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Proteomic Analysis Reveals Novel Molecules Involved in Insulin Signaling Pathway

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The binding of insulin to its receptor triggers a signaling cascade regulated by protein complexes via tyrosine phosphorylation events on a multitude of associated proteins. To search novel phosphotyrosine proteins or associated proteins involved in insulin signaling pathway, we employed a method in which Rat1 cells stably expressing the human insulin receptor were stimulated with or without insulin and sub-fractionated prior to enrichment of phosphotyrosine proteins by immunoprecipitation and analysis by LC−MS/MS. Bioinformatic analysis and manual confirmation of peptide phosphorylation site assignments led to identification of 35 phosphotyrosine sites derived from 31 protein groups. Over 50% of these proteins were reported for the first time as tyrosine phosphorylated, including gigaxonin, XIAP and CDK10. In addition, we also found that calcium/calmodulin-dependent protein serine kinase (CASK), a key protein in protein-targeting and vesicle transport in neurons, forms a complex with two unidentified phosphotyrosine proteins pp100 and pp95 in response to insulin-stimulation, though CASK is not itself tyrosine phosphorylated. Furthermore, insulin was able to decrease CASK nuclear location, as well as down-regulate the expression of CASK targeted genes. Our results imply CASK as a novel joint knot connecting CASK-mediated pathways with the insulin signaling. Our data provide a wealth of information potentially paving the way to identify new components in the insulin signaling network.

Keywords: insulin signaling pathway • subcellular fractionation • immuno-affinity purification • phosphotyrosine sites • CASK • T-element

Introduction

Insulin, the most potent anabolic hormone known, enhances the synthesis and storage of carbohydrates, lipids, and protein, while also inhibiting their degradation and release back into the circulation. Dysregulation of insulin secretion or signaling is associated with a variety of diseases, including diabetes, obesity, hypertension, polycystic ovarian syndrome, dyslipidemia, and atherosclerosis. At the cellular level, insulin action is characterized by diverse effects, including changes in vesicle trafficking, promotion of cellular growth and differentiation, and regulation of transcription.[1−4] These effects are initiated by insulin binding to the tetrameric insulin receptor (IR) consisting of two α- and two β-subunits.[1] Tyrosine kinase activity of the later catalyzes the phosphorylation of several intracellular substrates, including the insulin receptor substrate (IRS) proteins,[5] Shc,[6] PLCγ,[7] SIRP,[8] GAB-1,[9] APS,[10] p60^{DOK},[11] and c-Cbl.[12] Each of these substrates recruits a distinct subset of signaling proteins containing Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains, both of which interact specifically with sequences surrounding phosphotyrosine residues.[13−17] Moreover, each of these substrates can be confined to distinct locations within the cell by targeting sequences that direct association with other proteins or lipids. These phosphotyrosine proteins interact with other phosphotyrosine and nonphosphotyrosine proteins in this and other signaling pathways (cross-talk) to make up complex signalsomes, defined by the molecular components and/or cellular compartment. The makeup of signaling complex components and physical location forms the basis for selectivity and specificity of signal transduction.[4,18,19]

In the past, several signal pathways including the EGFR, FGFR, interferon-α, and other phosphotyrosine pathways have been systematically analyzed by enrichment of phosphotyrosine proteins or phosphotyrosine peptides using a combination of anti-phosphotyrosine immunoaffinity purification or/and immobilized metal affinity chromatography (IMAC), followed

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by identification through mass spectrometry.[20−24] These methods were successful in mapping the relationships between many novel phosphotyrosine proteins and/or phosphorylation sites and specific signaling pathways. While this type of approach has the advantage of efficient enrichment of phosphorylated targets, it carries the caveat that some interaction partners or low phosphorylated proteins are lost during the sample preparation. It is important to consider that some proteins, for example, some members of 14-3-3 and PKC family, while not themselves phosphotyrosine proteins, have nonetheless been confirmed as key components of signaling pathways through direct or indirect association with phosphotyrosine proteins.[25,26] Thus, to comprehensively understand signaling networks and their biological functions, direct analysis of both phosphotyrosine proteins, and the other components associated with their signaling complexes would be informative.

We have undertaken a novel approach whereby stimulated cells are first separated by sub-cellular fractionation before conventional analysis. In our system, Rat1 cells stably expressing the human insulin receptor (Rat1/hIR) were used as a cell model, as it has previously been validated as a suitable model for investigating the insulin signal pathway.[8] After stimulation with insulin, Rat1/hIR cells were fractionated into cyto., mem., and nucl. fractions. Insulin-induced phosphotyrosine protein complexes were then purified from these fractions by immunoprecipitation with anti-phosphotyrosine antibodies, specifically eluted by phenyl phosphate, and then analyzed by LC−MS/MS. Phosphopeptide profiling led to the identification of 31 unique peptides containing 35 phosphotyrosine sites, many of which were reported here for the first time. Moreover, we provide the first evidence linking the nonphosphotyrosine protein, calcium/calmodulin-dependent serine kinase (CASK), to the insulin signal pathway.

Experimental Section

Materials. Monoclonal antibody (mAb) 4G10, CSAK, and Na^+/K^+-ATPase were purchased from Upstate Biotech. Polyclonal antibody (pAb) against laminB was obtained from Santa Cruz Biotechnology. pAb CASK (ab3383) and blocking peptide (ab4961) was purchased from Abcam. mAb against beta-actin and secondary antibodies were purchased from Sigma. The pGL3 and pGL3−2T plasmids were a generous gift from Dr. Kai Schuh (University of Wuerzburg, Germany). The pRK5-CASK plasmid was kindly provided by Dr. Ben Margolis (Howard Hughes Medical Institute, University of Michigan Medical Center, USA). The GK domain of CASK (686−921aa) was cloned into the EcoRI and XbaI sites of pRK5. The sequences of the CASK RNAi oligonucleotides are as follows: CASK shRNA sense primer, 5′-GGGAAGCCAGTATCTGTCATAAAGCTTTATGACAGATACTGGCTTCCCTTTTTG-3′; CASK shRNA antisense primer, 5′-AATTCAAAAAGGGAAGCCATCTGTCATAAAGCTTTATGACAGATACTGGCTTCCC-3′. The sense and antisense oligonucleotides were annealed and cloned into the ApaI and EcoRI sites of pBS−U6 vector.

Cell Culture, Transfection, and Luciferase Assays. Rat1 cells stably expressing the human insulin receptor (Rat1/hIR) were cultured in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2.[8] Transient transfection was carried out with Lipofectamine 2000 reagent (Invitrogen). Each transfection used 2 μg of total DNA, comprised of 0.8 μg of control vector (pGL3) or plasmid with T-element promoter (pGL3−2T),[27] 1 μg of pRK5-CASK, pRK5-GK, pRK5, or CASK RNAi plasmid, and 0.2 μg of the pCMV-*lacZ* (Promega) as an internal control to monitor cell viability and to normalize transfection efficiencies among different experiments. Thirty-six hours post-transfection, cells were starved for an additional 12 h, stimulated with 100 nM insulin for a variety of time points, then harvested and lysed in 1× Reporter Lysis Buffer (Promega). Luciferase activity, normalized across different transfections using β−galactosidase activity, was determined using the Luciferase Assay System (Promega). Values shown represent the mean ± SEM. Each transfection was performed in duplicate and repeated at least three times.

Immunofluorescence. Rat1/hIR cell monolayers were grown to 80% confluence in complete medium before replacing the medium with serum free media. After 12 h, the cells were either left untreated or stimulated with insulin (100 nM) for 60 min. Cells were then fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton X-100. After incubation with anti-CASK, the slide was rinsed with TBST (50 mM Tris-HCl, pH7.5, 150 mM NaCl, and 0.05% Tween-20) and incubated with FITC-conjugated anti-mouse IgG antibody. Images were captured using a CCD camera (model DC350F; Leica) on a microscope (model DM5000B; Leica).

Fractionation, Immunoprecipitation, and Immunoblotting. Fractionation was performed using a modification of the previously published method.[28] Adherent cell cultures (150 mm) either left untreated or stimulated with 100 nM insulin for the indicated time, then rinsed twice with phosphate buffered saline (PBS) at 4 °C. Washed monolayers were then scraped from the dish into 750 μL of hypotonic buffer (10 mM Tris, 1 mM NaF, 10 mM IAA, pH 7.5) containing protease inhibitor cocktail (Roche) and incubated on ice for 20 min before the cells were passed through a 25-g needle approximately five times. The resulting lysate was centrifuged at 1000 rpm at 4 °C for 15 min, after which the pellet was re-suspended in 250 μL of hypotonic buffer, re-extracted, and centrifuged as before. Supernatants from the first and second spins were combined, adjusted to 0.25 M NaCl, and fractionated into cytosolic (supernatant) and membrane (pellet) fractions by centrifugation at 19 000 rpm (43 000 × *g*) for 90 min at 4 °C. The resulting pellet was re-suspended and further separated into nuclear (pellet) and Triton X-100 soluble (supernatant) fractions by centrifugation at 13 000 rpm. The Triton X-100 soluble and membrane fractions were combined. Samples from total cell lysate, membrane, and nuclear fractions were suspended in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholic acid sodium) and sonicated. Cleared cell lysates were incubated for 4 h with 30 μL of anti-phosphotyrosine-agarose (Sigma) at 4 °C. Precipitated immune complexes were washed three times with HNTG (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% Glycerol, pH 7.5) and then eluted with HNTG containing 100 mM phenyl phosphate (Sigma) at 4 °C. After SDS-PAGE separation, proteins were either visualized by silver staining or transferred to nitrocellulose membrane and subjected to western analysis with appropriate primary and horseradish peroxidase-conjugated secondary antibodies. Antibody labeling was visualized with ECL reagent (Pierce) according to the manufacturer's instructions. Image analysis of silver stained gels and immunoblots was performed using QuantityOne software (Bio-Rad).

Enzyme Digestion, Mass Spectrometry, and Protein Identification. The samples were digested by trypsin according to the published method.[29] Chromatography was performed using a surveyor LC system (Thermo Finnigan, SanJose, CA) on a C18 reversed-phase column (RP, 180 μm × 150 mm, BioBasic C18,

5 μm, Thermo Hypersil-Keystone). The pump flow rate was split 1:100 for a column flow rate of 1.5 μL/min. The column effluent was directly electrosprayed using an orthogonal metal needle source without further splitting. Mobile phase A was 0.1% formic acid in water and the B mobile phase was 0.1% formic acid in acetonitriacetonitrile. The separation of peptides obtained by enzymatic digest of sample was achieved with a gradient of 2−80% B over 480 min analyzed with an LCQ Deca XP ion-trap mass spectrometer. The micro-electrospray interface used a 30 μm metal needle orthogonal LCQ inlet. The instrument was set so that one full MS scan was followed by three MS/MS scans on the three most intense ions from the MS spectrum with the following Dynamic Exclusion settings: repeat count, 1; repeat duration, 0.5 min; exclusion duration, 3.0 min.

To identify phospho-proteins in the above sample, dta files were created using Bioworks3.1, with precursor mass tolerance 1.4 Da, minimum ion count 15. After MS/MS dta files created separately, spectra were searched with the SEQUEST algorithm against the nonredundant rat protein database from IPI (ipi-.RAT.v3.04), with a mass allowance of 3 Da, and 0.0000 fragment ion tolerance. All cysteine residues were searched as carboxamidomethycystein (+57.02 Da). Up to two internal cleavages sites were allowed for tryptic searches. Dynamic modifications were permitted to allow for the detection of oxidized Met (+15.9994 Da), and phosphorylated Tyr (+79.9799 Da). An accepted SEQUEST result required a ΔCn score of at least 0.1 (regardless of charge state) and Xcorr (one charge $\geq$ 1.9, two charges $\geq$ 2.2, three charges $\geq$ 3.75) for protein identification or Xcorr (one charge $\geq$ 2.0, two charges $\geq$ 2.5, three charges $\geq$ 4.0) for phosphotyrosine protein identification. Single peptides that alone identify a protein were manually validated after meeting the above criteria.

Bioinformatics Analysis. Proteins were categorized by molecular function according to Gene Ontology (GO) annotation terms extracted by Human Protein Reference Database (HPRD, *http://www.hprd.org*). The subcellular location of proteins was annotated by extraction from HPRD. The relationship between the phosphotyrosine proteins and insulin signaling pathway was confirmed by searching the interacting from HPRD database.

Results

Improvement of Affinity Purification in Combination with Subcellular Fractionation. To efficiently isolate a comprehensive collection of phosphotyrosine proteins and their associated protein of the insulin signaling pathway from different cellular compartments, we followed the approach schematized in Figure 1A. Because cells vary in their sensitivity to insulin, we optimized insulin treatment conditions. As shown in Figure 1B, at the concentration of 100 nM of insulin, 20 min treatment of cells seemed to induce the highest level of total tyrosine phosphorylation. Thus, 20 min treatment was used for the subsequent experiments.

Rat1/hIR cells stably overexpress the human insulin receptor, which becomes the predominant phosphotyrosine protein in response to insulin stimulation (Figure 1B). Results from pilot experiments and our previous work[8] have shown that a high level of phosphorylated IR β chain would titrate a large portion of the anti-phosphotyrosine antibody, thus prevent the antibody from binding to other phosphotyrosine targets in conventional immunoprecipitation reactions (Figure 1B,C and see Experimental Section). To achieve more representative phosphotyrosine protein precipitation, we subfractionated Rat1/hIR cells into membrane (Mem.), cytosolic (Cyto.), and nuclear (Nucl.) fractions in addition to using total cell lysates (TCL). Immunoprecipitation reactions were carried out with these individual sub-cellular fractions, and the resulting precipitated proteins were specifically eluted by the HNTG buffer containing 100 mM phenyl phosphate[30] (see Experimental Section) and resolved by SDS-PAGE. Total tyrosine phosphorylation in the fractions was evaluated by Western blot using anti-phosphotyrosine (4G10) antibody. As expected, overall tyrosine phosphorylation was enhanced by insulin treatment, with each fraction showing distinct patterns of phosphorylation (Figure 1C). The major tyrosine phosphorylation patterns of the total lysate, nuclear, and membrane fractions were relatively similar, with exception of a few phosphorylated bands (Figure 1C,D). In contrast, dramatic differences in total phosphotyrosine pattern were observed in the cytosolic fraction when compared to the others (Figure 1C, middle right panel).

To directly visualize total enriched proteins, regardless of whether they are phosphotyrosine proteins or associated complex proteins, one SDS-PAGE gel was silver stained. This analysis revealed differences not only between treated and untreated samples, but also among the various cellular fractions (Figure 1D). As anticipated, insulin receptor β and α subunits with MW of 100 and 130 kDa, respectively, were highly enriched in the membrane fraction and the TCL (Figure 1D). As above, the cytosolic fraction displayed a unique pattern of enriched proteins compared to the other fractions. Interestingly, while the general patterns of stimulated and unstimulated membrane fractions were similar, notable differences in protein density were observed (Figure 1D, middle left panel). It is possible that overexpression of IR in these cells led to increased a background level of IR autophosphorylation. It also explains the appearance of some phosphotyrosine signals in untreated membrane fractions (Figure 1C, middle left panel). We did not observe this effect in the Rat1 parental cell line (Supporting Information Figure 1).

Comparison of the patterns observed in the fractionated cell lysates highlighted the need for fractionation to improve the efficiency of immunoprecipitation, as well as to achieve more complete coverage of phosphotyrosine proteins and their associated complexes. While some of the fractions appeared similar to the total cell lysate, unique differences, which would have been otherwise masked by strong signals, were uncovered in the cytosolic fractions.

Identification of Phosphotyrosine Sites by LC−MS/MS. Phosphotyrosine proteins and their associated complexes precipitated from cellular fractions of Rat1/hIR cells treated or left untreated with insulin were analyzed by LC−MS/MS. In total, 700 protein groups (1100 proteins) were identified by mass spectrometry, including about one-third of proteins identified from at least two unique peptides (Supporting Information Table 2). According to the evaluation of the LC−MS/MS data, as well as manual confirmation of peptide phosphorylation site assignments, we found 35 phosphotyrosine sites derived from 31 unique peptides (Supporting Information Table 1).

All the phosphotyrosine proteins were categorized to three clusters; the first one includes previously reported insulin signaling proteins with identified phosphotyrosine sites (Supporting Information Table 1, Blue); the second contains the insulin signaling proteins involved in this pathway with new identified phosphotyrosine sites (Supporting Information Table

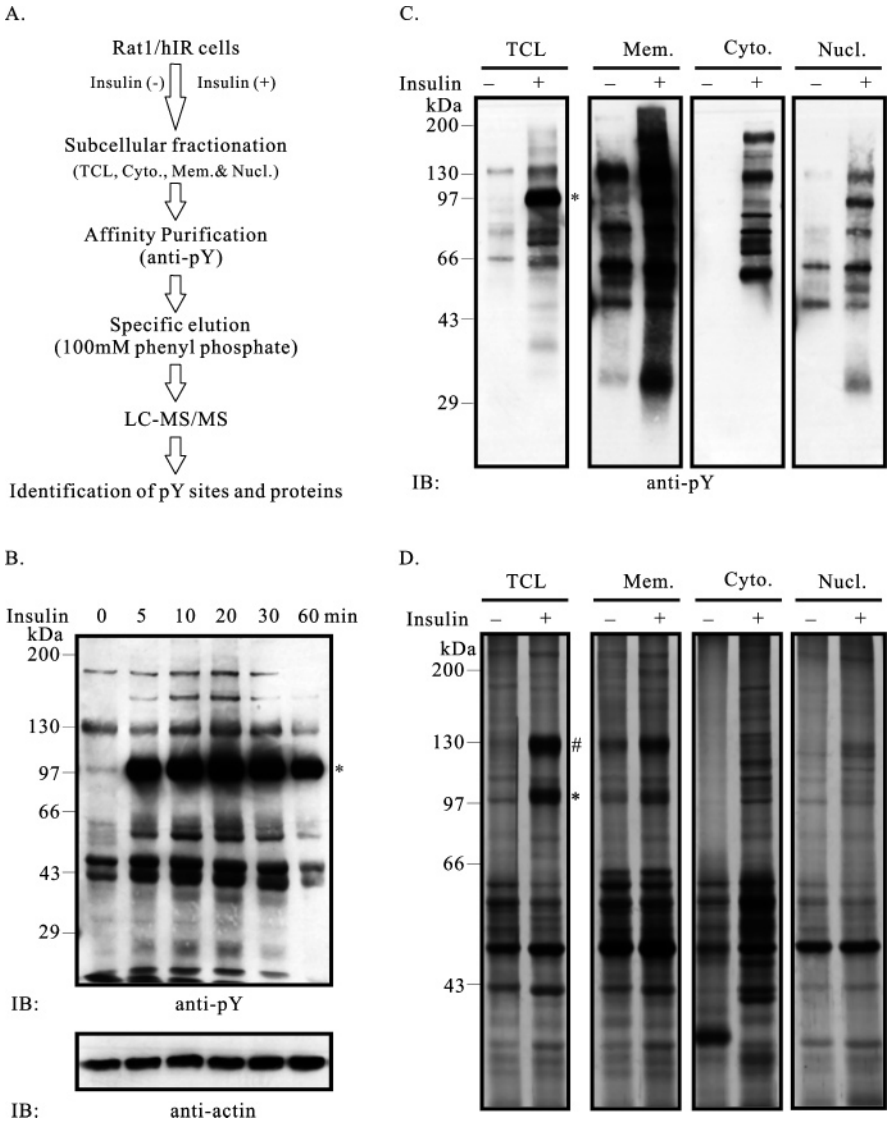

Figure 1. Enrichment of phosphotyrosine proteins by subcellular fractionation and immunoaffinity purification from Rat1/hIR cells. A diagram describing the experimental process is shown in (A). (B): Rat1/hIR cells were stimulated for a time course by 100 nM insulin. Total cell lysates were run on 10% SDS-PAGE and immunoblotted with anti-pY (4G10). Actin was used as a loading control. (C) & (D): Rat1/hIR cells were left un-treated or stimulated with 100 nM insulin for 10 min. The phosphotyrosine proteins were purified just as described and resolved on 10% (C) or 8% (D) SDS-PAGE and visualized by immunoblot with 4G10 (C) or silver staining (D). Because the level of phosphotyrosine from different fractions was vastly different, the different exposures were used. (*, IRβ; #, IRα).

1, Red); the third is that fully novel identified proteins based on their phosphotyrosine sites and involvement in insulin signal pathway (Supporting Information Table 1, Black). Through searching the HPRD database, about 51% (23/45, Supporting Information Table 1, Blue and Red) of proteins involved in insulin signal pathway whether from insulin stimulation or not including 21 unique peptides (21/31) involved in insulin signaling pathway (Supporting Information Table 1). Among these were LPRPEDTLTpY(477)ADLDM#VHLNR (Figure 2A) and EITQIQDTNDINDITpY(436)ADLNLPK (Figure 2B) peptides from SIRPα1, and LIEDNEpY(419)TAR (Figure 2C), derived from Yes kinase. The identification of these three peptides was expected as both proteins are widely known to be involved in insulin signal transduction.[8,31] In addition to these well-known proteins, we also report here for the first time many novel phosphoproteins and phosphorylated sites. These results are summarized in Supporting Information Table 1, and a few examples were shown in Figure 2. For example, a singly phosphorylated peptide IKEEpYEVSELGAPHGSASVR from interferon-inducible protein was identified by mass spectrometry (Figure 2D). From the spectral data one can see that the *b*- and *y*-ion series are consistent with the theoretically predicted peaks of the identified phosphopeptide from interferon-inducible protein. The peptide EPpYRKLVVPSPDVLK of KIAA1476 was also identified for the first time in our study (Figure 2E). MS/MS spectrum of the bivalently charged precursor ion (m/z 911.96), representing one of the identified phosphopeptides, was identified based on only MS/MS spectra and database searching. In addition to the above examples, phosphopeptides were identified from several interesting proteins that were not previously thought to contain phosphotyrosine residues, such as XIAP, gigaxonin, Rho-GTPase activating protein 24, and CDK10 (Supporting Information Table 1).

Each of the identified phosphotyrosine proteins was categorized by criteria such as molecular function and cellular location (Figure 3A, 3B, and Table 1). Figure 3A shows proteins

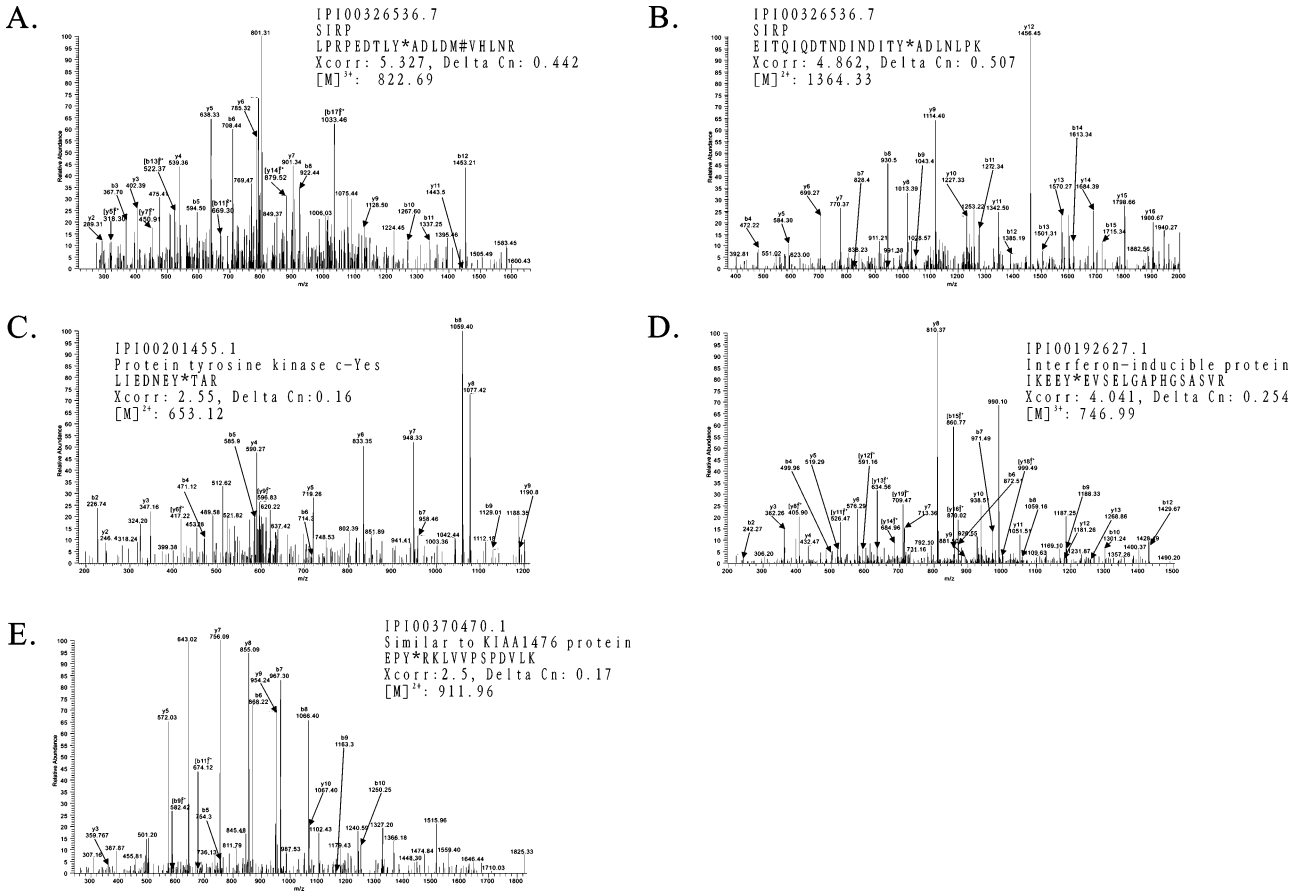

Figure 2. MS map of phosphotyrosine peptides. Shown is the LC−MS/MS analysis of tryptic peptides derived from some phosphotyrosine proteins identified in this study. The fragmentation pattern of the peptide is indicated (only *b*- and *y*- ions were indicated for simplicity). (A), (B), and (C) illustrate some of the phosphoproteins or phosphorylated sites reported in previous research; (D) and (E) show some of the phosphoproteins and phosphorylated that emerged for the first time in the present study. The *b*- and *y*-ion series used for the phosphopeptide identification are indicated. Y* represents the phosphotyrosine and M# represents oxidized Met.

classified by function using the Gene Ontology (GO) annotation terms as a standard. Of the identified phosphotyrosine proteins, 29 proteins containing 26 unique peptides were labeled by one annotation term within the GO molecular function category (Figure 3A and Supporting Information Table 1). Among these, about 90% (25/29) were involved in signal transduction, transcriptional regulation, and protein metabolism, which make up the main targets of the insulin signal pathway.[3,4,32] The subcellular location of identified phosphotyrosine proteins was also analyzed by reference of HPRD database, successfully mapping most proteins (31 or 70.5%) to at least one location (Figure 3B).

Insulin Inhibits CASK Nuclear Location and Activity of a T-Element-Regulated Reporter Vector. The proteins that emerged from our enrichment technique were presumably involved in the insulin-signaling network, though many had not previously been reported to be involved in insulin signaling. One of these, the adaptor protein CASK, was well-known to play an important role in protein targeting, vesicle transport, and cell polarity.[33−35] On the basis of these functions, we surmised that it might also be involved in the insulin signal pathway. We did not detect any tyrosine phosphorylation of CASK itself in the insulin-treated cells (Figure 4A); however, CASK was clearly associated with two phosphotyrosine proteins. One of these was found in a smear centered at 100 kDa (pp100), while the other one was resolved as a sharp band at 95 kDa (pp95) (Figure 4A). Western blot analysis revealed that insulin-stimulation notably enhanced the association of CASK with these precipitated phosphotyrosine proteins (Figure 4B).

Since cellular localization of CASK is important for carrying out its function,[33−37] CASK translocation in response to insulin was performed on sub-fractionated lysates (Figure 5A). Dynamic analysis of CASK location manifested the optimized insulin stimulation for 60 min (data not shown). Insulin-stimulation has no effect on CASK level in total cell lysate (Figure 5A, TCL). However, cytosolic and nuclear CASK levels were remarkably decreased after 60 min insulin stimulation (Figure 5A, Cyto. & Nucl.). In contrast, membrane CASK level was dramatically increased up to about 4.5-folds in comparison to the control level (Figure 5A, Mem.).

To visually track CASK translocation, we performed immunofluorescence analysis on CASK in Rat1/hIR cells stimulated with insulin for 60 min. In insulin-untreated cells CASK formed as spot signals, and distributed mainly in the cytoplasm, but in the nuclear and on the membrane as well (Figure 5B, left panel and data not shown). Consistent with the kinetic analysis of CASK in the cellular fractions, insulin-stimulation notably reduced nuclear localization of CASK by about 75% (Figure 5B and 5C). The enlarged pictures in Figure 5C middle panel clearly display the alteration of the insulin-induced CASK-translocation. The signal specificity of CASK immunofluorescence was proved by antigen block (Figure 5B, right panel).

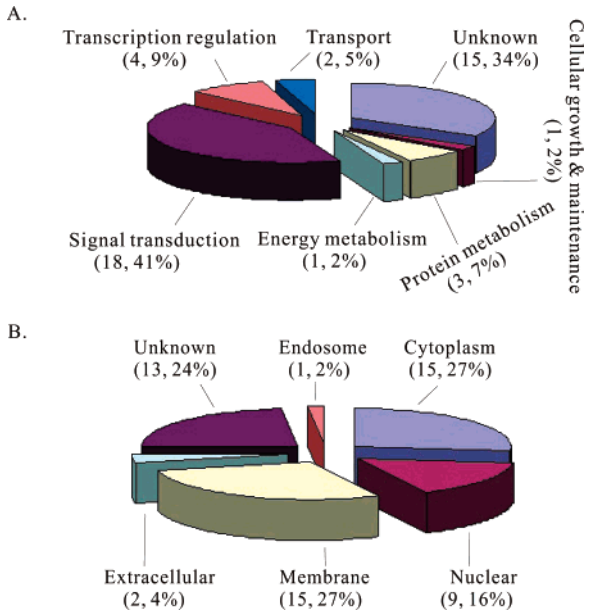

Figure 3. Summary of proteomics data. The identified phosphotyrosine proteins and distribution of molecular function (A) and cellular location (B) were mapped. The numbers and percentages (in parentheses) of proteins identified by LC−MS/MS are shown for each slice.

Nuclear CASK is well-known to be essential for regulation of T-element-containing gene expression, since it functions as a transcription co-activator for T-box transcription factor-1 (Tbr-1), which plays a critical role in brain development.[36,37] Thus, we wanted to determine whether insulin might have an effect on the transcriptional activation of a T-element-containing reporter vector. This was tested using a reporter vector containing two CASK-dependent T-elements in tandem orientation.[27] Insertion of the tandem elements resulted in a 20-fold increase in reporter activity compared to control vector in the Rat/hIR cells (Figure 5D, first and third columns). Cotransfection with CASK further enhanced about 50% of reporter activity of the tandem vector (Figure 5D, seventh column), which is consistent with the published results.[37] Insulin-stimulation significantly inhibited the reporter gene activity of both T-element tandem vector-only transfected, as well as CASK and tandem vector cotransfected cells, compared to un-stimulated cells (Figure 5D, fourth and eighth columns). However, insulin-stimulation had no effect on the activation of this reporter gene when cotransfected with a constitutive active mutant (GK) form of CASK.[37] Down-regulation of endogenous CASK by siRNA approach (Figure 5E) resulted in notable reduction of the reporter activity as well as losing response to insulin-stimulation (Figure 5D, fifteenth and sixteenth columns). These results demonstrated that CASK is a key regulator for the T-element reporter activity in the Rat1 cell model system. All together, activation of insulin-signaling pathway leads to decreasing the nuclear localization of CASK and also down-regulated the expression of its targeted genes.

Discussion

In the present study, we have analyzed the components of insulin-induced phosphotyrosine protein complexes through LC−MS/MS, following a sub-cellular fractionation and phosphotyrosine immunoprecipitation enrichment technique, leading to the identification of 35 phosphotyrosine sites. We successfully identified most of the known major substrates, as well as numerous key downstream molecules of insulin receptor signaling in the profile, including IRS2, Shc, Dok, PI3K, Akt, SIRP, SHIPs, Src family kinases (Src, yes), Ras, Raf, Crk, 14-3-3, Caveolin-1, Annexin, FAK (Supporting Information Table 2). Some of these were represented by relatively high account peptides, such as, Dock, SIRPα1, Src kinase, 14−3−3, Crk. LC−MS/MS was also able to detect 22 unique peptides from the insulin receptor, accounting for over 21% of the IR protein sequence (Supporting Information Table 2). These results indicate that activated IR and IR-initiated complexes were sufficiently enriched through our approach, substantiating the shotgun approach was a suitable methodology to identify the components of phosphotyrosine protein complexes. Compared to the reported methods,[21,22,24] our approach was able to identify more low abundant phospho-proteins, though not too sensitive to high abundant phospho-proteins. It was possible that the effective combination of these methods could render a more comprehensive phosphotyrosine profiling.

Profiling of Phosphotyrosine Sites Identified. Tyrosine phosphorylation is a key element for forming the functional protein complexes that mediate the insulin-regulated cellular actions.[1−4,32] Through bioinformatic analysis and manual confirmation of peptide phosphorylation site assignments, 35 phosphotyrosine sites were identified. Many novel phosphotyrosine proteins, which play important roles in different

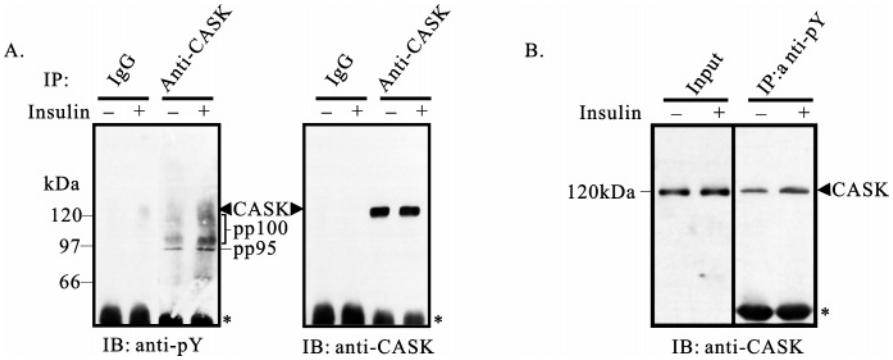

Figure 4. CASK was not tyrosine phosphorylated, but was associated with phosphotyrosine proteins in an insulin-stimulation dependent manner. Rat1/hIR cells were stimulated by 100 nM insulin for 20 min and sub-fractionated. Phosphotyrosine proteins were immunoprecipitated with mAb anti-CASK (A) or anti-pY antibodies (B) and then immunoblotted with mAb anti-CASK (A & B) or anti-pY (A). The bracket denotes the smear signal and an asterisk labels the IgG heavy chain, while pp100 represents the 100 kDa phosphotyrosine protein.

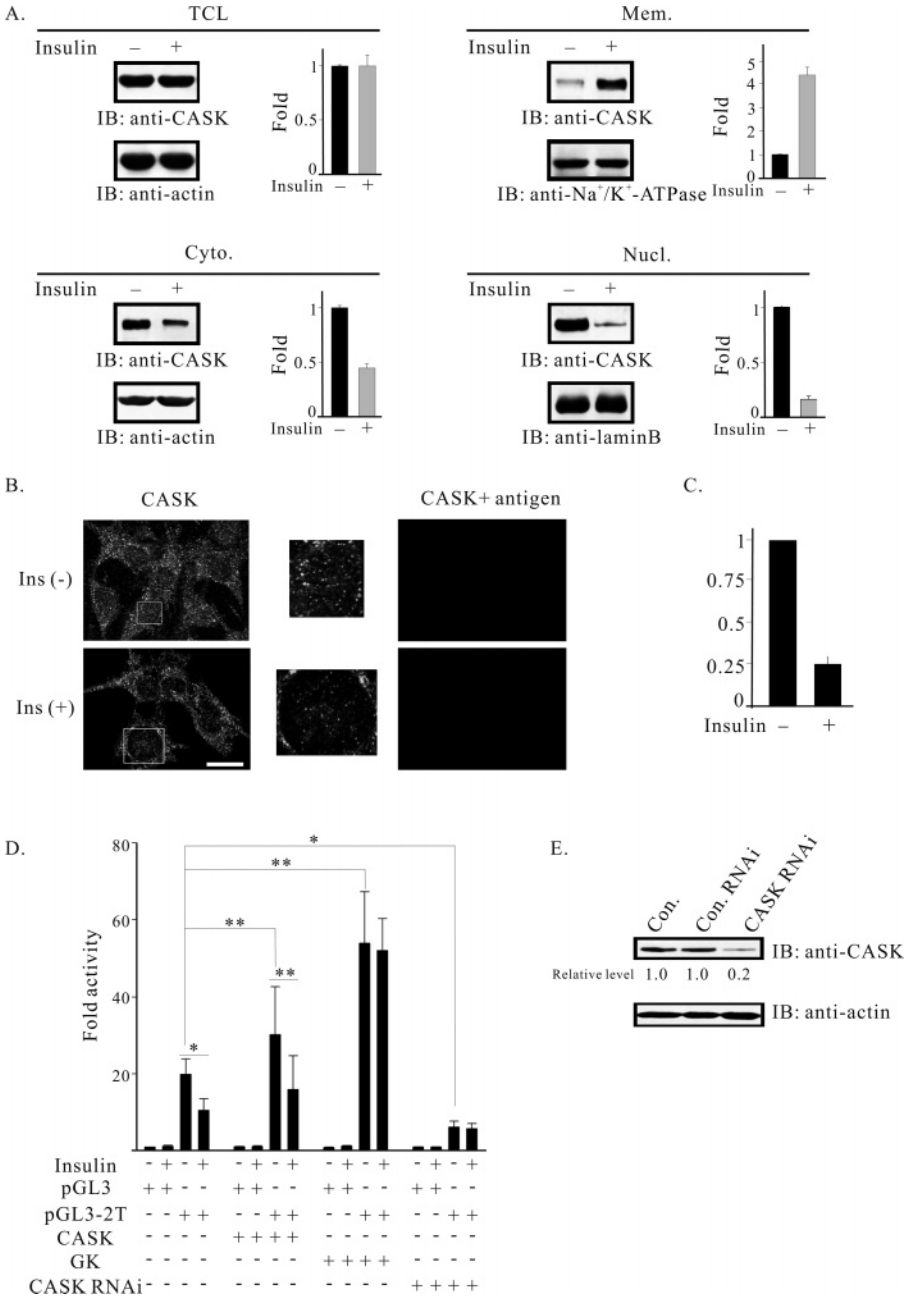

Figure 5. Insulin regulated the nuclear location of CASK and expression of CASK-targeted genes. Rat1/hIR cells were stimulated for 60 min by 100 nM insulin and fractionated into TCL, Cyto., Mem., and Nucl., (A). The cell lysates from different fractions were run on SDS-PAGE and immunoblotted with mAb anti-CASK or loading control antibodies (actin for TCL and Cyto.; Na^+/K^+-ATPase for Mem.; laminB for Nucl.). The immunoblot from Figure 5A and two other independent experiments was quantified and plotted. The initial relative level (CASK/control protein) of CASK from each fraction at 0 min was defined as 1, while the value in Figure 5A demonstrates the mean ± SEM from three independent experiments. Rat1/hIR cells were nonstimulated or stimulated by 100 nM insulin for 60 min, fixed and incubated with rabbit anti-CASK or anti-CASK and CASK antigen (B). The enlarged pictures were also shown. Bar: 30 μM. (C) Quantitative analysis of nuclear/total ratio of CASK immunofluorescence determined from confocal images. The initial ratio was defined as 1. The RNAi efficiency of CASK was tested (D). Rat1/HIR cells were transiently transfected with control vector (pGL3) or reporter gene (pGL3-2T) combined with CASK, GK or CASK RNAi plasmids. Before harvest, cells were stimulated by 100 nM insulin for 60 min. A luciferase assay was performed to compare the reporter activity of 60 min insulin-stimulated cells to that of the initial point (0 min) (student's t-test, $*p < 0.01$; $**p < 0.05$). (E) Rat1/hIR cells were transiently transfected by CASK RNAi plasmid or control plasmid and harvested after 48 h. The total lysate was run SDS-PAGE and immunoblotted.

signaling pathways, were identified, including gigaxonin, XIAP, and CDK10, suggesting these proteins may involve in this pathway in phosphotyrosine fashion except for the previous reported routine. Meanwhile, the other proteins provide a potential backup to be confirmed of insulin signaling network.

In addition to these novel finds, many anticipated phosphoproteins were also identified. These include two phosphotyrosine peptides from SIRP, as well as one from Src family kinase (Yes and Src), both of which were previously well documented in insulin signaling pathway.[8,38] However, most of the expected tyrosine phosphorylation sites from IR and its major substrates were missing, indicating that our approach

was not well suited for identification of phosphorylation sites, although it was able to identify the major protein targets of IR, as well as many novel phosphotyrosine proteins. For example, none of phosphopeptides of IR were found, although 114 peptide counts and 22 unique peptides of the insulin receptor were obtained. The following reasons may account for this phenomenon: first, the phosphorylation of tyrosine residues occurs to a lower extent than serine/threonine phosphorylation. For one phosphotyrosine protein, there are only a few tyrosine residues that actually become phosphorylated. Therefore, there are many more nontyrosine phosphorylated peptides than tyrosine phosphorylated ones identified by mass spectrometry. Second, high levels of nonphosphorylated peptide might mask the detection of low-abundance phosphopeptides, possibly leading to loss of phosphopeptides might be lost during the liquid chromatography steps. Third, not all phosphorylated residues may be amenable to identification by mass spectrometry. Some residues may be flanked by recognition motifs for trypsin and other proteases, thus producing peptides of sizes unsuitable for mass spectrometric detection. Moreover, phosphorylated residues may affect protease substrate recognition when this residue is close to a cleavage site such that the set of proteolytic peptides may be different between phosphorylated and unphosphorylated protein isoforms. Last, but not least, the detection of phosphoproteins and phosphopeptides by mass spectrometry (MS) is often hindered by low concentrations and poor ionization efficiencies.[39−41] It should be noted that due to methological limitations of our approach, more experiment needs to be done to further confirm whether and when these phosphorylation sites identified are induced by insulin stimulation. Therefore, we propose that enrichment by two-step affinity purification, based on both anti-phosphotyrosine and immobilized metal ion affinity chromatography (IMAC), combined with the shotgun and quantitative proteomics approaches[42,43] should be useful for the identification of phosphorylation sites and proteins involved in insulin signaling. On the other hand, nonphosphorylated proteins within the signaling complexes would be lost by those techniques. Information about these potentially important proteins, which may function as possible links or cross-talk points between the insulin signaling pathway and others, would be absent. In our study CASK, which will be discussed in detail later, is a good example of one of these proteins.

Insulin Regulates CASK Translocation Through an Unidentified Mechanism. Membrane-associated guanylate kinase (MAGUK) proteins are generally recognized as multidomain scaffolding proteins. Members of this family interact with transmembrane proteins and nucleate the formation of protein complexes linking transmembrane proteins and cytoskeletal and/or signaling molecules.[44] CASK, which has been shown to interact with several cellular proteins, contains multidomain modules, including a calmodulin-dependent protein kinase-like domain (CaMK), and PDZ, SH3, and guanylate kinase-like domains (GK).[37] A tripartite protein complex containing CASK/Lin-2, Mint-1/Lin-10, and Veli/Lin-7 has been identified in both *C. elegans* and mammals.[33−35] This complex is involved in vesicle trafficking, localization of NMDA receptors and EGFR, and vulval differentiation in *C. elegans*.[33−35,45,46] Mint-1 also contains a PTB domain,[33−35] therefore, CASK may indirectly interact with other phosphotyrosine proteins through the PTB domain of Mint, though the binding proteins to its PTB domain are currently unknown. It has been reported that the PDZ domain of CASK binds the C-terminal tail of syndecan-3 and PMCA.[27,47] Interestingly, Syndecan-3 is a transmembrane heparan sulfate proteoglycan of approximately 100 kDa, which was reported to be tyrosine phosphorylated by Src kinase.[48,49] The CASK-associated pp100 found in this study has a molecular weight similar to syndecan-3, as well as a smeared signal.[49] It is also well-known that activated IR enhances the activity of Src kinase, mediating a positive signal for insulin-induced glucose-uptake.[31] However, the exact molecular mechanism of Src in glucose-uptake remains unclear. It will be quite interesting to determinate whether insulin can induce tyrosine phosphorylation of syndecan-3, to further regulate the binding of sydecan-3 to CASK on the membrane. It is also possible that insulin signaling enhances the release of calcium, whereby leading to interaction between PMCA and CASK, which results in a reduction of CASK in the nucleus.[27,50] Exact mechanism, by which insulin regulates CASK-translocation, remains to be studied.

Insulin May Suppress Transcription of T-Element Containing Genes, via Regulation of the Nuclear Localization of CASK. In addition to association with transmembrane and cytoplasmic proteins, CASK enters the nucleus and binds to a specific DNA sequence (the T-element) in a complex with Tbr-1 that involves in forebrain development.[37] Recently, Wang and colleagues have reported that CASK also binds to CINAP, forming a complex with Tbr-1 to regulate transcription of T-element containing genes, including *reelin*, NMDA receptor subunit 2B (NR2B), NMDA receptor subunit 1 (NR1).[36,51] All the results imply nuclear CASK is essential for Tbr-1 controlled gene expression. Our data clearly established that insulin reduced nuclear localization of CASK. In addition, insulin stimulation decreased the activity of a T-element containing reporter gene (Figure 5D). Insulin could regulate many different kind of gene expression, for instance, Forkhead transcription factors targeted genes.[52] However, to our knowledge, our study is the first report to demonstrate that insulin may regulate expression of T-element containing genes.

Several proteins including PMCA, syndecan-3, and CINAP are known to affect CASK transcriptional activity through interaction with CASK.[27,36,37] PMCA and sydecan-3 interacted with PDZ domains of CASK, respectively, leading to reduction of nuclear CASK level, to end with decreasing T-element gene expression. These studies implicated that the regulatory domains of CASK play an important role in regulation of CASK cellular translocation. As mentioned above, the GK mutant, lacking the regulatory domains of CASK, was reported to be exclusively localized in nuclear, and to constitutively bind to Tbr-1, resulting in high transcriptional activation of the T-element-containing reporter vector.[37] In our work, insulin-stimulation was able to notably suppress CASK-enhanced T-element controlled reporter gene activity. However, insulin-stimulation had no any effect on the GK form-generated activation of the reporter gene (Figure 5D). In addition, when knock-down of endogenous CASK by transfection of its siRNA, insulin also lost its influence on regulation of the reporter gene activity. These results suggested that insulin signaling may regulate CASK cellular translocation through its regulatory domains, suppressing CASK/Tbr-1 targeted gene expression.

On the basis of CASK's known activities, we can envision two scenarios through which CASK functions in the insulin signal pathway. One is that insulin may regulate expression of T-element containing genes through controlling translocation of CASK in nuclear, which may link to cellular metabolism and/or other functions, for instance, *reelin*, which is one of the

major target genes of CASK/Tbr-1 and a protein that has features of an extracellular matrix protein, plays a critical role in brain development.[53] However, *reelin* function in nonneuron system is totally unknown. The other possibility is that insulin regulates vesicle transport through CASK in neurons[34,45] and/or other cells. Altogether, CASK may function as a novel effector in insulin receptor-initiated cellular processes.

Conclusion

In this work, we have systematically analyzed the molecules involved in the insulin-signaling pathway using an approach of subcellular fractionation, immunoaffinity purification/enrichment, and tandem mass spectrometry. We present a comprehensive dataset profiling known and novel players in the insulin receptor pathway. These newly identified phosphotyrosine proteins, as well as the new-found involvement of CASK in insulin signaling, may provide new insights into understanding the insulin signaling network and aid in the search for potential drug targets against metabolic diseases.

Abbreviations. TCL, total cell lysate; Cyto., cytosolic fraction; Mem., membrane fraction; Nucl., nuclear fraction; LC, liquid chromatography; MS, mass spectrometry; pY, phosphotyrosine; IR, insulin receptor; CASK, calcium/calmodulin-dependent serine kinase.

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Supporting Information Available: Thirty-one protein groups containing 35 phosphotyrosine sites and 700 groups containing 1100 proteins identified in this study are listed as Supporting Information Tables 1 and 2, respectively. The phosphotyrosine pattern of Rat1 and Rat1/hIR cells are shown as Supporting Information Figure 1. This material is available free of charge via the Internet at http://pubs.acs.org.

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