.D. (n = 5). (B) Confocal images of 2 in PBS with (right) or without (left) 20 mM glucose. The assemblies were visualized by doping DCVJ. (C) Cryo-TEM images of 2 in PBS with (right) or without (left) 20 mM glucose.

given that self-assemblies of small molecules are formed often through hydrophobic interaction, $\pi-\pi$ stacking, and hydrogen bonding. None of the compounds displayed formation of detectable particles only in the presence of glucose. However, repeated measurements identified 50 compounds that showed more than a twofold increase in both particle size and scattering intensity in the presence of 20 mM glucose.

To obtain more accurate data, the particle sizes were re-evaluated in the presence or the absence of 20 mM glucose by a single-assay dynamic light scattering instrument (Zetasizer Nano S). We selected the three most effective and reproducible compounds for further studies and subsequently found that WX-29D11 (**1**) exhibits the highest selectivity for glucose over galactose or mannose (Figures 1A and S1). Structural optimization led to the discovery of molecule **2**, in which the cyclopropylamine group of WX-29D11 (**1**) was replaced with a thiomorpholine (Figure S2). This dihydropyridopyrimidine molecule (**2**) showed the largest increase in particle size in the presence of 20 mM glucose and the highest selectivity for glucose (Figure 1A).

Analysis of Molecule 2. To validate the results of the dynamic light scattering, we examined the particles of molecule **2** by confocal microscopy and cryo-transmission electron microscopy. Aggregates of molecule **2** were stained with a viscosity-dependent fluorescent probe, DCVJ, and observed under a confocal microscope (Figure 1B). While small nm-sized particles were formed in a glucose-free solution, larger particles of approximately 2 μm in size were detected in a 20 mM glucose solution. Cryo-transmission electron microscopic images revealed that 20 mM glucose induced the formation of 1.5 μm particles, while only smaller nanoparticles of 400 nm were detected in the absence of glucose (Figure 1C). These results were in agreement with those obtained from dynamic light scattering.

Our initial hypothesis was that molecule **2** forms a coassembly with glucose. X-ray single-crystal structure determination of molecule **2** alone depicted a network of intermolecular interactions among all of the four-ring structures, including π stacking of m-tolyl groups (Figure S3 and Table S1). To our surprise, we were unable to obtain cocrystals of molecule **2** and glucose under any conditions. Powder X-ray diffraction (PXRD) measurements showed that the X-ray scattering patterns of the assembly powder obtained from a mixture of the compound and glucose were completely identical to those of the powder obtained from the compound alone (Figure S4). In fact, ^{1}H NMR signals of the powder dissolved in DMSO-d_6 displayed only the signal from the compound but no detectable peaks from glucose (Figure S5). These results suggest that glucose does not coassemble with

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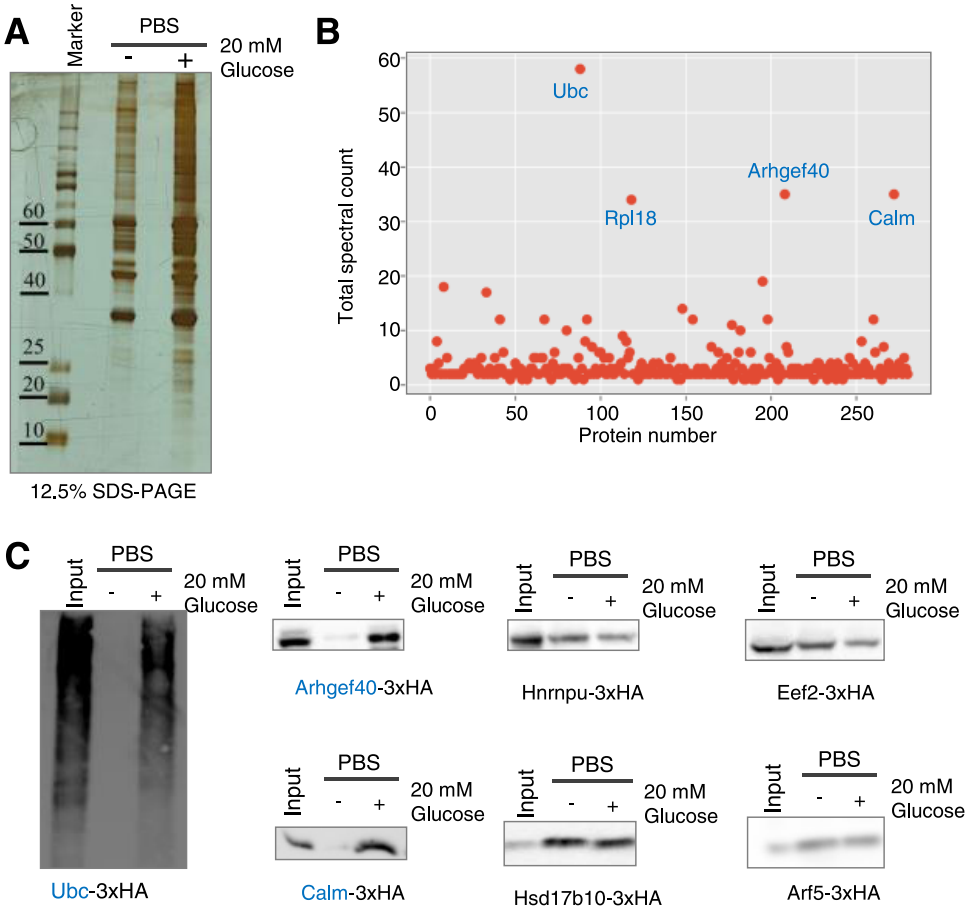

Figure 2. Proteomic analysis of glucose-induced precipitates. (A) Silver-stained SDS-PAGE gel of precipitated proteins with or without 20 mM glucose. Cell extracts of Hepa1-6 cells in PBS were incubated in the presence or the absence of 20 mM glucose for 24 h. After centrifugation, the precipitates were separated by SDS-PAGE. (B) Total spectral counts of 374 proteins that were precipitated selectively in the presence of 20 mM glucose. The identities of the four distinct proteins that showed a particularly robust glucose-dependent precipitation are shown. (C) Validation of the proteome analysis by western blotting analysis. Hepa1-6 cell lysates expressing each of the HA-tagged proteins were centrifuged in the presence or the absence of 20 mM glucose. The HA-tagged versions of polyubiquitin-C (Ubc), rho guanine nucleotide exchange factor 40 (Arhgef40), and calmodulin (Calm) exhibited glucose-dependent precipitation. Hnrnpu, Hsd17b10, Eef2, and Arf5 represent control proteins whose precipitations were detected equally in the presence and the absence of glucose in the proteome analysis; 10% inputs are also shown.

molecule 2 but rather promotes the aggregation of molecule **2** as a precipitant.

Such a salting-out-like effect of sugar is not completely new. Its high affinity for water increases the interfacial energy of water.[10,17] It has also been documented in the literature[18,19] that high-concentration glucose (*i.e.*, 50 g/L) displays a physicochemical effect called "sugaring-out," which drives water-soluble molecules out of the water into acetonitrile. However, these previous studies have all been performed under nonphysiological high concentrations of glucose. It was to our surprise that physiological concentrations of glucose have the intrinsic capability of aggregating particular small molecules. In the present study, we call this effect "glucosing-out," analogous to salting-out.

Proteome Screening under a High-Glucose Condition. The unexpected "glucosing-out" of certain small molecules prompted us to examine the possible existence of cellular proteins prone to form condensates under physiological glucose conditions. To search the proteome for any proteins that aggregate under glucose-rich environments, we decided to use proteomic lysates of hepatic cells. Hepatic cells are considered to be susceptible to glucose regulation because the liver is the central metabolic organ responsible for storing glucose and maintaining plasma glucose levels.

Cell extracts of mouse hepatocellular carcinoma cells, Hepa1-6, were incubated in the presence of 20 mM glucose for 24 h. After centrifugation, the precipitates were separated by SDS-PAGE and analyzed by silver staining (Figure 2A). The result showed more protein bands from the sample incubated in the presence of 20 mM glucose than from the glucose-free samples. LC/MS/MS analysis identified a total of 4745 precipitated proteins, 374 of which were precipitated selectively in the presence of 20 mM glucose (Figure S6A). Gene ontology (GO) analysis of the 374 proteins revealed that pathways related to protein phosphorylation were among the top pathways in high-glucose samples (Figure S6B). The enrichment levels of these proteins were ranked by their total spectral counts (Figure 2B and Supporting Excel file 1). Spectral counts refer to the total fragmentation spectra for peptides identified from a particular protein and provide a semiquantitative measure of protein abundance across numerous proteomic samples.[20] Four distinct proteins showed a markedly robust glucose-dependent precipitation: calmodulin (Calm), 60S ribosomal protein L18 (Rpl18), rho guanine

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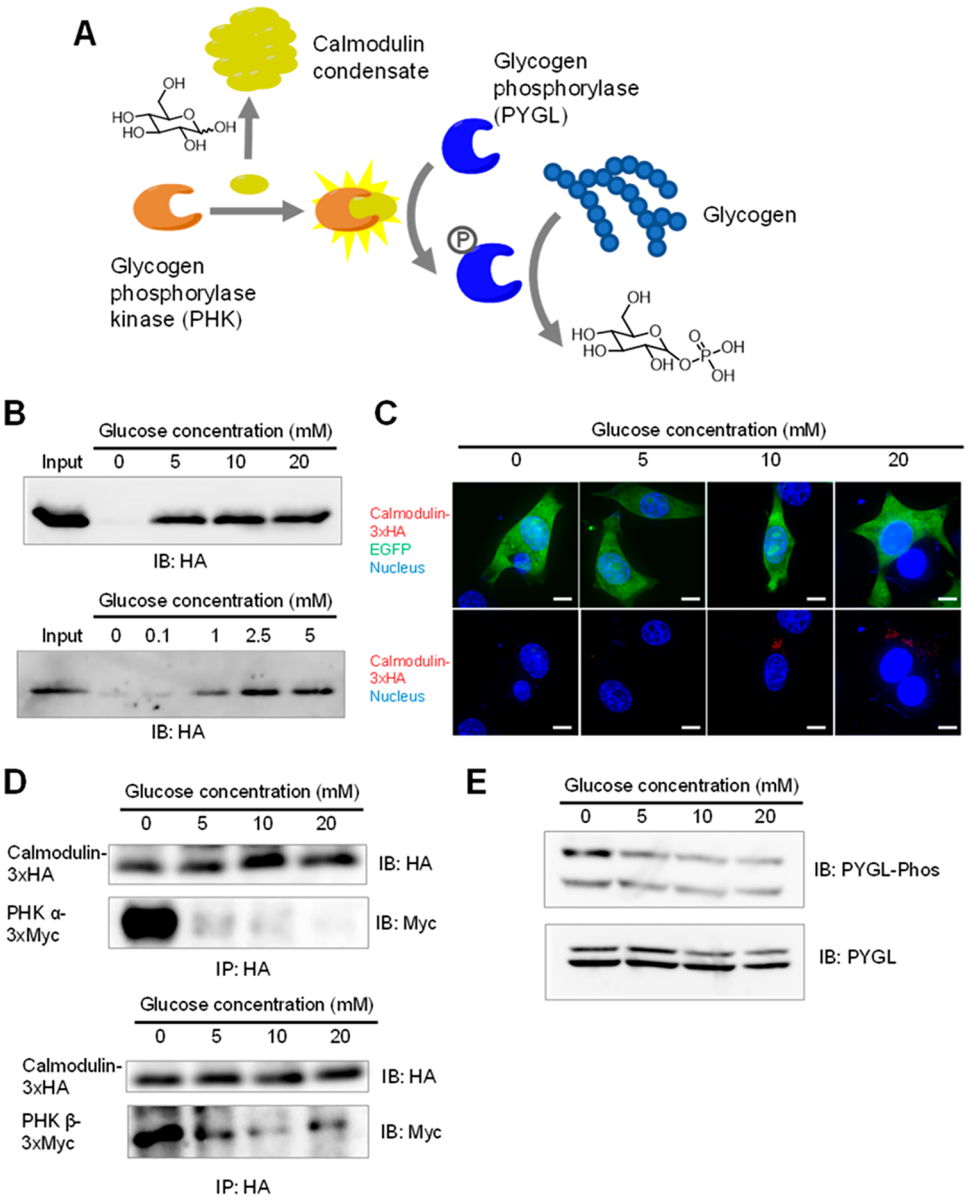

Figure 3. Glucose-dependent condensation of calmodulin *in vitro* and in cells. (A) Proposed role of calmodulin condensates in the regulation of glycogen degradation. (B) Glucose-dependent precipitation of HA-tagged calmodulin from Hepa1-6 cell lysates. (C) Microscopic observation of calmodulin condensates in Hepa1-6 cells. To locate transfected cells under a confocal microscope, EGFP was coexpressed in the cells. Localization of HA-tagged calmodulin was visualized using an anti-HA antibody. Scale bar, 10 μm. (D) Effects of glucose on the interaction between calmodulin and glycogen phosphorylase kinase (PHK). Lysates from HEK293 cells expressing calmodulin-3xHA together with PHK α-3xMyc or PHK β-3xMyc were incubated with glucose at the indicated concentrations, followed by coimmunoprecipitation using an anti-HA antibody. (E) Effects of glucose on phosphorylation levels of glycogen phosphorylase (PYGL). All samples were normalized by protein concentrations. The panels (B), (D), and (E) show representative results of at least two independent and reproducible experiments.

nucleotide exchange factor 40 (Arhgef40), and polyubiquitin-C (Ubc) (Figure 2B).

The glucose-dependent precipitations of the four proteins were corroborated by centrifugation and western blotting analysis of Hepa1-6 cell lysates expressing each of the HA-tagged proteins (Figure 2C). Although the expression of 60S ribosomal protein L18 was not detectable in Hepa1-6 cells for unknown reasons, the other three proteins were well expressed in Hepa1-6 cells and displayed a reproducible increase of precipitation in the presence of 20 mM glucose. Note that polyubiquitin-C is a linear ubiquitin that serves as a source of protein ubiquitination so that multiple bands for HA-tagged proteins were observed on an SDS-PAGE gel. In contrast, we detected no significant difference in precipitation extents for the HA-tagged versions of proteins whose precipitations were detected equally in the presence and the absence of glucose in the LC/MS/MS experiments (Figure 2C; Hnrnpu, Eef2, Hsd17b10, and Arf5). To rule out the effect of the tag on the protein properties, the experiments were also performed with the Myc-tagged proteins (Figure S7). The results were found to be similar to those obtained with the HA-tagged proteins. These results validated that 20 mM glucose induces the precipitation of at least those three proteins.

Glucose-Induced Condensation of Calmodulin. Of the three proteins, we focused on calmodulin, a calcium-binding protein that associates with and stimulates a class of protein

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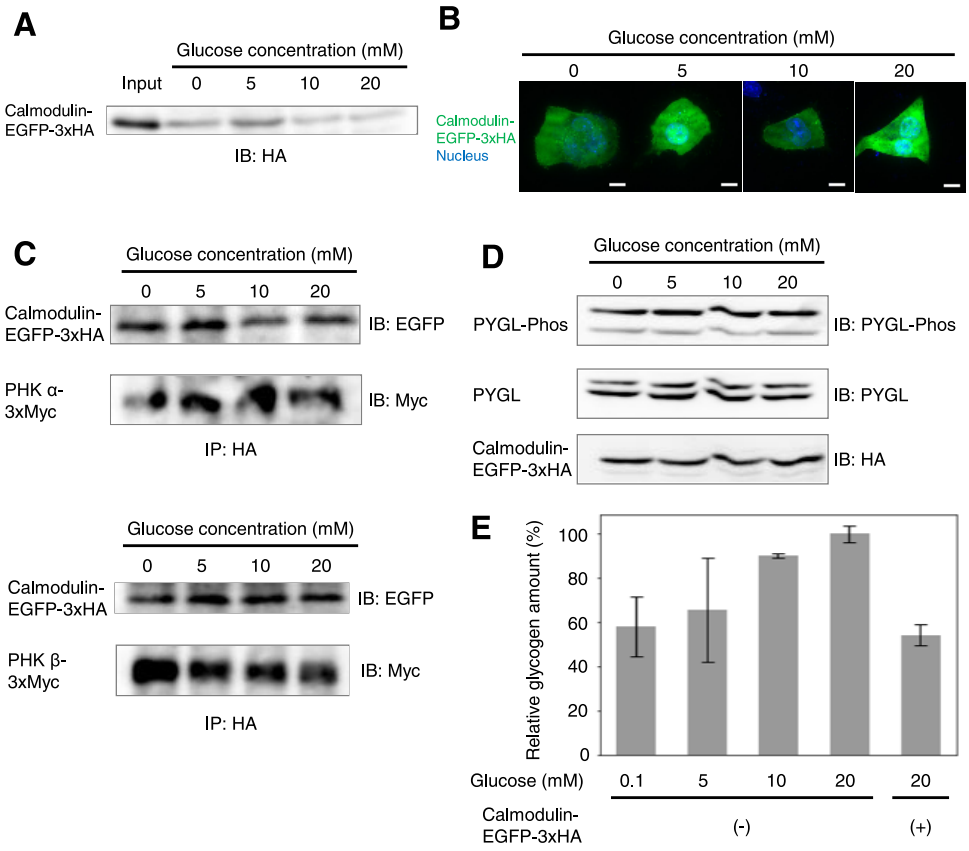

Figure 4. Calmodulin-EGFP-HA (calmodulin fused with EGFP and HA tag) as an aggregation-resistant calmodulin. (A) Western blotting analysis of precipitation of calmodulin-EGFP-HA. (B) Representative confocal images of calmodulin-EGFP-3xHA in cells. Scale bar, 10 μm. (C) Calmodulin-EGFP-3xHA interacts with PHK-3Myc even under high-glucose concentrations. Lysates from HEK293 cells expressing calmodulin-EGFP-3xHA together with PHK α-3xMyc or PHK β-3xMyc were incubated with glucose at the indicated concentrations, followed by coimmunoprecipitation using an anti-HA antibody. (D) Effects of glucose on phosphorylation levels of glycogen phosphorylase (PYGL) in cells overexpressing calmodulin-EGFP-3xHA. All samples were normalized by the protein concentration. (E) Glycogen amounts in Hepa1-6 cells. The cells were transfected by an expression vector encoding calmodulin-EGFP-3xHA or mock-transfected, and then incubated in DMEM containing varied concentrations of glucose for 8 h. All samples were normalized by protein concentrations. The panels (A), (C), and (D) show representative results of at least two independent and reproducible experiments.

kinases called calmodulin-dependent kinases in response to Ca^{2+} ions.[21] Its main role in hepatic cells is the stimulation of glycogen phosphorylase kinase (PHK), leading to the activation of glycogen phosphorylase (PYGL) that digests glycogen to regenerate glucose-1-phosphate (Figure 3A).[22,23] Although this glycogenolysis pathway in the liver has long been known to be regulated by calmodulin and intracellular Ca^{2+} in response to hormones,[24] it is not known whether glucose directly controls the calmodulin. We postulated that the glucose-dependent condensation may control the glycogenolysis-stimulating activity of calmodulin by cellular sequestration, as shown in Figure 3A.

We first investigated the glucose dependence of the calmodulin aggregation with concentrations of up to 20 mM glucose (Figure 3B). The data showed that HA-tagged calmodulin began to form precipitates at a concentration of 1 mM glucose in cell lysates and that the amount of precipitates increased along with the increase in the glucose concentration (Figure 3B). Interestingly, other monosaccharides (galactose and mannose) also induced precipitation of HA-tagged calmodulin at 20 mM (Figure S8). Glucitol, a glucose analog that lacks the aldehyde functionality, maintained the ability to elicit aggregation of HA-tagged calmodulin (Figure S9), ruling out the possibility that the imine adduct formation between calmodulin and glucose is responsible for the condensation. Comparison between the precipitated and supernatant fractions indicated that 20 mM glucose induced the precipitation of 13% of total calmodulin in the cell lysates (Figure S10). Considering such precipitation as an extreme form of protein condensation, we envisioned that glucose may influence the condensation status of a higher proportion of calmodulin.

Calmodulin, a small, highly conserved Ca^{2+} sensor, is composed of two domains, each with a pair of Ca^{2+} binding sites. We examined whether the calmodulin precipitation is affected by adding Ca^{2+} or EDTA (Figure S11). The results showed that addition of Ca^{2+} slightly increased the amounts of precipitation of HA-tagged calmodulin while that of EDTA had limited effects, suggesting that the Ca^{2+}-bound calmodulin is more prone to aggregate than Ca^{2+}-free calmodulin at least *in vitro*. RNA has been shown to tune the assembly of many phase-separating bodies.[25] RNase treatment had no detectable effects on the assemblies of HA-tagged calmodulin (Figure S11), suggesting that RNA is not required for the formation of the calmodulin assembly.

We next examined the ability of bacterially expressed, purified calmodulin to condensate *in vitro* in the presence of glucose (Figures S12 and S13). Although 1 μM calmodulin

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was apparently soluble in PBS, incubation with 20 mM glucose induced the time-dependent formation of Nile-Red-stained calmodulin condensates that are detectable under a confocal microscope, suggesting that the glucose-responsive precipitation of calmodulin does not require additional cellular factors. In line with this, cryo-transmission electron microscopic imaging of the Nile-Red-free samples (1 μM calmodulin, 20 mM glucose, 4 h) showed the formation of 200−600 nm particles of calmodulin (Figure S14).

To assess the intracellular formation of calmodulin condensates in response to glucose, we subjected Hepa1-6 cells overexpressing HA- or Myc-tagged calmodulin to high or low glucose conditions and performed fixed-cell protein immunofluorescence experiments (Figure 3C). To locate transfected cells under a confocal microscope, EGFP was coexpressed in the cells. HA-tagged calmodulin was dispersed under low glucose conditions (0−5 mM glucose). Under a high-glucose condition (10−20 mM glucose), in contrast, HA-tagged calmodulin formed numerous foci within the cells. Similar glucose-dependent assembly formation was observed with Myc-tagged calmodulin (Figure S15). Such puncta formation in glucose-rich conditions is not a common feature of cytoplasmic or nuclear HA- or Myc-tagged proteins, as a random sampling of other tagged proteins did not exhibit similar puncta formation in glucose-rich conditions (Figures S16 and S17). We also examined whether the increased focus numbers could be rescued by first subjecting cells to high glucose and subsequently culturing them in a low glucose medium. The result shows that glucose-induced calmodulin foci disappeared within 1 h (Figure S18). When the cells were cultured under a high-glucose condition again, calmodulin foci were restored rapidly, within 30 min (Figure S18). To accurately evaluate the size of the intracellular particles formed by calmodulin, the particles were observed under an ultrahigh-resolution fluorescence microscope. The tagged calmodulin was found to form particles of 200−500 nm diameter at 20 mM glucose (Figure S19). Taken together, these data suggest that calmodulin assembles into microscopically detectable foci in cells in response to glucose.

Potential Role of Calmodulin Condensates in Glycogenolysis. Calmodulin interacts with glycogen phosphorylase kinase to promote glycogen degradation.[26] To investigate the effect of glucose on this protein−protein interaction, we cotransfected HEK293 cells with expression vectors that encode HA-tagged calmodulin and Myc-tagged glycogen phosphorylase kinase and performed coimmunoprecipitation experiments as a function of glucose concentrations (Figure 3D). The results showed that the extent of coimmunoprecipitated glycogen phosphorylase kinase subunits (PHK-α and PHK-β) decreased in a glucose-dependent manner. Consistent with this result, we found that the phosphorylation levels of glycogen phosphorylase were significantly decreased in Hepa1-6 cells cultured in a high-glucose medium (Figure 3E). These results suggest that the glucose-induced condensation of calmodulin sequesters and insulates it from interacting with glycogen phosphorylase kinase, thereby decreasing phosphorylation levels of glycogen phosphorylase.

To confirm whether these glucose effects are mediated by aggregate formation of calmodulin, we searched for a calmodulin variant that is resistant to glucose-induced aggregation. Fortunately, calmodulin-EGFP-3xHA (HA-tagged calmodulin fused with EGFP) was found to exhibit no detectable glucose-dependent precipitates in Hepa1-6 cell lysates (Figure 4A). In line with this observation, calmodulin-EGFP-3xHA displayed no detectable particles in live-cell imaging even at 20 mM glucose (Figure 4B). Presumably, the fusion with high-molecular-weight EGFP (27 kDa) influenced the net physical properties of calmodulin to the extent that it can no longer be precipitated by glucose.

The aggregation-resistant calmodulin allowed us to evaluate the importance of calmodulin condensation in glycogen regulation. Calmodulin-EGFP-3xHA and Myc-tagged glycogen phosphorylase kinase were cotransfected into HEK293, followed by coimmunoprecipitation under varied glucose concentrations (Figure 4C). The results showed that calmodulin-EGFP-3xHA remained associated with glycogen phosphorylase kinases (PHK-α and PHK-β) regardless of glucose concentrations. Consistent with this, phosphorylation levels of glycogen phosphorylase were maintained at a high level in calmodulin-EGFP-3xHA-expressing Hepa1-6 cells cultured in a high-glucose medium (Figure 4D). Finally, we investigated the effect of calmodulin aggregate formation on cellular glycogen levels. Hepa1-6 cells were cultured under various glucose concentrations for 8 h. Quantification of cellular glycogen revealed that the amount of glycogen increased as the glucose concentration of the medium increased (Figure 4E). In contrast, the glucose-induced increase of glycogen was canceled by overexpressing calmodulin-EGFP-3xHA (Figure 4E). These results support the idea that the glycogen content in hepatic cells is regulated by glucose-dependent aggregation of calmodulin.

DISCUSSION

Our original intention of the chemical library screening was the search for compounds that form coassemblies with glucose. For such a goal, the screening concentration of the library compounds may ideally be arranged close to that of glucose. However, the generally poor water solubility of the library compounds enforced us to use 50 μM as a screening concentration, which is hundreds of folds lower than the glucose concentration (20 mM). This undesirable screening arrangement led to the unexpected detection of the compound that is aggregated by glucose rather than coassembled with glucose. Although such "sugaring-out" effects of high-concentration glucose (*i.e.*, 50 g/L) have been documented in the literature,[18,19] our small-molecule results indicate that physiological concentrations of glucose have the capability of aggregating particular small molecules. In the present study, the glucosing-out effects on small molecules served as a motivation for investigating the glucose-induced phase separation of proteins.

Protein phase separation is frequently triggered by cellular stresses. The best-documented example is stress granules,[27,28] 100−200 nm-sized dense condensates composed of proteins and RNA that are formed by a range of cellular stress including heat,[29] cold,[30] oxidative,[31] and hyperosmotic stress.[32−34] Other condensations of metabolic enzymes under nutrient deprivation have also been proposed. One of these intracellular assemblies is known as "purinosomes," which are condensed forms of six enzymes required for purine biosynthesis.[35] Other nutrient-sensitive membrane-less condensates are G-bodies (also called glucosomes), which are formed under hypoxic stress to colocalize glycolytic enzymes and enhance the rate of glycolysis.[36] The formation of G-bodies is controlled not directly by glucose but by the activation status of AMP-activated protein kinase (AMPK), an energy sensor kinase.[37]

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In the present study, we found the direct protein-condensation effect of glucose and proposed its role in glycogenolysis. Our study revealed that HA-tagged calmodulin began to form precipitates at 1 mM glucose and reached a plateau at 5 mM glucose in cell lysates (Figure 3B). The limitation of our study is that our study does not directly measure the intracellular glucose concentration in the cell line that we used (Hepa1-6 hepatic cells). Hepa1-6 cells express GLUT2, an insulin-independent and potent glucose transporter,[38] which may be translated to relatively high levels of intracellular glucose in Hepa1-6 cells. Although the intracellular glucose levels may be cell-type dependent, it has been reported that the glucose concentration in Hela cells can increase up to 3−4 mM, which is within the range of controlling the calmodulin condensation.[39,40] Our finding suggests an unprecedented mechanism of controlling energy metabolism and calmodulin through glucose-induced protein condensation. Our study does not preclude, and may even suggest, the possibility that other calmodulin-related biological events are controlled by intracellular glucose. This will remain a focus of the further investigation.

The present study raises other questions that will serve as a foundation for future in-depth investigations. Foremost, we do not understand why calmodulin condensation is highly responsive to glucose. Although calmodulin is a highly acidic protein, many of the acidic residues are engaged in coordinating the four calcium ions. The rest of the acidic residues, as well as other surface polar residues, are likely to be hydrated.[41,42] Glucose may possibly compete for hydrating water to decrease hydration levels of calmodulin. The decreased hydration enables surface polar residues to engage in intermolecular interactions during aggregation. It may also lead to protein precipitation by increasing surface exposure of hydrophobic regions.[43] Several X-ray crystal structures of calcium-loaded calmodulin have been determined, including those in the native unbound form and those with target-bound domains. Of these structures, two extreme conformations have been seen: one that is extended or dumbbell-shaped with the two lobes, each containing a pair of Ca^{2+} ions and a hydrophobic patch, separated by a long exposed "linker" helix,[41,42] and the other that is very compact and globular owing to the uncoiling of a segment of the linker helix that juxtaposes the two lobes.[44,45] Which of the two extreme structures or variations thereof is involved in the glucose-induced calmodulin aggregation is unknown, though it could favor the extended structure with the hydrophobic patches and more exposed polar groups.

Our study uncovered two other proteins (polyubiquitin-C and rho guanine nucleotide exchange factor 40) whose glucose-dependent aggregation is particularly robust. However, these distinct proteins share no obvious physical or functional properties with calmodulin. Clearly, more work will be required to determine the precise underlying mechanism of glucose-induced protein condensation. The biological roles of glucose-dependent aggregation of the other two proteins also remain unknown and are currently under investigation.

Another interesting issue is the relationship with ATP, a chaotropic molecule that is known to inhibit the formation of protein aggregates.[12,13] Our results suggest that glucose serves as a cosmotropic molecule and thus may counteract the effect of ATP. It is appealing to speculate that the balance between glucose and ATP, both of which are abundant in the cell, may regulate biomolecule aggregation in cells.

CONCLUSIONS

Our findings have revealed a novel role of glucose in driving protein phase separation that may have profound effects on cellular homeostasis. This role is exemplified by the discovery of the glucose-dependent condensates of calmodulin as a mechanism to control cellular events in hepatic cells. The glucose-dependent condensation may be relevant to a broad range of glucose-responsive cellular events. This underscores the need for future studies to address their physiological roles.

METHOD

LC/MS/MS Analysis of Glucose-Induced Protein Precipitations. Confluent Hepa1-6 cells (10 cm dish) were collected and washed with ice-cold 1× PBS, followed by sonication. The cell lysates were centrifuged (15 000 rpm, 4 °C, 30 min). The supernatant (200 μL) was incubated in PBS (400μL) with or without 20 mM glucose at 4 °C under mild rotary agitation overnight. The samples were centrifuged (15 000 rpm, 4 °C, 30 min). The pellets were mixed with a 6× sample buffer solution (Nacalai, product no. 09499-14), resolved on a 12.5% SDS-PAGE gel, and visualized by silver staining (Wako Silver Stain MS kit; 299-58901). For shot-gun LC/MS/MS analysis, gel lanes were excised and digested in-gel by trypsin. The digested peptides were desalted with in-house-made C18 stage-tips, dried under a vacuum, and dissolved in 2% acetonitrile and 0.1% formic acid. The peptides mixtures were then fractionated by C18 reverse-phase chromatography (3 μm, ID 0.075 mm × 150 mm, CERI). The peptides were eluted at a flow rate of 300 nL/min with a linear gradient of 5−35% solvent B over 40 min, and then to 90% for 5 min. The compositions of solvents A and B were 0.1% TFA in water and 100% acetonitrile, respectively. The molecular masses of the resulting peptides were searched against the mouse amino acid sequence data set (Uniprot Proteome mouse-20190611; 55,197 sequences, 22986518 residues) using the MASCOT version 2.6 (Matrix Science) with a false discovery rate (FDR) set at 0.01. Carbamidomethylation of cysteine was set as a fixed modification, and oxidation of methionine and acetylation of protein N-termini were included as variable modifications. Identified proteins were analyzed by Scaffold.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschembio.1c00849.

Methods for experiments, chemical synthesis, NMR and HRMS data (PDF)

Total spectral counts of 374 proteins (XLSX)

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Author Contributions

N.N. and M.U. conceived and designed this research; N.N. performed the majority of the experiments; Y.J, G.A., and S.S. assisted in the plasmid construction and analyses of precipitation experiments; N.N., T.O., and H.K. conducted cryo-TEM analyses; N.N., Y.M., and N.T. performed the crystallization and its data interpretation; M.H. performed the PXRD and its data interpretation; N.N. and F.I. performed the ultrahigh-resolution fluorescence microscopy analysis; and N.N. and M.U. cowrote the manuscript. All authors analyzed the data and checked the manuscripts.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ATP, adenosine triphosphate; DLS, dynamic light scattering; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DCVJ, 9-(2,2-dicyanovinyl)julolidine; Cryo-TEM, cryogenic-transmission electron microscopy; Arhgef, rho guanine nucleotide exchange factor 40; Calm, calmodulin; Ubc, polyubiquitin-C; Hnrnpu, heterogeneous nuclear ribonucleoprotein U; Eef2, elongation factor 2; Hsd17b10, 17β-hydroxysteroid dehydrogenase type 10/short-chain L-3-hydroxyacyl-CoA dehydrogenase; Arf5, ADP-ribosylation factor 5; LC/MS/MS, liquid chromatography with tandem mass spectrometry; PYGL, glycogen phosphorylase; PHK, glycogen phosphorylase kinase; EDTA, ethylenediaminetetraacetic acid; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; HA, hemagglutinin; Myc, myelocytomatosis virus; EGFP, enhanced GFP; GFP, green fluorescent protein

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