Global Phosphoproteomics Identifies a Major Role for AKT and 14-3-3 in Regulating EDC3*

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Insulin plays an essential role in metabolic homeostasis in mammals, and many of the underlying biochemical pathways are regulated via the canonical phosphatidylinositol 3-kinase/AKT pathway. To identify novel metabolic actions of insulin, we conducted a quantitative proteomics analysis of insulin-regulated 14-3-3-binding proteins in muscle cells. These studies revealed a novel role for insulin in the post-transcriptional regulation of mRNA expression. EDC3, a component of the mRNA decay and translation repression pathway associated with mRNA processing bodies, was shown to be phosphorylated by AKT downstream of insulin signaling. The major insulin-regulated site was mapped to Ser-161, and phosphorylation at this site led to increased 14-3-3 binding. Functional studies indicated that induction of 14-3-3 binding to EDC3 causes morphological changes in processing body structures, inhibition of microRNA-mediated mRNA post-transcriptional regulation, and alterations in the protein-protein interactions of EDC3. These data highlight an important new arm of the insulin signaling cascade in the regulation of mRNA utilization.  

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Insulin plays an essential role in regulating fuel homeostasis, cellular differentiation, and growth (1). One of the primary signaling cascades activated by insulin is the PI3K1/AKT pathway. This pathway mediates many of the metabolic actions of insulin via phosphorylation of key metabolic substrates such as glycogen synthase kinase-3 for glycogen synthesis (2), FOXO1 for transcription (3), and TSC2 for protein synthesis (4). Intriguingly, insulin also plays a major role in mRNA decay and translation repression; however, the signaling molecules linking insulin with these pathways have not been fully elucidated (5–9). Several important mechanisms exist by which cells target mRNAs for post-transcriptional regulation including AU-rich element-binding proteins and through the actions of microRNAs (miRNAs) (10–12). The targeted mRNAs are then regulated by degradation or by inert reversible storage in cytoplasmic protein complexes called processing bodies (p-bodies) (13, 14). Such stored complexes may serve as a readily releasable reservoir of mRNA that could theoretically be rapidly mobilized for translation under anabolic conditions such as insulin stimulation. Enhancer of mRNA decapping 3 (EDC3) is a p-body protein that has previously been shown to have a role in regulating the removal of the mRNA 5' cap structure (15). To achieve this function, EDC3 interacts with a number of other proteins through its three domains (16, 17). The N-terminal Lsm domain is responsible for p-body targeting of EDC3 and can mediate interactions with DCP1A/B (16, 17). The central FDF domain has no known function but enables the interaction of EDC3 with the RNA helicase DDX6 and the decapping enzyme DCP2, which functions in conjunction with DCP1A/B to remove the 5' cap structure from mRNA (16, 17). The C-terminal Yjef homology domain is responsible for the homodimerization of EDC3 that may be important for its function (17, 18). Once the 5' cap structure is removed from the mRNA, it is quickly degraded by exonucleases such as XRN1, which is also present in p-bodies (19).

The insulin-regulated AKT substrate repertoire identified to date reflects the diversity of the actions of insulin in relevant target cells (20). Many AKT substrates undergo 14-3-3 binding upon phosphorylation, and so these two processes may be linked in modulating protein function (21). In the present study, we took advantage of the high affinity of 14-3-3 for AKT substrates combined with quantitative mass spectrometry methods based on stable isotope labeling with amino acids in cell culture (SILAC) (22, 23) to search for novel actions of insulin. Here, we report the identification of a novel insulin-regulated phosphoprotein, EDC3, a component of p-bodies...
(13, 16), thus revealing a novel action of insulin on mRNA post-transcriptional regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—NIH-3T3 murine fibroblasts, L6 fibroblasts, and Chinese hamster ovary (CHO) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen, Mycological-Plus FCS was from Trace Scientific (Melbourne, Australia), and antibiotics were from Invitrogen. Paraformaldehyde was from ProSciTech (Thuringowa Central, Australia). Insulin was obtained from Calbiochem, and BSA was from USB Corp. (Cleveland, OH). Bicinchoninic acid (BCA) reagent, Supersignal West Pico chemiluminescent substrate, and protein G-agarose beads were from Pierce. Lipofectamine and Plus Reagent were from Invitrogen. PVDF membrane was from Millipore (Billerica, MA). Trypsin was from Promega (Madison, WI). C_{18} cartridges were from Michrom Bioresearches, Inc. (Auburn, CA). Magic C_{18} material was from (Alltech, Deerfield, IL). Complete protease inhibitor mixture tablets were from Roche Applied Science.

All other materials were obtained from Sigma. Antibodies were kindly provided by Dr. Jens Lykke-Andersen (EDC3 and DCP1A; University of Colorado). Antibodies were purchased from Novus Biologicals (EDC3; Littleton, CO), MBL International (DDX6; Woburn, MA), Santa Cruz Biotechnology (Heldis, HMGA-2, and pan-14-3-3; Santa Cruz Biotechnology), Cell Signaling Technology (phospho-AKT substrate, AKT, AKT Ser(P)-473, and GST; Danvers, MA), and Sigma (FLAG M2). Antibodies against AS160 have been described previously (24). Cy5- and Cy3-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa Fluor 680-conjugated secondary antibodies were obtained from Invitrogen. IrDye 800-conjugated secondary antibodies were obtained from Rockland Immunochemicals (Gilbertsville, PA). HRP-conjugated secondary antibodies were from GE Healthcare. Active AKT2 for the in vitro kinase assay was from Cell Signaling Technology.

**Production of Rabbit EDC3 Ser(P)-161 Antibody**—The antibody specific against EDC3 Ser(P)-161 was produced by 21st Century Biochemicals. Briefly, rabbits were inoculated with EDC3 Ser(P)-161 peptides encoding amino acids 154–169 of mouse EDC3 conjugated to keyhole limpet hemocyanin carrier protein five times over a 72-day period. Subsequently, the phosphospecific antibody was affinity-purified by removal of antibodies that bind the non-phosphorylated peptide and then affinity purification using the phosphorylated peptide.

**Cell Culture**—NIH-3T3 fibroblasts (25), L6 cells (26), HEK 293T, and CHO IR/IRS-1 cells (27) were cultured as described previously. Briefly, NIH-3T3 cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37 °C in 10% CO₂ and passaged at 60% confluence. To establish basal conditions before use, cells were incubated in serum-free DMEM for 16 h at 37 °C in 10% CO₂. L6 myoblasts were cultured in minimal essential medium α supplemented with 10% FCS, 2 mM L-glutamine, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37 °C in 10% CO₂ and passed at ~60% confluence. To establish basal conditions before use, cells were incubated in serum-free DMEM for 16 h at 37 °C in 10% CO₂. L6 myoblasts were cultured in minimal essential medium α supplemented with 10% FCS, 2 mM L-glutamine, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37 °C in 10% CO₂ and passaged at ~60% confluence. For differentiation into myotubes, cells were cultured in minimal essential medium α supplemented with 2% FCS, 2 mM L-glutamine, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37 °C in 10% CO₂ and were maintained in this medium postdifferentiation. Myotubes were cultured in serum-free DMEM for 16 h at 37 °C in 10% CO₂. L6 myoblasts were cultured in minimal essential medium α supplemented with 10% FCS, 2 mM L-glutamine, 100 units/liter penicillin, and 100 μg/liter streptomycin at 37 °C in 10% CO₂. CHO IR/IRS-1 cells, NIH-3T3, and HEK 293T cells were transiently transfected with DNA constructs using Lipofectamine LTX according to the manufacturer's instructions. For specific inhibition of PI3K in cells, 100 nM wortmannin was added to the basal cell culture medium 30 min prior to stimulation of cells with insulin.

**Production of Thrombin-cleaved Human 14-3-3s in E. coli for 14-3-3 Pulldown Experiments—GST-14-3-3β was prepared as described previously (28) and when bound to glutathione beads was incubated with 1000 units/ml thrombin in 50 mM Tris-HCl, pH 8, and 1 mM CaCl₂ overnight at room temperature. The supernatant was buffer-exchanged three times into bead coupling buffer using Amicon 10-kDa molecular mass cutoff ultrafiltration devices (Millipore). The concentrated 14-3-3 protein was then coupled to cyanogen bromide-activated Sepharose 4B (GE Healthcare) according to the manufacturer’s instructions at a concentration of 5 mg of 14-3-3 coupled per 1 ml of packed beads. After extensive washing with TBS, the beads were stored at 4 °C as a 50% slurry in TBS containing 0.02% sodium azide.

**14-3-3 Pulldown from SILAC-labeled L6 Cells or Mouse Quadriceps—Frozen mouse quadriceps (isolated under the approval of the Garvan/St. Vincent’s animal ethics committee) were lysed using a Polytron mixer (Kinematica, Lucerne, Switzerland) for 30 s in IP buffer (1% Nonidet P-40, 50 mM Tris-Cl, pH 7.4, 1% glycerol, 150 mM NaCl, Roche Applied Science Complete protease inhibitor mixture, 2 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM ammonium molybdate, and 10 mM sodium fluoride) and incubated for 30 min at 4 °C. L6 myotubes were lysed in IP buffer by scraping down the cells on ice and syringing the lysate six times with a 22-gauge needle followed by three times with a 27-gauge needle. The lysate was centrifuged for 30 min at 18,000 × g at 4 °C. Equal protein amounts of each sample were then incubated with 14-3-3 beads that had been washed once in IP buffer (50 μg of 14-3-3/1 mg of lysate) and incubated overnight at 4 °C. Beads were then washed once with IP buffer, once with high salt (0.5x NaCl) IP buffer, and finally once more in IP buffer. Beads were transferred to a spin column (Pierce) and centrifuged dry at 500 × g for 5 min. The eluate was collected by centrifugation at 500 × g for 1 min at room temperature.

**SDS-PAGE and Immunoblotting**—All samples were subjected to SDS-PAGE analysis on 10% resolving gels according to Laemmli (29) but with the addition of 50 mM tris(carboxyethyl)phosphine (Bond-Breaker TCEP neutral pH solution, Pierce) in the sample buffer. Equal amounts of protein were loaded for each sample in a single experiment with 10 μg/lane. For mass spectrometric identification, SYPRO Ruby staining was performed according to the manufacturer’s instructions. For Western blotting, separated proteins were electrophoretically transferred to PVDF membrane, blocked with 5% nonfat skim milk in 0.1% Tween 20 in TBS (TBST), and incubated with primary antibody in 5% BSA in TBST overnight at 4 °C. After incubation, membranes were washed three times in TBST and incubated with HRP-labeled or Alexa Fluor 680/IRDye 800-labeled secondary antibodies in 5% nonfat skim milk in TBST. Proteins were visualized using Supersignal West Pico chemiluminescent substrate and imaged with x-ray film (Fuji) for HRP-labeled secondary antibodies or a Licor Odyssey imager for Alexa Fluor 680/IRDye 800-labeled secondary antibodies.

**Production of Human GST-14-3-3β in E. coli and 14-3-3 Overlay Assay—GST-14-3-3β was prepared as described previously (28) and stored at 1 mg/ml in PBS containing 0.02% sodium azide. For the 14-3-3 overlay assay, proteins were transferred to PVDF membranes as described above for immunoblotting. Membranes were blocked in 5% skim milk TBS and 0.1% Tween 20 for 1 h at room temperature. Membranes were then washed twice in TBS and 0.1% Tween 20 and
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incubated overnight at 4 °C in 5% BSA TBS and 0.1% Tween 20 containing 1 μg/ml GST-14-3-3-38. Membranes were then detected as described above using a GST-specific primary antibody.

Immunoprecipitation of FLAG-tagged EDC3 from CHO IR/IRS-1 Cells—CHO IR/IRS-1 cells transiently transfected with TagRFP alone or TagRFP-FLAG-EDC3 were lysed after 24 h in IP buffer and centrifuged at 18,000 × g for 30 min at 4 °C. Anti-FLAG antibody and protein G beads were added to the supernatant and incubated for 1 h at 4 °C with mixing. The beads were then washed three times with IP buffer, and FLAG-EDC3 complexes were eluted by incubation of beads in 0.2 mg/ml 3× FLAG peptide (Sigma) in TBS for 1 h at 4 °C.

Immunoprecipitation of SILAC-labeled FLAG-tagged EDC3 from HEK 293T Cells—SILAC-labeled HEK 293T cells were transiently transfected with either a TagRFP-only negative control (light label), TagRFP-FLAG-EDC3 S161A-R18 (medium label), or TagRFP-FLAG-EDC3 S161A-R18-KK (heavy label) (supplemental Fig. 5S). After 24 h, cells were lysed in IP buffer and centrifuged at 18,000 × g for 30 min at 4 °C. Anti-FLAG antibody and protein G beads were added to the supernatant and incubated for 1 h at 4 °C with mixing. The beads were then washed three times with IP buffer, and FLAG-EDC3 complexes were eluted by incubation of beads in 0.2 mg/ml 3× FLAG peptide (Sigma) in TBS for 1 h at 4 °C. As each lysate is mixed prior to immunoprecipitation, low affinity interactions will allow exchange of the stable isotope labels and induce a decrease in the medium over light (M/L) and heavy over light (H/L) SILAC ratios for those low affinity binding proteins. To avoid disregarding these low affinity binders, we used a low stringency (M/L or H/L SILAC ratio >1) cutoff in our data analysis with MaxQuant in the elimination of contaminant nonspecific binding proteins. This yields excellent removal of environmental contaminants (keratins, trypsin, antibody, etc.) that do not incorporate stable isotopes and therefore have a very low M/L or H/L SILAC ratio. But this method will not remove some experimental contaminants such as STK38, which may have M/L and H/L SILAC ratios close to 1.

Dual-Luciferase Assay for Analysis of let-7-mediated mRNA Repression—The Dual-Luciferase assay was performed according to the method of Clancy et al. (30). Briefly, NIH-3T3 cells stably expressing FLAG-tagged EDC3 constructs or vector alone were transiently transfected with firefly and Renilla luciferase plasmids as described (30). After transfection, cells were incubated in medium containing 10% serum for 16 h and then assayed for luciferase expression as described (30).

Immunoprecipitation of EDC3 Phosphorylated at Ser-161—NIH-3T3 cells were lysed in IP buffer and centrifuged at 18,000 × g for 30 min at 4 °C. Anti-EDC3 Ser(P)-161 antibodies were coupled to cyanogen bromide-activated Sepharose 4B Beads (GE Healthcare) according to the manufacturer’s instructions. The cell lysate was incubated with the antibody-coupled beads in the presence of a 10-fold molar excess of either Ser(P)-161 phosphopeptide or Ser-161 nonphosphopeptide overnight at 4 °C. Beads were washed three times in IP buffer and boiled in SDS-PAGE sample buffer.

Retroviral Transfection of NIH-3T3 Cells for Generation of Stable Cells—NIH-3T3 cells were grown to 20% confluency, and then pMXSC-ires-GFP (pMIG) retrovirus constructs produced in Plat-E cells were added to NIH-3T3 fibroblasts cultured as described above on glass coverslips. The cells were serum-depleted for 16 h at 37 °C after which they were incubated in the absence or presence of 31 μl insulin for 30 min. Cells were then fixed with 3% paraformaldehyde in PBS. Fixed cells were washed with PBS, and free aldehyde groups were quenched with 50 mM glycine in PBS. The cells were then processed for immunolabeling by permeabilization and labeling in PBS containing 0.1% saponin and 2% BSA using standard procedures. Primary antibodies were detected with Alexa Fluor 488- or Cy3-conjugated secondary antibodies. Optical sections were analyzed by epifluorescence microscopy on a Zeiss Observer inverted microscope. Images were generated by the maximum projection of a z-stack taken from the top to the bottom of the cell monolayer. The contrast was adjusted for all images with the same settings. Quantitation of p-body intensity was performed in ImageJ software using a peak threshold setting, which was applied equally across all images, and calculation of the integrated intensity by the Analyze Particles function.

In Vitro AKT2 Kinase Assay Using [γ-32P]ATP—TagRFP-FLAG-EDC3 wild type or S161A mutant were expressed by transient transfection in CHO IR/IRS-1 cells, and FLAG was immunoprecipitated as described above. Eluted TagRFP-FLAG-EDC3 proteins were incubated with 1 μCi/ml [γ-32P]ATP, 1 mM cold ATP, 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl and in the presence or absence of 10 ng/ml active AKT2 (Cell Signaling Technology) for 10 or 100 min at room temperature.
RESULTS

Identification of Insulin-responsive 14-3-3-binding Proteins Using SILAC and Mass Spectrometry—In the present study, we devised a strategy to identify novel insulin-regulated AKT substrates in insulin-responsive L6 myotubes (supplemental Fig. S1). By combining the high specificity for AKT substrates provided by 14-3-3 affinity chromatography and the inherent quantitative capability of SILAC (22), we identified from among the large cohort of intracellular 14-3-3-binding proteins those that do so in an insulin- and PI3K-dependent manner, thus providing a unique glimpse of the phosphoproteome. Analysis of these experiments using MaxQuant (33) software yielded >400 identified proteins in L6 myotubes of which 28 showed a significant (significance (B) <0.05) change in 14-3-3 binding after insulin stimulation (Table I and supplemental Table S1). Of these, we identified five previously unknown insulin-responsive proteins on which we chose to focus our study (supplemental Table S2). The majority of novel insulin-responsive proteins were wortmannin-sensitive, indicating that they were downstream of PI3K (Table I). Immunoblotting using specific antibodies was used to verify several of the novel insulin-responsive proteins. Beclin, EDC3, and DNA methyltransferase 1 underwent rapid insulin-dependent phosphorylation as demarcated by 14-3-3 binding in a wortmannin-sensitive manner (Fig. 1, a and c). Moreover, these proteins were also found to undergo increased 14-3-3 binding in mouse quadriceps in vivo under physiological conditions such as the fasted/fed transition (Fig. 1b). Bioinformatics analysis using Scan-site (38) and Eukaryotic Linear Motifs (ELM) (39) software packages revealed that EDC3, TRIP12, and MYCBP2 contained consensus AKT phosphorylation motifs (supplemental Table S2).

The identification of these proteins potentially unveils important actions of insulin in a variety of novel pathways. Based on the key role of EDC3 in mRNA repression and the fact that an effect of insulin on this pathway has not been described previously, we prioritized this protein for further analysis and characterized the role of insulin-dependent phosphorylation of this protein in a range of model systems. Although all of the proteins identified in our screen contain 14-3-3 binding and AKT phosphorylation consensus sites (supplemental Table S2), EDC3 contains one Scan-site high stringency AKT site at Ser-161 (in the top 0.008% of all sites in the Swiss-Prot vertebrate database) and two medium stringency AKT sites at Ser-262 and Ser-231. ClustalX alignment showed that the

### Table I

Novel insulin-responsive proteins identified by combined SILAC and 14-3-3 affinity analysis

| UniProt accession no. | Protein name | Protein description | Average (insulin/basal)* | S.D. (insulin/basal) | Average (wort + insulin/basal)* | S.D. (wort + insulin/basal) |
|-----------------------|--------------|---------------------|--------------------------|---------------------|--------------------------------|-----------------------------|
| Q8K2D3                | EDC3        | Enhancer of mRNA-decapping protein 3 | 5.96                   | 0.80                 | 1.99                           | 0.52                        |
| O8B380                | TRIP12      | Thyroid hormone receptor interactor 12 | 5.35                   | 0.19                 | 0.88                           | 0.15                        |
| Q9W4H4                | FOXO3       | Forkhead box O3a    | 4.83                   | 0.96                 | 1.22                           | 0.59                        |
| Q9D1F4                | PRAS        | Proline-rich AKT1 substrate 1 | 4.41                   | 0.40                 | 1.25                           | 0.04                        |
| Q62433                | NDRG1       | Protein NDRG1        | 4.20                   | 0.07                 | 1.23                           | 0.16                        |
| Q8B8L0                | ARH GAP2    | Rho GTPase-activating protein 22 | 3.33                   | 0.20                 | 0.69                           | 0.07                        |
| Q9EP53                | TSC1        | Tuberous sclerosis 1 | 2.93                   | 0.56                 | 1.32                           | 0.15                        |
| Q8C570                | MRNP41      | mRNA export factor   | 2.83                   | 0.32                 | 1.52                           | 0.15                        |
| Q99MS7                | EBP1        | EH domain-binding protein 1-like protein 1 | 2.80           | 0.97                 | 0.97                           | 0.04                        |
| P23949                | BRF2        | Butyrate response factor 2 | 2.77                   | 0.35                 | 2.03                           | 0.58                        |
| Q9Z158                | GAB2        | Growth factor receptor-bound protein 2-associated protein 2 | 2.63 | 1.08 | 1.02 | 0.11 |
| P23950                | BRF1        | Butyrate response factor 1 | 2.55                   | 0.38                 | 2.08                           | 0.58                        |
| Q61037                | TSC2        | Tuberous sclerosis 2  | 2.54                   | 0.52                 | 1.10                           | 0.18                        |
| Q7TPH4                | MYCBP2      | Probable E3 ubiquitin-protein ligase MYCBP2 | 2.49 | 0.00 | 1.62 | 0.23 |
| O08908                | PIK3R2      | Phosphatidylinositol 3-kinase regulatory subunit \(\gamma\) | 2.43 | 0.20 | 2.32 | 0.30 |
| Q95JO3                | BECN1       | Beclin 1             | 2.35                   | 0.12                 | 1.17                           | 0.10                        |
| Q148W8                | DUSP27      | Inactive dual specificity phosphatase 27 | 2.21 | 0.01 | 1.38 | 0.02 |
| Q9WXT5                | SKP1        | S phase kinase-associated protein 1 | 2.16 | 0.58 | 1.52 | 0.41 |
| Q6PDH0                | PHLDB1      | Pleckstrin homology-like domain B member 1 | 2.10 | 0.08 | 1.03 | 0.25 |
| O6Z098                | LARP1       | La-related protein   | 1.98                   | 0.08                 | 1.09                           | 0.05                        |
| A0T1J8                | LMO7        | LIM domain only 7    | 1.83                   | 0.28                 | 1.46                           | 0.13                        |
| Q8BBC8                | PI4KB       | Phosphatidylinositol 4-kinase \(\beta\) | 0.61 | 0.01 | 0.86 | 0.16 |
| A0ULR7                | AHNAK       | AHNAK nucleoprotein isoform 1 | 0.59 | 0.14 | 0.80 | 0.08 |
| Q6PN50                | MTSS1L      | MTSS1-like protein   | 0.58                   | 0.04                 | 0.58                           | 0.11                        |
| Q9JM13                | RABGEC1     | Rab5 GDP/GTP exchange factor | 0.56 | 0.13 | 0.83 | 0.18 |
| Q6AXD2                | ABI2        | Abi interactor 2     | 0.54                   | 0.02                 | 0.59                           | 0.02                        |
| O3S063                | KIF13B      | Kinesin family member 13B | 0.54 | 0.17 | 1.09 | 0.10 |
| Q8CI08                | SLAIN2      | SLAIN motif-containing protein 2 | 0.32 | 0.02 | 0.54 | 0.07 |
| P42128                | FOXK1       | Forkhead box protein K1 | 0.38 | 0.01 | 0.81 | 0.05 |

* Average ratio of -fold change in 14-3-3 binding with insulin stimulation or wortmannin (wort) and insulin stimulation compared with basal.
sequence surrounding Ser-161 is highly conserved in vertebrates (Fig. 1d). Strikingly, those residues that make EDC3 Ser-161 a high scoring AKT site in the Scansite algorithm were preserved throughout vertebrate evolution. Phosphorylation and 14-3-3 binding have been shown previously to be strongly associated with disordered regions of proteins (40). Using the GlobPlot algorithm (41), we analyzed the disorder around the putative 14-3-3/AKT sites in our subset of proteins (supplemental Table S2). These data revealed that EDC3 Ser-161 lies in the middle of a disordered region of the protein between its N-terminal Lsm-like domain and its central FDF domain (16) (Fig. 1e).
Identification of EDC3 Phosphorylation Sites and Interacting Partners—To identify phosphorylated 14-3-3 binding sites within EDC3, we expressed either TagRFP alone or a TagRFP-FLAG-tagged human EDC3 construct by transient transfection of CHO cells that stably express the insulin receptor and IRS-1 (CHO IR/IRS-1). Cells were either left unstimulated, stimulated with 100 nM insulin for 30 min, treated with the PI3K inhibitor wortmannin prior to insulin stimulation, or treated with AKT inhibitor prior to insulin stimulation. Analysis of EDC3 phosphorylation and 14-3-3 binding at Ser(P)-161. a, SDS-PAGE and SYPRO Ruby staining of immunoprecipitated (IP) TagRFP-FLAG-EDC3 WT or TagRFP vector alone transiently transfected into CHO IR/IRS-1 cells. Arrows indicate 14-3-3 identified by mass spectrometry; Edc3 marks the TagRFP-FLAG-EDC3 protein. b, immunoblotting of immunoprecipitated FLAG-EDC3 constructs transiently transfected with the indicated constructs and serum-starved prior to analysis. c, analysis of in vitro AKT phosphorylation of TagRFP-FLAG-EDC3 constructs in the presence of [γ-32P]ATP and active AKT2 kinase for the indicated times. d, immunoblotting of total cell lysates from NIH-3T3 fibroblasts that were stimulated with 1 μM insulin for the indicated times. Immunoblots and images are from a representative experiment (n = 3). pAkt, phospho-Akt; Myr, myristoylated.
and several other bands with molecular masses of ~55 and 68 kDa were also evident (Fig. 2a). Of note, two bands with an approximate molecular mass of 30 kDa showed increased abundance in the immunoprecipitate from insulin-stimulated cells and were not present in the vector control. Bands from each lane were excised and subjected to tryptic digestion and analysis by LC-MS/MS. These data revealed that the most abundant protein corresponded to human EDC3 containing five phosphopeptides, consistent with phosphorylation of EDC3 at Ser-161, Ser-131, Thr-173, Ser-109, and Tyr-225 (supplemental Table S3 and Fig. S2 for MS/MS spectra of phosphopeptides). Analysis of the entire gel lane from both the vector control and the TagRFP-FLAG-EDC3 immunoprecipitation revealed the presence of DCP1A/1B and DDX6, previously described EDC3 interactors, as well as six 14-3-3 isoforms within the 30-kDa bands from the EDC3 immunoprecipitates (supplemental Table S3). Together these data show that recombinant EDC3 co-immunoprecipitates with endogenous 14-3-3 isoforms in response to insulin in vivo and is phosphorylated at the putative 14-3-3 binding and AKT phosphorylation site Ser-161.

EDC3 Ser(P)-161 Is a 14-3-3 Binding Site and Is Phosphorylated by Akt—To determine whether EDC3 Ser(P)-161 is indeed the 14-3-3 binding site, we generated an Ser-161 alanine mutant (S161A) in FLAG-tagged EDC3 and again analyzed FLAG immunoprecipitates from CHO IR/IRS-1 cells expressing either vector, FLAG-EDC3 WT, or FLAG-EDC3 S161A by transient transfection. Cells expressing these EDC3 constructs were either left unstimulated or stimulated with 100 nm insulin for 30 min. Immunoblots and images are from a representative experiment (n = 3). pAkt, phospho-Akt.

FIG. 3. Generation of EDC3 constructs that constitutively bind 14-3-3. a, domain structure of FLAG-EDC3 constructs showing the location of the AKT phosphorylation and 14-3-3 binding site Ser(P)-161, the R18 or R18-KK peptide insertion site, and the known functions of EDC3 protein domains. b, immunoblotting of immunoprecipitated FLAG-EDC3 constructs from transiently transfected CHO IR/IRS-1 cells. Cells were either left unstimulated or stimulated with 100 nm insulin for 30 min. Immunoblots and images are from a representative experiment (n = 3). pAkt, phospho-Akt.

Expression of EDC3 Constructs with Altered 14-3-3 Binding Alters mRNA Processing Body Structure and Function—We next wanted to test the functional consequences of EDC3 phosphorylation and 14-3-3 binding. Previous studies have identified a small peptide motif (R18) that binds 14-3-3 strongly without requiring phosphorylation (42). We exploited this motif by cloning it into the EDC3 protein sequence three amino acids downstream of the S161A mutation (Fig. 3a). This generated a construct (S161A-R18) that could not be phosphorylated at Ser-161 but displayed constitutive 14-3-3 bind-
ing (Fig. 3b). We also made an additional construct that has a mutated R18 sequence that could not bind 14-3-3 (S161A-R18-KK) (27). To show that EDC3 WT binds 14-3-3 directly and that the S161A-R18 construct displays constitutive 14-3-3 binding, we used a bacterially produced GST-14-3-3β protein in a far-Western (overlay) analysis (Fig. 3b). These data showed that the S161A and S161A-R18-KK constructs did not bind 14-3-3, whereas the R18 construct bound ~20–30 times more 14-3-3 than the insulin-treated WT construct. Furthermore, each EDC3 construct displayed 14-3-3 co-immunoprecipitation to the same degree as the 14-3-3 far-Western (overlay) analysis (Fig. 3b).

14-3-3 proteins often play roles in altering protein subcellular distribution and protein-protein interactions (43, 44). To qualitatively investigate whether phosphorylation of Ser-161 and subsequent 14-3-3 binding had an effect on p-body morphology, we transiently transfected NIH-3T3 cells with TagRFP-tagged constructs that expressed vector alone, EDC3 WT, S161A, S161A-R18, or S161A-R18-KK. The morphology of p-bodies within these cells was examined by immunofluorescence microscopy using antibodies specific for the endogenous p-body markers DDX6 or Hedls. Consistent with previous studies, p-bodies were evident as small puncta scattered randomly throughout the cytoplasm (supplemental Fig. S4). Insulin increased apparent p-body size/brightness and number substantially in both vector and EDC3 WT cells. This was not observed in cells expressing EDC3 S161A or S161A-R18-KK. Strikingly, cells expressing EDC3 S161A-R18 showed numerous large/bright p-body structures regardless of insulin stimulation. These data clearly indicate that insulin treatment and 14-3-3 binding to EDC3 modulate p-body morphology.

To quantify the functional consequences of 14-3-3 binding to EDC3, we generated a series of NIH-3T3 stable cell lines infected with retroviral vectors expressing FLAG-tagged EDC3 WT, S161A, S161A-R18, or S161A-R18-KK linked to IRES-GFP or an empty vector as control. These cell lines were sorted for GFP by fluorescence-activated cell sorting, enabling us to normalize for the expression of the different constructs (Fig. 4a), and displayed stable expression of these retroviral constructs for more than 10 passages. Analysis of FLAG immunoprecipitates from these cells showed that as expected the S161A and S161A-R18-KK constructs did not bind 14-3-3 and the R18 construct bound significantly more 14-3-3 than the insulin-treated WT construct (Fig. 4b). These data also showed that DDX6 did not change in its association with each of these constructs, indicating that 14-3-3 binding does not control the EDC3-DDX6 interaction. The morphology of p-bodies within each of these cell lines was examined by immunofluorescence microscopy (Fig. 4c) and found to be similar to that observed in transiently transfected cells. Quantification of the intensity of the p-bodies in these images emphasized the marked increase in DDX6 staining intensity after insulin stimulation of vector and EDC3 WT stable cells (Fig. 4d). It also showed the total lack of response in EDC3 S161A and EDC3 S161A-R18-KK stable cells. In contrast, EDC3 S161A-R18 cells showed high intensity DDX6 p-body staining regardless of insulin stimulation.

To investigate whether the altered p-body morphology present in these cells correlated with altered miRNA function we used a Dual-Luciferase reporter assay to analyze repression by the let-7 miRNA (30, 45). Previous studies have shown that the let-7 miRNA is expressed in most cell types (46), and we exploited this by transiently transfecting plasmids expressing Renilla luciferase with either three WT or three mutant let-7 binding sites in its 3′-untranslated region. Only the mRNA containing the WT let-7 binding sites is a target for the let-7 miRNA. Each of these constructs was co-transfected with a firefly luciferase plasmid, which acted as a transfection control. By calculating the ratio of the firefly-corrected Renilla luciferase activity in cells transfected with the wild type let-7 binding plasmid or the mutant let-7 binding plasmid, we could evaluate the level of post-transcriptional regulation by let-7 in these cells. We found that the EDC3 S161A-R18 cells displayed an inhibition of this post-transcriptional regulation compared with the other stable cell lines tested (Fig. 4e). These data indicate that constitutive 14-3-3 binding to EDC3 is inhibitory for its role in miRNA function.

**EDC3 Bound to 14-3-3 Displays a Distinct Protein Interaction Repertoire**—To determine the mechanism by which 14-3-3 binding to EDC3 may alter its function, we comprehensively analyzed the interaction partners of EDC3 constructs either constitutively bound to 14-3-3 (S161A-R18) or deficient in 14-3-3 binding (S161A-R18-KK). This analysis was performed using SILAC (47) technology, which enabled quantitative analysis of regulated EDC3-interacting partners (supplemental Table S4 and Fig. S5). This analysis was performed by transient transfection into SILAC-labeled cells of either a vector-only negative control (light label), TagRFP-FLAG-EDC3 S161A-R18 (medium label), or TagRFP-FLAG-EDC3 S161A-R18-KK (heavy label) (supplemental Fig. S5). These studies revealed that EDC3 deficient in 14-3-3 binding co-immunoprecipitated ~10-fold more poly(A)-binding protein 1 (PABP1), 7-fold more Y-box-binding protein 1, and ~5-fold more IGF-2 mRNA-binding protein 1 compared with EDC3 with 14-3-3 constitutively bound (Table II and supplemental Table S4). Some of these interactions may be indirectly bridged by mRNA, and previous studies have shown that PABP or PABP-mRNA complexes may associate with various p-body components (48, 49). These observations are exciting because they may indicate that the interaction of EDC3 with 14-3-3 decreases the mRNA binding of the EDC3 complex. As expected the EDC3 S161A-R18 construct was able to co-immunoprecipitate 10-fold more 14-3-3 compared with the EDC3 S161A-R18-KK construct. The interaction between EDC3 and previously identified interaction partners such as DDX6 and DCP1A was not markedly altered between the two EDC3 constructs.
Akt Regulation of EDC3

(a) pMIG Empty WT S161A S161A R18 R18-KK
FLAG-Edc3
Tubulin
14-3-3

(b) Insulin + - + + +
pMIG Empty WT S161A S161A S161A R18 R18-KK
FLAG-Edc3
DDX6
14-3-3

(c) pMIG Empty
WT
S161A
S161A R18
S161A R18-KK

(d) % of HeLa p-EDC3

(e) Relative mRNA Repression

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Akt Regulation of EDC3

**TABLE II**
Differential protein-protein interactions of the EDC3 and 14-3-3 protein complex

| UniProt accession no. | Protein name | Protein description | Average (KK/R18) | S.D. (KK/R18) |
|------------------------|--------------|---------------------|------------------|---------------|
| P11940                 | PABPC1       | Polyadenylate-binding protein 1 | 9.37 | 4.53 |
| P67809                 | YBX1         | Nuclease-sensitive element-binding protein 1 | 6.55 | 2.62 |
| Q9NZI8                 | IGF2BP1      | Insulin-like growth factor 2 mRNA-binding protein 1 | 4.99 | 1.83 |
| P08107                 | HSPA1A       | Heat shock 70-kDa protein 1 | 2.56 | 0.37 |
| P11142                 | HSPA8        | Heat shock cognate 71-kDa protein | 2.40 | 0.22 |
| P23396                 | RPS3         | 40 S ribosomal protein S3 | 2.75 | 0.24 |
| P07900                 | HSP90A1      | Heat shock protein 90-kDa α | 2.38 | 0.39 |
| P08238                 | HSP90A2      | Heat shock protein 90-β | 2.48 | 0.59 |
| P07437                 | TUBB         | Tubulin β chain | 1.67 | 0.20 |
| Q15208                 | STK38        | Serine/threonine-protein kinase 38 | 1.49 | 0.27 |
| P26196                 | DDX6         | Probable ATP-dependent RNA helicase DDX6 | 1.45 | 0.21 |
| Q9NP16                 | DCP1A        | mRNA-decaping enzyme 1A | 1.54 | 0.48 |
| A8K674                 | UBC          | Ubiquitin C | 0.94 | 0.36 |
| Q8IZD4                 | DCP1B        | mRNA-decaping enzyme 1B | 1.10 | 0.49 |
| P62258                 | YWHAE        | 14-3-3 protein ε | 0.13 | 0.12 |
| P31946                 | YWHA8        | 14-3-3 protein β/α | 0.13 | 0.13 |
| P61981                 | YWHA9        | 14-3-3 protein γ | 0.13 | 0.12 |
| Q04917                 | YWHAH        | 14-3-3 protein η | 0.12 | 0.12 |
| P27348                 | YWHAQ        | 14-3-3 protein θ | 0.11 | 0.11 |
| P63104                 | YWHAZ        | 14-3-3 protein δ | 0.12 | 0.12 |

*Average ratio of protein-protein interaction with EDC3 S161A-R18-KK (no 14-3-3 binding) compared with EDC3 S161A-R18 (14-3-3 constitutively bound).

DISCUSSION

In the present study, we used quantitative mass spectrometry methods based on SILAC to delineate additional elements of the insulin signaling pathway downstream of PI3K. In addition to well known AKT substrates, we identified several novel insulin-regulated proteins including EDC3, DNA methyltransferase 1, TRIP12, and Beclin. The identification of these phosphoproteins points to novel actions of insulin and other growth factors that activate PI3K/AKT, including regulation of autophagy (50), DNA methylation (51), and protein ubiquitylation (52). In this study, we further interrogated EDC3 and unveiled a novel action of insulin on mRNA post-transcriptional regulation, a pathway that plays an essential role in controlling protein expression in eukaryotic cells most notably via miRNAs (53–57). This discovery opens up new avenues of research in trying to identify the cellular basis of metabolic disease.

The canonical PI3K/AKT pathway plays an essential role in regulating numerous biological pathways downstream of tyrosine kinase growth factor receptors. In the context of insulin action, AKT has been shown to be critical in many if not most of the metabolic actions of insulin, including glucose transport and protein synthesis. Most of these pathways were mapped using a reverse discovery process whereby the metabolic actions of insulin were first identified and subsequently used as an end point to define the upstream biochemistry. Here, we describe a forward discovery process whereby identification of novel insulin-responsive 14-3-3-binding proteins can be used to define novel actions of insulin, and we predict that such an approach can be used to identify many more actions of this and other hormones. The approach takes advantage of the unusually high affinity of 14-3-3 proteins for AKT substrates and quantitative mass spectrometry using SILAC. The latter was a particularly important adaptation of the technique, allowing us to define from within the large repertoire of 14-3-3 proteins those that bind in an insulin- and PI3K-regulated manner. One of the important advantages of this technique compared with other techniques is that it overcomes the need to identify specific phosphopeptides in the mass spectrometer, which is limited by poor phosphopeptide separation, ionization, and fragmentation in LC-MS/MS (58, 59). To quantify proteins in this study, we were not dependent on the identi-
fication of specific peptides; rather any peptide from a target protein will be sufficient for generation of quantitative data for that protein. Furthermore, our technique can also allow the identification of insulin-responsive protein complexes where only one protein in the complex may bind to 14-3-3. Although the use of 14-3-3 preferentially selects for isolation of AGC family kinase substrates, we may overlook insulin-regulated phosphoproteins that do not interact with 14-3-3. Further studies using complementary technologies such as combined strong cation exchange/titanium dioxide chromatography or affinity chromatography using other phosphoprotein binding domains will be needed for a comprehensive analysis.

The observation that EDC3 is phosphorylated in response to insulin via the AGC kinase AKT is exciting because this reinforces the possibility that functions associated with p-bodies can be rapidly regulated (60). To control mRNA decay or translation repression, EDC3 binds to several core p-body components, and so it is well positioned as a key regulatory target (16–18, 62, 63). Intriguingly, other mRNA regulatory proteins such as BRF-1 (37, 64) and tristetraprolin (65) are also regulated by insulin and other growth factors via AKT-dependent phosphorylation, suggesting that this may represent a coordinated regulation of many components of the mRNA decay/repression process via AKT.

In this report, we show that AKT-dependent phosphorylation of EDC3 occurred within a disordered region between its Lsm and DFD domains. Phosphorylation at this site triggered 14-3-3 binding, and five observations suggest that this modulates EDC3 function. First, insulin treatment of cells significantly increased both the number and size of p-bodies. Second, the use of a 14-3-3 binding peptide (R18) engineered into EDC3 mediated constitutive 14-3-3 binding when expressed in cells, and this, like insulin, exaggerated the formation of large/bright p-bodies. Third, expression of an EDC3 mutant incapable of insulin-regulated phosphorylation and 14-3-3 binding in cells blocked this insulin effect. Fourth, 14-3-3 binding to EDC3 was associated with a significant inhibition of let-7 miRNA-induced mRNA repression. Finally, 14-3-3 binding to EDC3 resulted in a significant decrease in the binding of several key mRNA regulatory factors such as PABP and Y-box-binding protein 1 to EDC3. Collectively, these findings suggest that in the absence of insulin the mRNA decay/repression pathway is active and that this pathway corresponds with morphologically small p-bodies. Insulin may then inhibit this decay/repression pathway by phosphorylation of EDC3 and other proteins in the same pathway, including BRF-1, leading to 14-3-3 binding. Consistent with this, it has been shown that mRNA decay and miRNA-mediated mRNA repression can occur in the absence of visible p-body structures (66–70). It seems likely based on our data that 14-3-3 binding rather than phosphorylation per se is the key regulator of EDC3 function. We speculate based on the bivalent nature of 14-3-3 that the transition from active to inactive mRNA decay/repression is mediated by the displacement of EDC3 and probably other factors from functional p-bodies into larger aggregate structures (18, 61). This mechanism would suggest that insulin has the capacity to increase protein synthesis via multiple mechanisms: transcription, mRNA stability, mRNA accessibility to translation machinery, and translation efficiency.

Our studies have considerable implications for the regulation of protein synthesis in eukaryotic cells. In view of the tissue specificity of miRNA expression and growth factor regulation, one can envisage that this pathway might rapidly regulate the expression of a unique repertoire of proteins that play a unique role in a particular tissue. Thus, in the context of insulin action, this pathway may regulate the expression of proteins involved in the adipogenic program in adipocytes, whereas in other cell types, different growth factors may utilize this same pathway to regulate the expression of a unique subset of proteins via an alternate family of cell-specific miRNAs. This provides the basis for a combinatorial mechanism of cell-specific regulation involving this canonical AKT/p-body signaling pathway. Hence, it will be of interest to determine the impact of this pathway in disease states such as diabetes, cardiovascular disease, and cancer that at least in part are characterized by alterations in PI3K signaling.

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