We devised a strategy of 14-3-3 affinity capture and release, isotope differential ($d_0/d_4$) dimethyl labeling of tryptic digests, and phosphopeptide characterization to identify novel targets of insulin/IGF1/phosphatidylinositol 3-kinase signaling. Notably four known insulin-regulated proteins (PFK-2, PRAS40, AS160, and MYO1C) had high $d_0/d_4$ values meaning that they were more highly represented among 14-3-3-binding proteins from insulin-stimulated than unstimulated cells. Among novel candidates, insulin receptor substrate 2, the proapoptotic CCDC6, E3 ubiquitin ligase ZNRF2, and signaling adapter SASH1 were confirmed to bind to 14-3-3s in response to IGF1/phosphatidylinositol 3-kinase signaling. Insulin receptor substrate 2, ZNRF2, and SASH1 were also regulated by phorbol ester via p90RSK, whereas CCDC6 and PRAS40 were not. In contrast, the actin-associated protein vasodilator-stimulated phosphoprotein and lipolysis-stimulated lipoprotein receptor, which had low $d_0/d_4$ values, were not. In contrast, the actin-associated protein vasodilator-stimulated phosphoprotein and lipolysis-stimulated lipoprotein receptor, which had low $d_0/d_4$ scores, bound 14-3-3s irrespective of IGF1 and phorbol ester. Phosphorylated Ser19 of ZNRF2 (RTRAYpS19GS), phospho-Ser493 of lipolysis-stimulated lipoprotein receptor (RPRARpS493LD) provide one of the 14-3-3-binding sites among 14-3-3-binding proteins from insulin-stimulated cells. Among novel candidates, insulin receptor substrate 2, the proapoptotic CCDC6, E3 ubiquitin ligase ZNRF2, and signaling adapter SASH1 were also regulated by phorbol ester via p90RSK, whereas CCDC6 and PRAS40 were not. In contrast, the actin-associated protein vasodilator-stimulated phosphoprotein and lipolysis-stimulated lipoprotein receptor, which had low $d_0/d_4$ scores, bound 14-3-3s irrespective of IGF1 and phorbol ester. Phosphorylated Ser19 of ZNRF2 (RTRAYpS19GS), phospho-Ser493 of lipolysis-stimulated lipoprotein receptor (RPRARpS493LD) provide one of the 14-3-3-binding sites on each of these proteins. Differential 14-3-3 capture provides a powerful approach to defining downstream regulatory mechanisms for specific signaling pathways. Molecular & Cellular Proteomics 8: 2487–2499, 2009.

Activated tyrosine kinase receptors generally drive cells to assimilate nutrients; regulate partitioning of the assimilate to make storage polymers and biosynthetic precursors and for energy production; and promote cellular survival, growth, division, movement, and differentiation. From this spectrum, each cell displays a specific subset of responses depending on the hormone, specific receptors, cross-talk from other signaling pathways, metabolic conditions, and cellular complement of effector proteins. For example, insulin stimulates glucose uptake and glycogen synthesis in skeletal muscle, whereas IGF1 promotes survival, growth, and proliferation of many cell types (1, 2).

Many of these cellular responses are mediated via PI 3-kinase, which generates phosphatidylinositol 3,4,5-trisphosphate, promoting the activation of AGC protein kinases such as PKB/Akt and other signaling components (3, 3). PI 3-kinase is activated by binding to tyrosine-phosphorylated receptors such as the platelet-derived growth factor receptor or via adaptor molecules such as insulin receptor substrates, which are phosphorylated by the activated insulin receptor. Dereguated PI 3-kinase and downstream signaling has been linked to problems with wound healing, immune responses, neurodegeneration, and cardiovascular disease; decreased PI 3-kinase signaling may underlie insulin resistance and type II diabetes; and this pathway is often activated in human tumors (4, 5). To help pinpoint drug targets for these diseases we must define the mechanisms linking PI 3-kinase and other signaling pathways to downstream effectors and understand specificity with respect to different hormone/cell type combinations.

Many missing substrates of PI 3-kinase/AGC kinases must be found to explain all the cellular responses to insulin and growth factors (3). Several targets of PI 3-kinase/PKB signaling, including TSC2 (6), PRAS40 (7), AS160 (8), and FYVE domain-containing phosphatidylinositol 3-phosphate 5-kinase (9) were
identified using the anti-PAS antibody, which loosely recognizes the minimal phosphorylated consensus for PKB, which is RXRXP(S/P) where pS is phosphoserine and pT is phosphothreonine. Another helpful feature for identifying new downstream targets is that phosphorylation by PKB sometimes creates binding sites for 14-3-3s, which are dimeric proteins that bind to specific phosphorylated sites on target proteins. Thus PKB promotes the binding of 14-3-3s to proteins including PFKFB2 cardiac PFK-2 (10, 11), BimEL (12), B-catenin (13), p27(Kip1) (14), PRAS40 (7), FOXO1 (15), Miz1 (16), TBC1D4 (AS160) (17, 18), and TBC1D1 (19). Functionally 14-3-3s can trigger changes in the conformations of their targets and alter how targets interact with other proteins. Consistent with 14-3-3/target interactions being important in cellular responses to growth factors and insulin, reagents that compete with targets for binding to 14-3-3s inhibit the IGFl-stimulated increase in the glycolytic stimulator fructose-2,6-bisphosphate (10) and PKB-dependent cell survival (20).

Some 14-3-3-binding sites on the above named proteins can also be phosphorylated by other basophilic protein kinases (21). For example, AS160 and TBC1D1 are two related RabGAPs (GTPase-activating protein for Rabs) regulated by multisite phosphorylation that regulate trafficking of GluT4 transporter to the plasma membrane for uptake of glucose. The two 14-3-3-binding sites on AS160 can be phosphorylated by PKB, p90RSK, serum- and glucocorticoid-inducible kinase, and other kinases, whereas one of the 14-3-3-binding sites on TBC1D1 is also a substrate of the energy-sensing kinase AMP-activated protein kinase (17–19). Thus, the relative sensitivity of glucose trafficking to insulin and AMP-activated protein kinase (17–19). Thus, the relative sensitivity of glucose trafficking to insulin and AMP-activated protein kinase activators in different tissues may depend on the distribution of 14-3-3 and TBC1D1. Other insulin-regulated 14-3-3 targets, such as myosin 1C (22), are also convergence points for phosphorylation by more than one AGC and/or Ca2+/calmodulin-dependent protein kinase.

Here many more proteins than those already identified were found to display 14-3-3 and/or PAS binding signals when the PI 3-kinase pathway was activated in cells against a “background” of other proteins whose 14-3-3 and PAS binding status was unaffected by PI 3-kinase signaling. We aimed to pick out the PI 3-kinase-regulated proteins, which was challenging given the hundreds of 14-3-3 binding partners in mammalian cells (10, 23–27). We used 14-3-3 affinity capture and release, identified phosphopeptides, and devised a quantitative proteomics approach in which 14-3-3-binding proteins from insulin-stimulated versus unstimulated cells were labeled with formaldehyde containing light or heavy isotopes, respectively. Biochemical checking of candidates from these screens, which included proteins with links to diabetes, cancers, and neurodegenerative disorders, confirmed the identification of novel downstream targets of PI 3-kinase, some of which are also convergence points for regulation by MAPK/p90RSK signaling.

**Experimental Procedures**

**Materials**—Synthetic peptides were from Graham Bloomberg (University of Bristol). Oligonucleotides were from MWG-Biotech. IGF1 was from BIOSOURCE. Microcystin-LR was from Linda Lawton (Robert Gordon’s University, Aberdeen, Scotland, UK). Vivaspin concentrators were from Vivascience. Tissue culture reagents were from Invitrogen. Protease inhibitor mixture tablets (catalog number 1697498) and sequencing grade trypsin were from Roche Applied Science. Precast SDS-polyacrylamide gels were from Invitrogen. Protein G-Sepharose and chromatographic matrices were from GE Healthcare. Formaldehyde-d3 and -d4 were from Sigma-Aldrich, and unless stated other chemicals were from BDH Chemicals or Sigma-Aldrich.

**Stimulation and Extraction of HeLa and HEK293 Cells**—To our knowledge, HeLa S3 and HEK293 cells do not express insulin receptors, although they have IGFl receptors that bind insulin with lower affinity than the cognate receptor (28). Insulin, which is less expensive than IGFl, was therefore used for large scale experiments with HeLa S3 cells, which were cultured in suspension in Dulbecco’s modified Eagle’s medium containing 10% FCS, 1% penicillin-streptomycin, and 1% sodium pyruvate. Trial experiments indicated that serum starvation of HeLa S3 cells for 4 h followed by 20-min stimulation with 50 milliunits/ml (300 nM) insulin gave maximal phosphorylation of PKB (Thr(P)308 and Ser(P)473) and AS160 (Thr(P)642). After 36 h, cells were rinsed with warm PBS and serum-starved in Dulbecco’s modified Eagle’s medium for 8 h. Where indicated, cells were preincubated with PI-103 (1 μM for 30 min) and BI-D1870 (10 μM for 30 min) and stimulated for 20 min with 50 ng/ml IGF1 and 100 ng/ml PMA. Cells were lysed in 0.5 ml of 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (by volume) 2-mercaptoethanol, “Complete” protease inhibitor mixture (one tablet/50 ml). Cell lysates were clarified by centrifugation at 4 °C for 20 min at 15,000 rpm.

**14-3-3 Affinity Chromatography**—14-3-3 affinity chromatography involved binding of protein to 14-3-3-Sepharose (mixed BMH1 and BMH2, the 14-3-3 isoforms from Saccharomyces cerevisiae) and elution of specifically bound proteins by competition with the 14-3-3-binding synthetic ARAApSAPA phosphopeptide (Fig. 2) as in Pozuelo Rubio et al. (10) except that the high salt wash was only 500 ml and the mock peptide elution was omitted.

**Western Blots, 14-3-3 Far-Western Overlays, and Immunoprecipitations**—Sheep anti-IGFl was raised against the peptide YPYDPDYA, and sheep anti-AS160 was raised against KAKIGNKP (17). Anti-phospho-Erk1/2 (Thr(P)202/Tyr(P)204), anti-phospho-Thr208 PKB, and anti-PKB/Akt were from Cell Signaling Technology. For Western blots the indicated antibodies were used at 1 μg/ml. Western blots and 14-3-3 overlays (using digoxigenin-labeled 14-3-3s in place of primary antibody) were visualized by ECL reagent or the Odyssey Infrared Imaging System (LI-COR, Inc.) as indicated. For immunoprecipitations with anti-IGFl, 4 μg of antibody/mg of lysate was mixed at 4 °C for 1 h, and then Protein G-Sepharose (30 μl of a 50% suspension in lysis buffer) was added and mixed for a further 1 h. The suspension was centrifuged at 12,000 × g for 1 min between washes.

**Tryptic Digestion, Dimethylation, and Phosphopeptide Enrichment**—14-3-3-binding proteins that had been purified from unstimulated or insulin-stimulated HeLa cells were denatured in lithium do-decyl sulfate sample buffer (Invitrogen) containing 10 mM DTT at
95 °C for 5 min, cooled, and alkylated with 50 mm iodoacetamide for 30 min in the dark at room temperature. The protein samples were loaded on adjacent lanes of a NuPAGE 4–12% gradient gel (Invitrogen) and electrophoresed at 160 V for 60 min, and the gel was stained with colloidal Coomassie Blue (Invitrogen). The gel lanes were each cut into seven equal sections (with band 1 at the top of the gel) that were washed successively with 50 mM triethylammonium bicarbonate (twice); and acetonitrile (15 min each wash) before drying in a SpeedVac (Eppendorf). Trypsin (5 μg/ml trypsin gold; Promega) in sufficient 25 mM triethylammonium bicarbonate to cover the gel pieces was added for 12 h at 30 °C. The supernatant was transferred to a fresh tube to which two 50% acetonitrile washes of the gel pieces were also added. The digested samples were split into two equal fractions and dried in a SpeedVac. One half was enriched for phosphopeptides using titanium dioxide, and the other half was dimethylated with formic acid using a modified version of the procedure described previously (29). Individual trypsinic digests were redissolved in 2 μl of 25 mM sodium acetate buffer, pH 5.5, 30 mM sodium cyanoborohydride containing 0.2% (v/v) formaldehyde (for the preparation from insulin-stimulated cells and d2 for the preparation from unstimulated cells) and incubated at room temperature for 15 min. The dimethylated digests were mixed pairwise for corresponding gel sections, diluted 25-fold with strong cation exchange (SCX) loading buffer (25% acetonitrile, 0.2% formic acid), and loaded onto 5 μl of Poros 50 HS beads equilibrated in the same buffer. The slurry was loaded on a Millipore SCX ZipTip and washed three times with 60 μl of SCX loading buffer. Peptides were eluted with 2 x 40 μl of 50% isopropanol, 0.2 mM ammonium hydroxide and dried under vacuum.

For phosphopeptide enrichment, tryptic digests of the preparations from insulin-stimulated cells were dissolved in 200 μg/ml 2,3-dihydrobenzoic acid (Sigma-Aldrich) in 80% (v/v) acetonitrile, 5% (v/v) TFA (loading buffer). Titaniumspheres (5 mg of 5-μm spheres; Hichrom Ltd.) equilibrated in loading buffer were added to each digest and agitated for 10 min. The slurry was loaded in a C18 StageTip (Pierce); washed three times with 80% (v/v) acetonitrile, 5% (v/v) TFA; eluted with 40 μl of 1 M ammonium hydroxide, 50% (v/v) acetonitrile; 40 μl of 50% (v/v) acetonitrile; and 40 μl of 0.5% (v/v) formic acid, 50% (v/v) acetonitrile; combined; and dried under vacuum.

LC-MS Analysis—Tryptic digests were analyzed using Ultimate 3000 nanoflow chromatography (LC Packings) coupled to an LTQ-Orbitrap (Thermo Finnigan) mass spectrometer equipped with a dynamic NanoSpray source (Optron). The dimethylated peptide mixtures were separated using an LC Packings Integrated System ( Dionex, Camberley, UK) consisting of a WPS3000T microautosampler, FLMM3200 microcolumn switching module, UltiMate LPG3600 micropump, a PepMap C18 column (75 μm, 15 cm; LC Packings), and mobile phases of 2% acetonitrile, 0.1% formic acid in water (A) and 90% acetonitrile, 0.085% formic acid in water (B). The column was equilibrated in 2% B at a flow rate of 300 nl/min.

The dried digests were resolubilized in 50% (v/v) acetonitrile, 0.1% (v/v) TFA; diluted 10-fold with 0.1% (v/v) TFA; and loaded onto a C18 capillary trap (Michrom Bioresources, Auburn, CA) equilibrated in buffer A at a flow rate of 20 μl/min. After 8 min, the capillary cartridge was switched in line with the analytical column and eluted with the following gradient: 2–50% buffer B (8–80 min), 50–85% B (80–85 min), 85–2% B (85–90 min), and 2% B (90–100 min). The column eluate was electrosprayed with a voltage of 1200 V applied to a Picotip (FS360-50-15-N, New Objective, Woburn, MA).

Mass spectra were acquired using two different acquisition methods. For protein identification, the LTQ-Orbitrap was programmed to perform two FT scans (60,000 resolution) on 300–800- and 800–1800-amu mass ranges with the top five ions from each scan selected for LTO-MS/MS. FT spectra were internally calibrated using a single lock mass (445,1200 atomic mass units). For phosphopeptide analysis, the same two FT scans were performed, but multistage activation was performed on the selected ions with a neutral loss of 97.98, 48.99, 32.66, and 24.50. For these two methods, target ion numbers were 500,000 for FT full scan on the orbitrap and 10,000 MS" on the LTQ.

Peptide and Protein Identification—Raw files were converted to peak lists in Mascot generic format (MGF) files using raw2msm v1.7 software (Matthias Mann) using default parameters and without any filtering, charge state deconvolution, or deisotoping. MGF files were searched using a Mascot 2.2 in-house server against the Internation Protein Index human 3.26 database (57,846 sequences; 26,015,783 residues). For the quantitative dimethyl labeling experiments, search parameters were as follows: digestion with trypsin; two missed cleavages permitted; fixed modification, carbamidomethyl cysteine; variable modifications, oxidized methionine, dimethyl N terminus, and dimethyllsine; a precursor mass tolerance of 10 ppm with a possible wrong picking set to two isotopes; and an MS/MS mass tolerance of 0.8 Da. The Mascot integrated decoy database search calculated a false discovery rate of 1.39% (38 reverse database peptide matches from a total of 2719 peptide matches) when searching was performed on the concatenated MGF files with an ion score cutoff of 20 and a significance threshold of p < 0.05. Only peptides with ion scores over 20 were considered, and only proteins with at least one unique peptide (red bold in Mascot) were considered. This ion score threshold is enough to keep the false discovery rate under 2%. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. When a protein was identified with only one peptide or with only one unique peptide (one red bold peptide), the MS2 spectrum was manually inspected and annotated (supplemental data).

For the phosphorylation site mapping experiments, Mascot search parameters were the same except for variable modifications, which included oxidized methionine and phosphorylation of serine/threonine/tyrosine. The Mascot integrated decoy database search calculated a false discovery rate of 0.45% (12 reverse database peptide matches for a total number of 2675 peptide matches) when searching was performed on the concatenated MGF files with an ion score cutoff of 20 and significance threshold of p < 0.05. Only phosphoproteins with ions scores over 20 were considered. This ion score threshold is enough to keep the false discovery rate under 2%. For more confidence, MS2 spectra were manually inspected and annotated (given in the supplemental figures).

Isotope-based Quantification—Quantification was done manually using Qual Browser v2.0.7 software (Thermo Finnigan). Only unique peptides (red bold peptides in Mascot) with signal intensity over 10^5 counts/s were used for the quantification. The maximum height of the extracted ion chromatogram with a mass tolerance of 10 ppm was used to evaluate the ratio of each calculated light/heavy dimethylated peptide. The reaction incorporates two molecules of formaldehyde per labeling, resulting in a mass difference of 4,025 Da per labeling comparing light and heavy peptides. d2/d0 ratios for three (when possible) peptides were used to rank each protein listed in supplemental Table 1.

RESULTS

Visualization of Proteins Whose Phosphorylation and Binding to 14-3-3s Is Enhanced by IGF1 and Inhibited by PI 3-Kinase Inhibitors—A 14-3-3-Sepharose-binding fraction from extracts of unstimulated cells contained many proteins that displayed 14-3-3 and PAS binding signals (Fig. 1). The number and intensities of such signals increased for preparations from......
cells stimulated with serum and IGF1 and decreased to the basal levels when the PI 3-kinase signaling pathway was blocked with the inhibitors LY294002 (LY; 100 μM for 1 h) prior to stimulation. Cells were lysed in 0.3 ml/dish ice-cold lysis buffer, and 3 mg of each extract was added to 100 μl of a 50% (v/v) slurry of 14-3-3-Sepharose and mixed end over end for 4 h. After washing, the protein bound to 14-3-3-Sepharose was extracted into SDS sample buffer, separated by SDS-PAGE using a 4–12% gradient gel, and analyzed by Far-Western 14-3-3 overlay (red), which detects proteins on the blot that can bind directly to digoxigenin-labeled 14-3-3 proteins. Western blotting was also performed with the anti-PAS antibody (green).

**Fig. 1.** Proteins that bind to 14-3-3s in response to IGF1 in a PI 3-kinase-dependent manner. HEK293 cells cultured on 10-cm-diameter dishes in medium containing 10% (v/v) serum (labeled not serum-starved) were serum-starved for 4 h (unstimulated) and then stimulated as indicated with IGF1 at 50 ng/ml for 15 min and serum at 10% (v/v) for 15 min. Where indicated, cells were incubated with LY294002 (LY; 100 μM for 1 h) prior to stimulation. Cells were lysed in 0.3 ml/dish ice-cold lysis buffer, and 3 mg of each extract was added to 100 μl of a 50% (v/v) slurry of 14-3-3-Sepharose and mixed end over end for 4 h. After washing, the protein bound to 14-3-3-Sepharose was extracted into SDS sample buffer, separated by SDS-PAGE using a 4–12% gradient gel, and analyzed by Far-Western 14-3-3 overlay (red), which detects proteins on the blot that can bind directly to digoxigenin-labeled 14-3-3 proteins. Western blotting was also performed with the anti-PAS antibody (green).

Differential Screen to Identify Proteins Whose Phosphorylation and Binding to 14-3-3s Is Stimulated by Insulin—To identify the unknown targets of insulin/IGF1 signaling indicated by Fig. 1, we devised a strategy based on capturing 14-3-3-binding proteins in the cell extracts and releasing them specifically by competition with a 14-3-3-binding phosphopeptide followed by SDS-PAGE, in-gel protease digestion, isotope differential dimethyl labeling of N-terminal and lysine amine groups on peptides, and quantitative analysis of the $d_0/d_4$ ratios of corresponding peptides from the two preparations (Fig. 2A).

In several trial experiments, approximately twice as much protein was isolated by 14-3-3 capture from extracts of insulin- or serum-stimulated cells compared with serum-deprived, unstimulated cells. Although by eye we could not spot any proteins that looked selectively regulated on SDS-PAGE, Western blots revealed that AS160 was more abundant in the preparations from insulin-stimulated cells (Fig. 3, A, B, and C), whereas KLC2 was approximately equal in both preparations (not shown). These findings are consistent with 14-3-3 binding of AS160 being stimulated by insulin (17, 18) and findings that KLC2 binds to 14-3-3s irrespective of insulin stimulation.2 We therefore proceeded to digest proteins with trypsin to generate peptides for dimethyl labeling and analysis as outlined in Fig. 2A.

Supplemental Tables 1 and 4 list the 296 proteins that were identified and ranked in order of their $d_0/d_4$ ratios where high $d_0/d_4$ ratios indicate proteins that appear to be more enriched in the preparation of 14-3-3-binding proteins from insulin-stimulated cells. The $d_0/d_4$ (±insulin) ratios are also summarized in Fig. 3D with examples of $d_0/d_4$ data for two proteins given in Fig. 3E. Two proteins with low $d_0/d_4$ scores were kinesin heavy chain (KIF5B), which binds 14-3-3 indirectly via phosphorylated kinesin light chains (KLCs), and EML3 (ELP95) (Fig. 3D). In other projects, we have no indication of insulin regulation of KLCs or EML3.3 Strikingly, however, four proteins that are already known to bind to 14-3-3s in response

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2 N. Wood, B. Wong, and C. MacKintosh, unpublished data.
3 N. Wood, B. Wong, K. Dissanayake, K. Geraghty, and C. MacKintosh, unpublished data.
to insulin/IGF1 via PI 3-kinase signaling, namely PFKFB2 (cardiac PFK-2), AKT1S1 (PRAS40), TBC1D4 (AS160), and MYO1C (myosin 1C) (7, 11, 17, 18, 22) scored relatively high $d_0/d_4$ ratios, meaning that these proteins were more highly represented among the 14-3-3-binding proteins from insulin-stimulated compared with unstimulated cells (Fig. 3D). This clustering of known “positives” was encouraging, and instead of refining the primary screen at this stage, we decided to test its predictive power by examining the cellular regulation of individual proteins with high and low $d_0/d_4$ ratios, respectively. The results are given later. We also used a second screening approach.

Phosphorylated Residues on Proteins Isolated by 14-3-3 Affinity Capture from Lysates of Insulin-stimulated Cells—In the second screen, we identified phosphorylated residues in 14-3-3-binding proteins from lysates of insulin/IGF1-stimulated cells using the strategy in Fig. 2B. The full results are in supplemental Tables 2 and 3 with a summary in Fig. 4. Of 221 phosphorylated residues identified, 82 (37%) were derived from RXX(pS/pT) motifs, including 14 (6.3%) that conform to the RXXXX(pS/pT) pattern. Basic residues at position 1 are characteristic of sites phosphorylated by basophilic AGC and Ca$^{2+}$/calmodulin-dependent protein kinases and are often found in 14-3-3-binding sites. We also note some LXXRxX(pS/pT) sites, which can be phosphorylated by kinases on several branches of the Ca$^{2+}$/calmodulin-dependent protein kinase subfamily (31, 32), as well as phosphopeptides with Leu at position 4, which is preferred by AMP-activated protein kinase and other Ca$^{2+}$/calmodulin-dependent protein kinases (33). We identified 58 (26%) (Ser(P)/Thr(P))-Pro motifs, which are not generally associated with 14-3-3 binding. The remaining 81 phosphosites (37%) varied but included sequences with Pro at +2 as did the peptides in the RXX(pS/pT) and RXX(pS/pT) categories. Although not essential for 14-3-3 binding, a +2 Pro is found in the canonical 14-3-3-binding motifs where it adopts a cis conformation that twists the peptide out of the docking site (34, 35). Only 17 proteins, represented by 44 phosphopeptides, were found in both the differential dimethyl labeling ($d_0/d_4$) and phosphopeptide screens, showing that these analyses are far from saturated (Table I).

Although the features of many of the identified phosphopeptides were suggestive, the next question was whether any of the identified sites are actually phosphorylated by basophilic kinases downstream of PI 3-kinase and/or responsible for these proteins binding to 14-3-3. A literature survey showed that several of the RXX(pS/pT) motifs identified here are known 14-3-3-binding sites that are phosphorylated by PKB and other AGC kinases, including sites on PFKFB2 (cardiac PFK-2), BAD, AKT1S1 (PRAS40), and NEDD4L (Nedd4-2) (Refs. 7, 11, 36, and 37; supplemental Table 3). FOXO1 also has PKB-phosphorylated 14-3-3-binding sites that were not identified here, although we identified a (Ser(P)/Thr(P))-Pro site on this protein. The phosphorylated residue we identified on TSC2 is also known, although whether 14-3-3s bind to this site has been controversial (38, 39). We also identified phosphorylated residues from proteins reported previously to bind 14-3-3s in response to other or unknown signaling path-
ways, namely M110/MYPT1, GIT1, Raf1, A-RAF, KSR, PI4KB, PTPH1, KLC2, ELM3 (ELP95), HDAC4, HDAC7A, MAP3K2, DOCK7, CRTC2, YAP1, and WWTR1 (Taz) (Refs. 10, 23, 26, and 40–49; supplemental Table 3). For example, phosphorylated Ser 294 (LNRTNpSQP) on phosphatidylinositol 4-kinase III/PI4KB has been pinpointed as a 14-3-3-binding site that is phosphorylated by protein kinase D and critical for regulation of Golgi trafficking (42). The yeast homologue PIK1 also binds to 14-3-3s at an analogous phosphorylated site (LKRTApSNP) (50).

Cellular Regulation of insulin receptor substrate 2 (IRS2), CCDC6, ZNRF2, SASH1, PRAS40, Vaspodilator-stimulated Phosphoprotein (VASP), and Lipolysis-stimulated Lipoprotein Receptor (LSR)—We next determined whether there were any novel targets of PI 3-kinase signaling among the candidates with high \(d_0/d_4\) ratios (Figs. 2 and 3 and supplemental Table 1) and/or for which basophilic phosphorylated sites were identified (Fig. 4 and supplemental Table 2). Far-Western 14-3-3 overlay assays were used to determine whether tagged forms of candidate proteins extracted from transfected HEK293
Fig. 4. Phosphopeptides derived from 14-3-3-binding proteins isolated from insulin-stimulated cells. The phosphorylated sites were classified according to whether they are found within motifs that conform to RXRXXpS/pT, RX(pS/pT) other than RXRXX(pS/pT), (pS/pT)P, or none of these (other). One phosphorylated site that matches both RX(pS/pT) and (pS/pT)P is included in the RX(pS/pT) group.

FIG. 4 . Phosphopeptides derived from 14-3-3-binding proteins isolated from insulin-stimulated cells. The phosphorylated sites were classified according to whether they are found within motifs that conform to RXRXXpS/pT, RX(pS/pT) other than RXRXX(pS/pT), (pS/pT)P, or none of these (other). One phosphorylated site that matches both RX(pS/pT) and (pS/pT)P is included in the RX(pS/pT) group.

The IRS2 had a $d_0/d_4$ ratio of 9, and phosphopeptides were identified (Fig. 3D, Table I, and supplemental tables). IRS1 is known to bind to 14-3-3s indirectly via intermediary phosphoproteins (10, 26). In addition to IGF1 (in the presence or absence of the dual PI 3-kinase/mammalian target of rapamycin inhibitor PI-103) we stimulated cells with PMA (in the presence or absence of the p90RSK inhibitor BI-D1870) as a first step to testing the responsiveness of targets to other signaling pathways.

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The LSR also had a low $d_0/d_4$ score of 1.2, and phosphorylated sites were identified for this protein, including an RXRXXpS/pT site (RPRARpS493LD; Fig. 3D, Table I, and supplemental tables). HA-LSR gave a 14-3-3-binding signal even when extracted from unstimulated cells, and the signal was no stronger from cells stimulated with IGF1 and PMA (Fig. 5 and data not shown). Nevertheless the binding of LSR to 14-3-3s was abolished by S493A mutation within the identified RXRXXpS motif (Fig. 5). We note that Ser493 has the characteristics of a potential site of phosphorylation by various kinases in the Ca$^{2+}$/calmodulin-dependent protein kinase subfamily, and that 13 phosphorylated residues have been identified in mouse LSR (54). Thus, insulin-independent kinases promote the 14-3-3 binding of LSR.

**DISCUSSION**

14-3-3s bind to hundreds of phosphoproteins, so defining which signaling pathways stimulate the phosphorylation of which 14-3-3-binding sites opens exciting opportunities for understanding the global regulation of diverse cellular processes. Here we combined 14-3-3 affinity capture and proteomics strategies to visualize and identify the subset of proteins whose 14-3-3 binding responds to insulin/IGF1. These screens have proven predictive for those proteins that we tested with several novel validated targets of the insulin/IGF1/PI 3-kinase signaling pathway identified (Fig. 5). Further mining of the data sets should be fruitful, and the many candidates that remain to be checked include proteins with intriguing links to cancer, diabetes, and neurodegenerative disorders.
### Table I

14-3-3 affinity-captured proteins identified by both their $d_3/d_4$ ratios and phosphopeptides

Seventeen proteins, represented by 44 phosphopeptides, were found in both the $d_3/d_4$ (± insulin) ratio screen (supplemental Table 1) and phosphorylation site identification screen (marked by * beside the gene symbols in supplemental Tables 1 and 3). The sequence within the square brackets indicates the phosphopeptide identified by mass spectrometry, whereas the extended sequence outside of the brackets is shown to aid identification of motifs that may be recognized by the protein kinases that phosphorylate these sites.

| Approved gene symbol | $d_3/d_4$ (± insulin) ratio | Phosphorylated residues as in supplemental Tables 2 and 3 with oxidized methionines in lowercase letters |
|----------------------|----------------------------|--------------------------------------------------------------------------------------------------|
| AKT1S1 (PRAS40)      | 9.9                        | LPRPR[LNIIDQFK]a/QQYAK[LPVSVVPWGFK]                                                              |
| CAD                  | 6.7                        | R[HRhSDPGLPAEPEK]/P[HRhSDPGLPAEPEK]                                                              |
| CCDC6                | 7.1                        | K[LDQPVSPAPPS][D/R][LPQEKLQPVSPAPPS][D/R]                                                       |
| DUT                  | 1.4                        | M/[PCEPTAPasPSK][A]                                                                             |
| EML3                 | 1.6                        | KLSRK[AsISANLVR][S/KLSR][K1asISANLVR][S AGPAPATPSR][PaspLLSPLSVL]                             |
| FAM122B              | 3.5                        | DPKELSKP[RDFTVPasPAPSP][TR]                                                                      |
| FOXK1                | 0.3                        | PLSSR[aspASPTPHLmSP]                                                                             |
| HSPB1                | 2.7                        | RALSRRQ[LSGVEIR][H]                                                                              |
| IRS2                 | 9.0                        | RSYR[RVsGDAADLDLR][G]                                                                            |
| LMO7                 | 2.1                        | GMR[RGsLDNLSP][S/RRQ][A]                                                                       |
| LSR                  | 1.2                        | K[NLALSRESLV]-[R/GPLATIPRDEEWWWGHS][PSR][S/RRQ][A]                                              |
| LMCALL1              | L = only found in insulin preparation |                                                                                                  |
| PFKFB2               | 6.2                        | VEQpM[POasPGLAPR][T/PVRMR][RNSFTPLSSNTIR][a]                                                   |
| TPI1                 | 3.2                        | KMNGR[QRsLGEITLNAAK][V]                                                                         |
| WDR20                | 1.5                        | K[FALTslHR][K]                                                                                  |
| YAP1                 | 10.6                       | KHSR[QAASDGATAGALTPOHVR]/PPEPK[SHsRQASDAGTAGALTPOHVR]                                           |
| ZNRF2                | 2.6                        | RTR[AYsGSDLPSGSSGANGTAGGG[GA][A]                                                               |

*a RXRX(pS/pT) motifs.

In contrast to SILAC procedures where differential labeling is done at the cell stage, dimethyl labeling was performed later on in our procedure, although with adequate controls this should not be a problem, and our methods are applicable to cell and tissue types that cannot be conveniently SILAC labeled. Refining the quantitative aspects of the screens, defining changes in phosphorylation stoichiometries, filtering out proteins that show minor responses to stimuli, and so on will require many reiter-
ative comparisons backed up by biochemical analyses such as those in Fig. 5. We noticed for example that most of the proteins identified had a $d_0/d_4$ ($\equiv$ insulin) ratio greater than 1 (Fig. 3B), suggesting that unknown underlying trends might have skewed the data. Possibilities are selective proteolysis of the 14-3-3-binding proteins from unstimulated cells and/or an insulin-stimulated modification of the endogenous 14-3-3s that lowers their avidity so that targets are more easily released and better able to bind to the 14-3-3-Sepharose.

We also note that the data fell into a range of $d_0/d_4$ rankings, not a discrete division into high and low ranked (insulin-stimulated binding or not) categories. This gradation could relate to the fact that 14-3-3s are dimers, which often bind to two phosphorylated sites on the same target. Thus, in some cases insulin could stimulate phosphorylation of both 14-3-3-binding sites, whereas other proteins might already have one site phosphorylated in the unstimulated state. For example, basal binding of 14-3-3s to TBC1d4 (AS160) due to phos-
phorylation of Ser\textsuperscript{341} is markedly enhanced when insulin stimulates phosphorylation of both Ser\textsuperscript{341} and Thr\textsuperscript{642} of AS160 (17). In 2002, Yaffe (55) introduced the concept of one phosphorylated site on a target providing a high affinity 14-3-3 binding “gatekeeper” that facilitates docking of a second lower affinity site on the same target into the other side of the 14-3-3 dimer. In such a configuration, a 14-3-3 dimer behaves as a logic gate, a device in which either (“or”) or both (“and”) of two inputs are needed to trigger an output. Comparing the data in Fig. 5 gives a sense of how targets differ in the “digital logic” of how their two phosphorylated sites engage with a 14-3-3 dimer presumably dependent on differences in affinities and avidities. For example, phospho-Ser\textsuperscript{19} appears to be a gatekeeper site that is dominant for binding of ZNRF2 to 14-3-3. In contrast, 14-3-3 displays one mode of binding to SASH1 that is not regulated by IGF1 and PMA but that is enhanced by phosphorylation of the IGF1/PI 3-kinase- and PMA/p90RSK-responsive Ser\textsuperscript{50}. In cases where different kinases phosphorylate each site, 14-3-3s effectively become “coincidence detectors” for two different inputs. Perhaps the binding and functional effect of 14-3-3s can also depend on the temporal order of phosphorylation and dephosphorylation of the relevant sites. It will be interesting to explore the full range of digital behaviors of 14-3-3s more widely in the future.

Overall our aim to identify novel targets of the PI 3-kinase signaling pathway was achieved. Newly validated insulin-responsive targets include a protein involved in the insulin/PI 3-kinase signaling pathway itself (IRS2), a proapoptotic protein (CCDC6), an E3 ubiquitin ligase implicated in synaptic vesicle trafficking (ZNRF2), and a signaling adapter protein (SASH1). We also confirmed direct binding of 14-3-3s to CCDC6, 14-3-3s (24, 27) and is a known target of growth factor signaling by virtue of its fibroblast growth factor-stimulated phosphorylation on a tyrosine residue (MRVLpYYMEK where pY is phosphotyrosine) (71). Levels of this protein are elevated in certain colon cancers (72), whereas knockdown of LSR increases motility of bladder cancer cells (73). LSR is a transmembrane protein with extracellular immunoglobulin-like domains and phosphorylated sites in the putative intracellular region (Pfam database). The name LSR refers to its proposed role as a cell surface lipoprotein receptor that is activated by free fatty acids and clears dietary triglyceride-rich lipoproteins from the blood into liver and other tissues (74–76). LSR is critical for liver and embryonic development (77). The other name for this protein, liver-specific basic helix-loop-helix leucine zipper transcription factor (LISCH7), appears to be a misnomer. LSR is encoded by one of three related human genes (LSR, C1orf32, and ILDR1), and genetic variation in the mouse form of C1orf32, named Lisch-like, was recently linked genetically to type II diabetes in mice. These findings give exciting prospects of insights into how insulin and growth factors control cell behavior.

CCDC6 is best characterized as a transforming fusion with the tyrosine kinase RET or phosphatase PTEN in papillary thyroid tumors (56–59) and tyrosine kinase domain of the platelet-derived growth factor \( \beta \) receptor in certain leukemias (60, 61), suggesting that the CCDC6 gene has a high propensity for recombination (62, 63). The normal CCDC6 protein was identified as a substrate of the kinase ataxia telangiectasia mutated in response to DNA damage (64). In wild-type cells, etoposide and ionizing radiation promoted phosphorylation on Thr\textsuperscript{343} by ataxia telangiectasia mutated, which stabilized the nuclear buildup of CCDC6 and promoted apoptosis (64, 65). We hypothesize that binding of 14-3-3 to CCDC6 in response to PI 3-kinase signaling may inhibit its proapoptotic activity.

Zinc and ring finger 2 is one of two E3 ubiquitin ligases (ZNRF1 and ZNRF2) with a zinc finger adjacent to a ring finger (66). E3 ligases generally act as specificity modules that bring substrates to the E2 for ubiquitylation. In yeast two-hybrid experiments, ZNRF2 was one of several E3 ubiquitin ligases that bind to UBC13, the active component of the E2 ligase for Lys\textsuperscript{63}-linked ubiquitylation (67), and we found that UBC13 co-purifies with ZNRF2 from extracts of transfected HEK293 cells (data not shown) indicating a physiological partnership between ZNRF2 and UBC13. ZNRF1 and ZNRF2 are both highly expressed in nerve cell presynapses, and the catalytically inactive proteins inhibit Ca\textsuperscript{2+}-dependent exocytosis in PC12 cells (66, 68). Serine 19 of ZNRF2, which is phosphorylated and binds to 14-3-3s in response to both IGF1/PI 3-kinase and phorbol ester, is N-terminal to a MAGE domain of unknown function (69), suggesting that regulated binding of 14-3-3s to ZNRF2 may affect Lys\textsuperscript{63} ubiquitylation via effects on the MAGE domain of ZNRF2.

SASH1 is a little characterized, ubiquitously expressed member of the sterile \( \alpha \) motif- and SH3 domain-containing SLY1 family of signaling proteins whose down-regulation in colon cancers was found to have negative prognostic significance for metastases and survival (70).

For LSR, 14-3-3s bind to at least one phosphorylated site (phospho-Ser\textsuperscript{345} within RPRARpS\textsuperscript{435}/VDAL), and although the relevant kinase is unknown, AMP-activated protein kinase is a candidate. LSR had been isolated previously by its affinity for 14-3-3s (24, 27) and is a known target of growth factor signaling by virtue of its fibroblast growth factor-stimulated phosphorylation on a tyrosine residue (MRVLpYYMEK where pY is phosphotyrosine) (71). Levels of this protein are elevated in certain colon cancers (72), whereas knockdown of LSR increases motility of bladder cancer cells (73). LSR is a transmembrane protein with extracellular immunoglobulin-like domains and phosphorylated sites in the putative intracellular region (Pfam database). The name LSR refers to its proposed role as a cell surface lipoprotein receptor that is activated by free fatty acids and clears dietary triglyceride-rich lipoproteins from the blood into liver and other tissues (74–76). LSR is critical for liver and embryonic development (77). The other name for this protein, liver-specific basic helix-loop-helix leucine zipper transcription factor (LISCH7), appears to be a misnomer. LSR is encoded by one of three related human genes (LSR, C1orf32, and ILDR1), and genetic variation in the mouse form of C1orf32, named Lisch-like, was recently linked genetically to type II diabetes (78). C1orf32 has a serine (ESRAHS\textsuperscript{469}GFYQ) corresponding to Ser\textsuperscript{435} in LRS that although not in an RXRXXpS motif does look like a potential AMP-activated protein kinase site, but there is no matching serine residue in ILDR1. Given that elevated levels of fatty acids and triglyceride-rich lipoproteins are linked to inflammation and insulin resistance (79, 80), we need closer examination of the roles and regulation of this family of proteins.

In summary, 14-3-3 capture and quantitative proteomics led us to discover new downstream targets of insulin signaling with roles in apoptosis, vesicle trafficking, metabolism, and cancer. Future work on these proteins should lead to exciting mechanistic insights. Although insulin/PI 3-kinase/AGC sig-
naling has a dramatic effect in regulating many 14-3-3-binding proteins (Fig. 1), of the targets that we followed up only CCDC6 and PRAS40 were selectively responsive to PI 3-kinase, whereas IRS2, 2NRF2, and SASH1 are convergence points for the PI 3-kinase and MAPK/p90RSK pathways (Fig. 5). Understanding how these two pathways exert widespread controls over cells is a fascinating problem made all the more pressing because drugs that target these pathways are promising anticancer therapies, although interplay between these two pathways is emerging and must be understood to devise optimal combination therapies. One ambitious goal is therefore to use 14-3-3 capture combined with sensitive mass spectrometry to define how the global 14-3-3-binding phosphoproteome responds to different extracellular stimuli and inhibitors. Such overviews of the changing 14-3-3-linked phosphoproteome of cells could be used to pinpoint biomarkers whose 14-3-3 binding status shows which signaling pathways are active in healthy and diseased tissues and how they respond to drugs.

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