

A Novel Proteomic Approach for Specific Identification of Tyrosine Kinase Substrates Using [¹³C]Tyrosine*

Received for publication, October 24, 2003, and in revised form, January 20, 2004
Published, JBC Papers in Press, January 21, 2004, DOI 10.1074/jbc.M311714200

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Proteomic studies to find substrates of tyrosine kinases generally rely on identification of protein bands that are “pulled down” by antiphosphotyrosine antibodies from ligand-stimulated samples. One can obtain erroneous results from such experiments because of two major reasons. First, some proteins might be basally phosphorylated on tyrosine residues in the absence of ligand stimulation. Second, proteins can bind non-specifically to the antibodies or the affinity matrix. Induction of phosphorylation of proteins by ligand must therefore be confirmed by a different approach, which is not always feasible. We have developed a novel proteomic approach to identify substrates of tyrosine kinases in signaling pathways studies based on *in vivo* labeling of proteins with “light” (¹²C-labeled) or “heavy” (¹³C-labeled) tyrosine. This stable isotope labeling in cell culture method enables the unequivocal identification of tyrosine kinase substrates, as peptides derived from true substrates give rise to a unique signature in a mass spectrometry experiment. By using this approach, from a single experiment, we have successfully identified several known substrates of insulin signaling pathway and a novel substrate, polymerase I and transcript release factor, a protein that is implicated in the control of RNA metabolism and regulation of type I collagen promoters. This approach is amenable to high throughput global studies as it simplifies the specific identification of substrates of tyrosine kinases as well as serine/threonine kinases using mass spectrometry.

Phosphorylation and dephosphorylation are two major regulatory mechanisms controlling the activity of proteins. A number of signaling pathways rely on the activity of tyrosine kinases for transmitting signals from the cell surface to the nucleus (1). For instance, ligand binding to receptor protein-tyrosine kinases activates their kinase activity and promotes their autophosphorylation. This creates docking sites for substrates that either possess intrinsic enzymatic activity or serve as adapter molecules that mediate the interaction between

signaling molecules and the receptors (2). Activation of downstream tyrosine kinases enables the propagation of these signals by modifying the activity of downstream intermediates and inducing the assembly or disassembly of signaling multi-protein complexes.

Identification of substrates of tyrosine kinases is a major step in the analysis of signaling pathways. Enrichment of tyrosine-phosphorylated proteins with antiphosphotyrosine antibodies is a necessary step given the low stoichiometry of tyrosine phosphorylation and the complexity of the samples such as cell lysates. In traditional approaches, immunoprecipitated protein samples derived from untreated or ligand-stimulated cells are separated in parallel by gel electrophoresis and visualized by gel staining. The differentially stained protein bands are subsequently analyzed by mass spectrometry (3–7). Non-specific binding and binding of basally tyrosine-phosphorylated proteins to antibodies and the affinity matrix are major obstacles to visualizing proteins whose tyrosine phosphorylation is induced by the ligand. Because identification of the candidate proteins depends on visualization of the difference in staining intensity between the two samples, this method is limited to identification of proteins that exhibit a substantial increase in tyrosine phosphorylation and do not overlap with other, often more abundant, contaminating proteins. Identification of substrates is time consuming as comigration of real substrates and “contaminants” in the same band makes it necessary to independently validate each candidate protein. Such validation usually involves repeating the experiment using a specific antibody, followed by gel separation of the immunoprecipitated protein and Western blotting with antiphosphotyrosine antibody. The validation of each of the protein candidates is not always possible, especially for novel proteins, as it depends on the availability of good antibodies.

Strategies in proteomics that allow one to label proteins could be devised such that subsequent validation is not necessary. Several labeling methods have been developed based on the incorporation of a light or a heavy tag to the different experimental protein samples (for reviews see Refs. 8–11). This enables their distinction and quantification in the mass spectrum in a mixed sample because the labeled peptide will present as pairs with an expected mass difference. We have previously demonstrated stable isotopic labeling in cell culture (SILAC)¹ for labeling proteins *in vivo* (12). In this study, we explore the use of a different amino acid, tyrosine-labeled with ¹³C, to identify substrates of tyrosine kinases in the insulin signaling pathway which is a well studied tyrosine kinase pathway (13–17). By using this method and coupling it to

* This work was supported in part by NHLBI Grant HV-28180 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: SILAC, stable isotope labeling in cell culture; LC-MS/MS, liquid chromatography/tandem mass spectrometry; PRTF, polymerase I and transcript release factor.

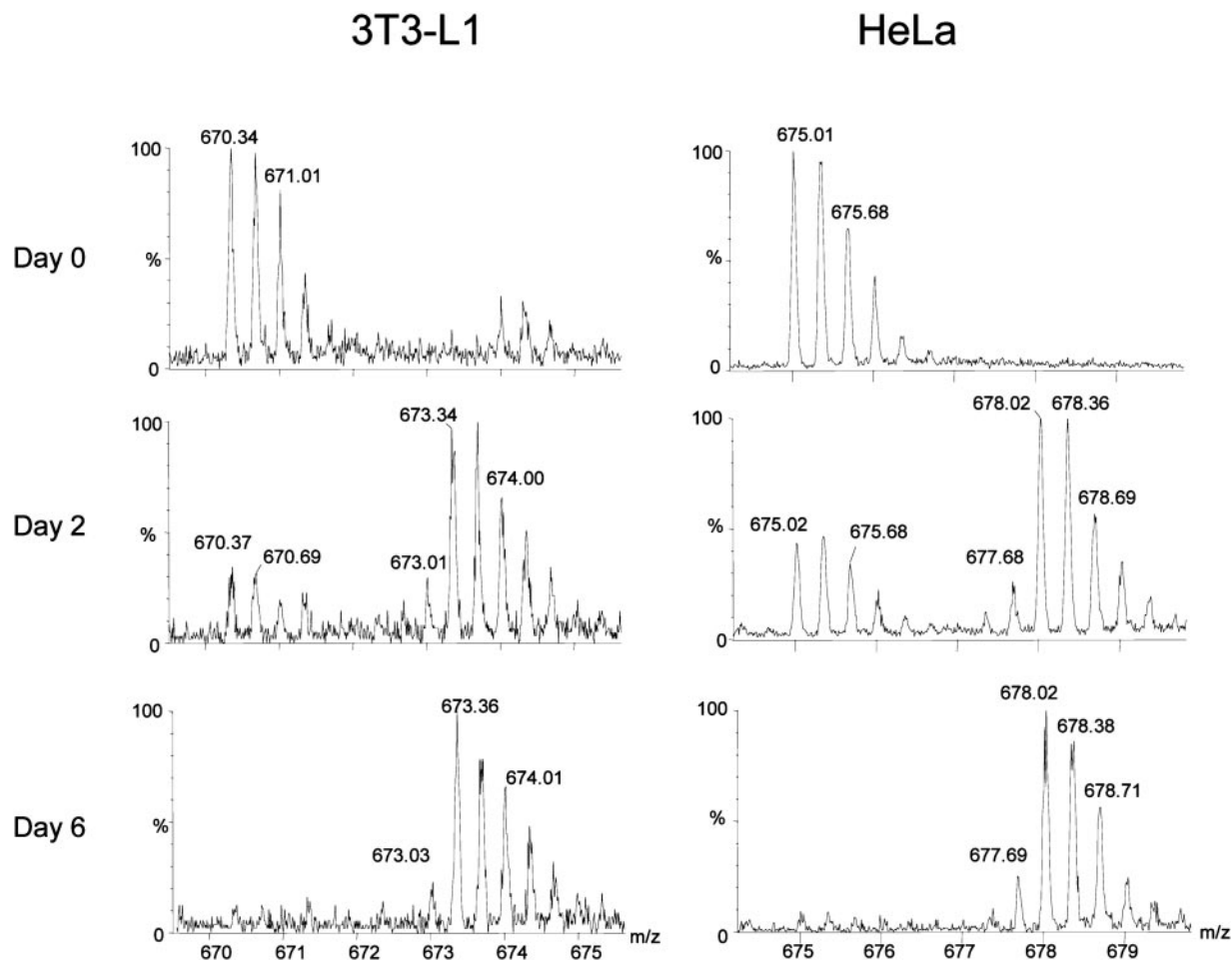


FIG. 1. Incorporation of [^{13}C]tyrosine in mammalian cell lines. 3T3-L1 and HeLa cells growing in normal medium were switched to grow in media containing [^{13}C]tyrosine. Cells lysates were collected either at the start of the experiment (day 0) and day 2 or 6 after the adaptation was begun. Protein pools were resolved by SDS-PAGE and stained. One protein band was excised, trypsin-digested, and analyzed by mass spectrometry. The figure shows the mass spectra of peptides derived from transketolase containing one tyrosine residue in 3T3-L1 and HeLa cells harvested on different days as indicated.

enrichment with antiphosphotyrosine antibodies, we can specifically identify substrates and distinguish them from non-specifically bound proteins using mass spectrometry. Thus our approach simplifies the identification of tyrosine kinases substrates and is amenable to high throughput identification of kinases substrates in any signaling pathway.

MATERIALS AND METHODS

Media, Growth Factors, and Antibodies—Custom Dulbecco's modified Eagle's medium without tyrosine was obtained from Invitrogen, supplemented with [^{12}C]- or [^{13}C]tyrosine (Cambridge Isotope Laboratories, Andover, MA) and 10% fetal bovine-dialyzed serum. Dexamethasone and 3-isobutyl-1-methyl-1-xanthine were obtained from Sigma, and recombinant human insulin was obtained from Roche Diagnostics. Antiphosphotyrosine antibodies, 4G10, agarose-conjugated and RC20-Biotin, were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and BD Biosciences (San Jose, CA), respectively. Streptavidin-agarose-conjugated was obtained from Upstate Biotechnology, Inc.

Cell Culture and Immunoprecipitation—3T3-L1 or HeLa cells were grown in the specially synthesized Dulbecco's modified Eagle's medium as described above supplemented with either [^{12}C]tyrosine or [^{13}C]tyrosine plus 10% fetal bovine dialyzed serum. Dialyzed serum is necessary to ensure complete incorporation of amino acids present in the medium. To assess the incorporation of [^{13}C]tyrosine into proteins, 3T3-L1 or HeLa cells that were grown in the above media were lysed in modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 0.25% sodium deoxycholate) at different time points. The cell extracts were separated by one-dimensional gel electrophoresis and visualized by silver staining. Bands were then excised and digested

using trypsin as described previously (18). The tryptic peptides were analyzed by mass spectrometry.

To differentiate 3T3-L1 into adipocytes, confluent cultures were grown for 2 days before the addition of the differentiation inducing mixture. After 48 h of incubation in 390 ng/ml dexamethasone, 115 $\mu\text{g}/\text{ml}$ 3-isobutyl-1-methyl-1-xanthine and 10 $\mu\text{g}/\text{ml}$ insulin (DMI), the medium was replaced, and cells were grown for an 2 additional days in insulin-containing medium. The cells were subsequently grown in medium containing 10% fetal dialyzed serum for 3 days and starved overnight before insulin treatment. 3T3-L1 adipocytes labeled with [^{13}C]tyrosine were treated for 5 min with 10 $\mu\text{g}/\text{ml}$ human recombinant insulin. Twenty percent of the [^{12}C]tyrosine-labeled adipocytes was also treated with insulin to ensure the detection of a basal level of tyrosine phosphorylation in the mass spectrometry analysis. After treatment, the cells were lysed in modified RIPA buffer. The lysates from untreated and treated were then mixed and immunoprecipitated with a mixture of 4G10 and RC20 monoclonal antiphosphotyrosine antibodies and streptavidin-agarose conjugate for 6 h at 4 $^{\circ}\text{C}$. Elution was performed with 100 mM phenyl phosphate, and the eluate was resolved by one-dimensional gel electrophoresis after dialysis. The gel was silver-stained for visualizing protein bands.

Mass Spectrometric Analysis—Protein bands were excised from the gel and digested. Tryptic peptides were analyzed by automated LC-MS/MS. The LC-MS setup consisted of an injector (1100 Microwell plate auto sampler, Agilent), a pump (1100 CapLC, Agilent) running a gradient of 8% B to 45% B in 35 min (Buffer A: 0.4% acetic acid, 0.05% heptafluorobutyric acid; Buffer B: 90% acetonitrile, 0.4% acetic acid, 0.05% heptafluorobutyric acid), a 75- μm inner diameter/100-mm, pore size 12 nm, particle diameter 5–15 μm C_{18} trap column (ODS-A, YMC), and an analytical C_{18} column 75 μm inner diameter/100 mm, particle

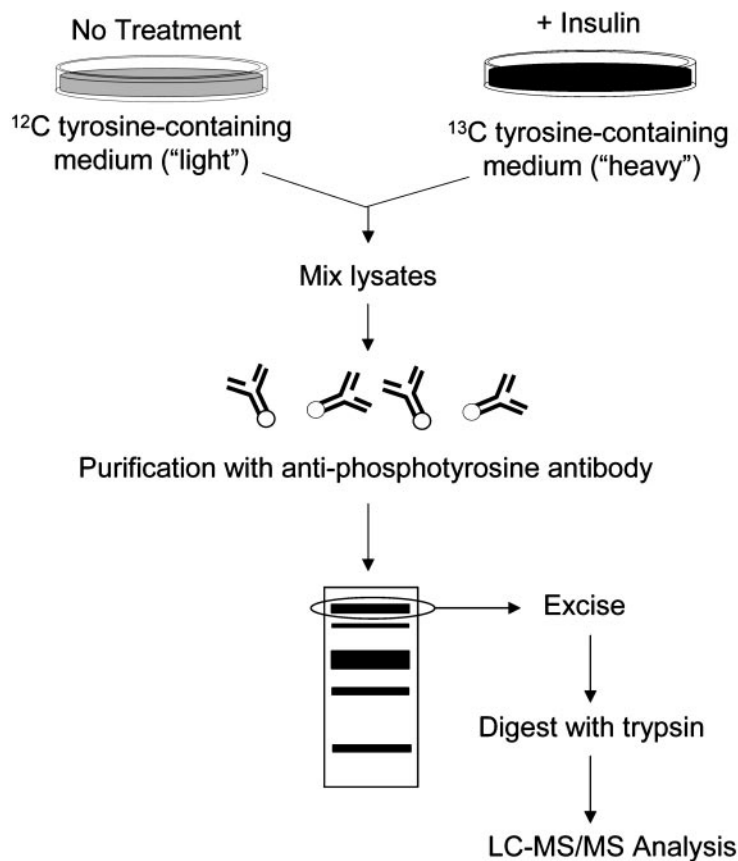


FIG. 2. A schematic of the ^{13}C tyrosine labeling method. Two cell populations are labeled by growing them in media containing normal $^{12}\text{C}_9$ tyrosine (light) or $^{13}\text{C}_9$ tyrosine (heavy). After treatment of the cell populations labeled with heavy tyrosine with a ligand (e.g. insulin), the cell lysates are mixed and immunoprecipitated with antiphosphotyrosine antibodies. The immunoprecipitated proteins are separated by gel electrophoresis and stained. The protein bands are excised, digested with trypsin, and analyzed by LC-MS/MS. An illustrative mass spectrum where the isotopic distribution of a peptide is represented as a single vertical black bar is shown at the bottom of the figure. Two types of peptide pair distributions that could be found are shown. The arrows indicate the mass shift due to the presence of a heavy tyrosine residue.

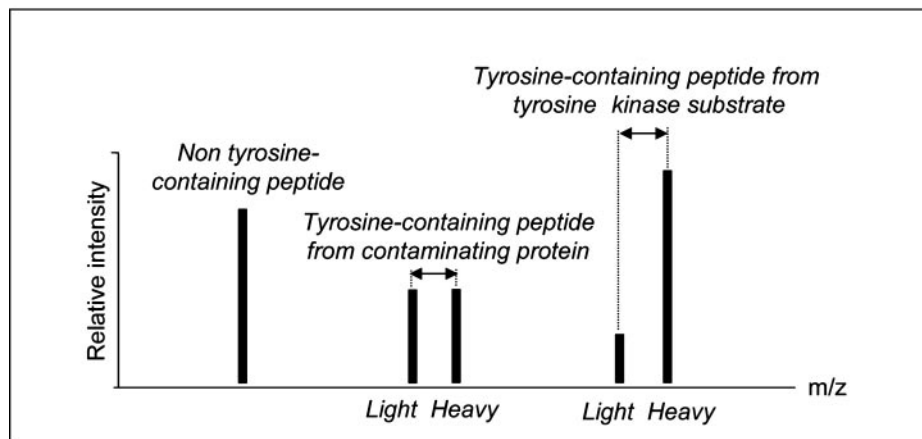


TABLE I
Tyrosine kinase substrates in the insulin signaling pathway

Protein name	Accession no.	Ratio
Insulin receptor	NP_034698	3.1
ERK1	NP_036082	2.9
IRS-1	NP_034700	2.9
APS	NP_061295	2.8
ERK2	NP_036079	2.8
p38	NP_036081	2.0
Syncrin	NP_062770	2.0
Gab1	NP_067331	1.8
PRTF	NP_033012	1.6

diameter 5 μm (218MS, Vydac). MS analysis was carried out using a quadrupole time-of-flight mass spectrometer (Micromass QTOF, US-API) equipped with a nano electrospray source (Proxeon A/S, Denmark). Prior to injection, the digested samples were lyophilized to a volume of ~ 20 μl . After MS analysis but prior to data base searching, MS/MS data files from all the analyses were merged using a Perl script that was written for this purpose. The merging of MS/MS data files resulted in a

higher sequence coverage due to protein overlap between neighboring excised gel bands. The merged file was searched against NCBI nr data base using the Mascot search engine (19), using $^{13}\text{C}_9$ tyrosine and $^{13}\text{C}_9$ phosphotyrosine as variable modifications (Matrix Science, UK).

The results were analyzed as follows: peptides containing tyrosine were identified from the data base output, and a selected ion monitoring chromatogram (width at half-height of the monoisotopic ion and $^{13}\text{C}_9$ -Y ion) was generated for the heavy (insulin-treated) and light (untreated) form of each tyrosine-containing peptide. The ratio between the heavy and light tyrosine-containing peptides was calculated using the maximum intensities from the ion traces.

RESULTS AND DISCUSSION

Stable Labeling of Cellular Proteins with ^{13}C -containing Tyrosine—Mass spectrometric analysis can be used for relative quantitation of proteins if any of a number of methods to “tag” proteins is employed. One such technique involves the metabolic labeling of cellular proteomes by growing the cells in media containing stable isotope containing amino acids (SILAC) (12). The mass difference between the light and heavy versions of amino acids

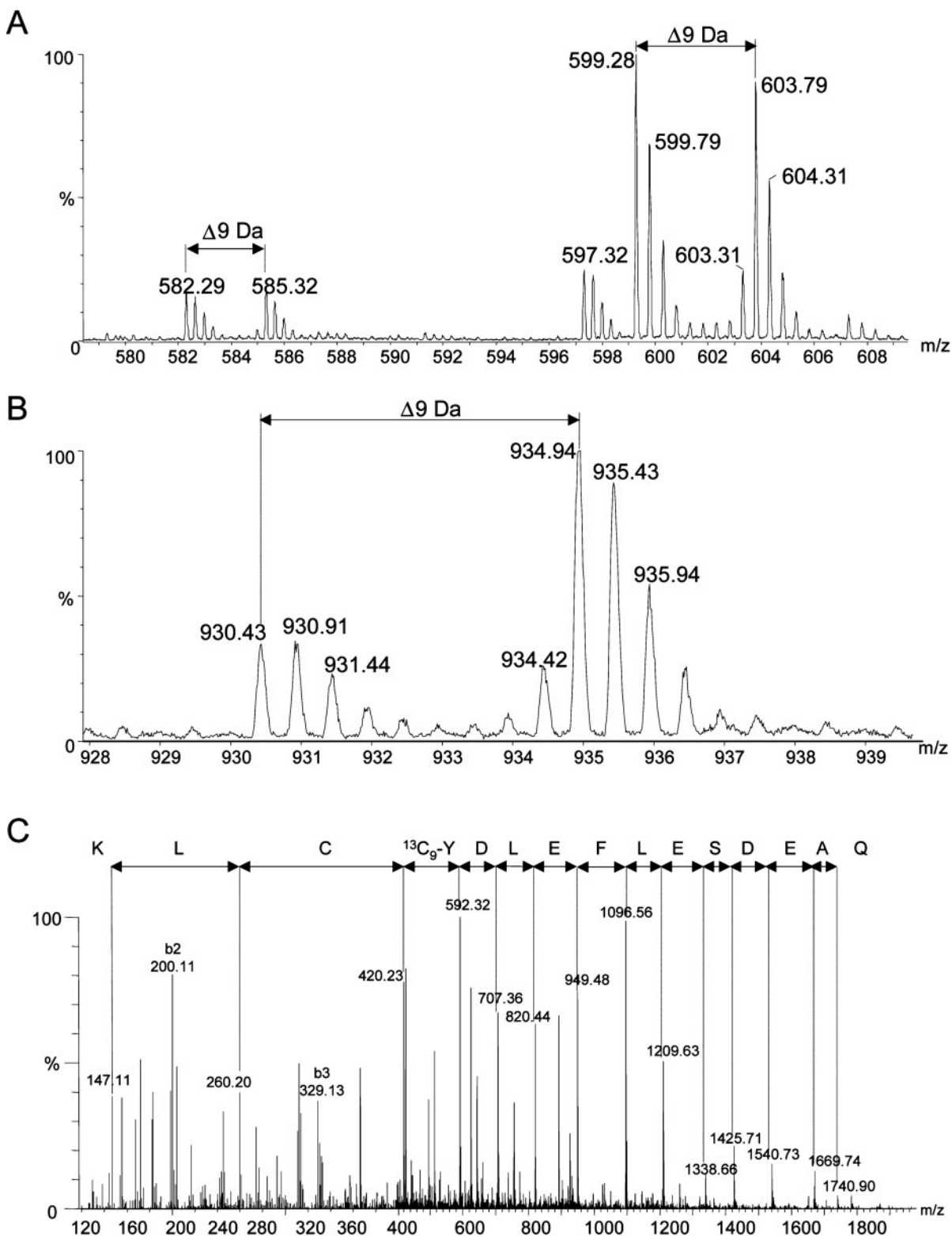


FIG. 3. Profiling tyrosine kinase substrates by ^{13}C tyrosine labeling. 3T3-L1 cells were labeled with $^{12}\text{C}_9$ tyrosine (*light*) or $^{13}\text{C}_9$ tyrosine (*heavy*) and differentiated into adipocytes. Adipocytes labeled with heavy tyrosine were stimulated with insulin for 5 min, and lysates from the two cell populations were mixed, immunoprecipitated with antiphosphotyrosine antibody, and resolved by gel electrophoresis. The protein bands were excised, digested with trypsin, and analyzed by mass spectrometry. *A*, the mass spectrum shows two pairs of peptides containing a single tyrosine residue and one peptide lacking tyrosine residues. The two peptide pairs differ in mass by 9 Da because of the incorporation of a $^{13}\text{C}_9$ tyrosine. *B*, MS of a peptide pair derived from insulin receptor. *C*, MS/MS spectrum of the heavy peptide shown in *B*. The sequence of the peptide is indicated, and the position of the heavy tyrosine is shown as $^{13}\text{C}_9\text{-Y}$. To better visualize the lower m/z range, the spectrum from m/z values between 120 and 400 is magnified and shows b2 and b3 ions as indicated.

enables the distinction of the labeled peptides derived from two different experimental cell populations in the same mass spectrum, where they will show as pairs differing by an expected

mass. Quantitation of the relative abundance of the proteins derived from two different cell populations is then possible by comparison of the intensities of the light and heavy versions of

TABLE II
A partial list of proteins found as contaminants

Protein name	Accession no.	Ratio
14-3-3 protein γ	NP_061359	1.0
Acetyl-coenzyme A acetyltransferase 1	NP_659033	1.0
Adenine nucleotide translocase-2	NP_031477	1.0
Citrate synthase	NP_080720	0.9
Fatty-acid synthase	NP_032014	1.0
Heat shock protein 8	NP_112442	1.0
Nucleolin	NP_035010	1.0
Perilipin	NP_783571	1.0
Perlecan	NP_032331	1.0
PSF (PTB-associated splicing factor)	NP_076092	0.9
Pyruvate carboxylase	NP_032823	1.0
Ribosomal protein S2	NP_032529	0.9
60 S ribosomal protein L4	NP_077174	1.1
RNA-binding protein FUS	NP_631888.1	0.8
RuvB-like protein 2	NP_035434	1.1
Succinate dehydrogenase complex, subunit A	AAH11301	1.0
Tubulin α 1	NP_035783	1.0
Voltage-dependent anion channel 1	NP_035824	0.9

the peptides. Additionally, this procedure avoids experimental errors associated with separate manipulations of the samples because the samples are mixed in the first step.

We have shown previously that D3-labeled leucine amino acid can be efficiently incorporated into the cell proteome (12). Labeling with this isotope is limited by the fact that only peptides containing leucine will be labeled and that the mass spectrometric separation between peptides in a pair is adequate only when the peptides are in their singly charged state. When identifying substrates of tyrosine kinases, peptides containing tyrosine are of special interest as they contain the potential phosphorylation sites. The 9-Da mass difference between [$^{12}\text{C}_9$]- and [$^{13}\text{C}_9$]tyrosine isotopes provides enough resolution in a mass spectrum to detect peptide pairs in the doubly and triply charged states as well. Thus, we explored the possibility of using [$^{13}\text{C}_9$]tyrosine to label experimental cell populations.

As a first step in our study, we assessed the incorporation efficiency of [$^{13}\text{C}_9$]tyrosine into proteins in cultured cells. We monitored the extent of incorporation of [$^{13}\text{C}_9$]tyrosine in proteins in 3T3-L1 and HeLa cell lines over a period of 6 days by mass spectrometry. Two populations of 3T3-L1 and HeLa were switched from growing in media containing normal tyrosine to media containing [$^{13}\text{C}_9$]tyrosine. Cells were harvested before the switch of media (day 0) and after 2 and 6 days of culture in labeling media. The lysates were resolved by SDS-PAGE, and several protein bands were digested with trypsin. On day 2, the mass spectrum of tryptic peptides from one of the bands from 3T3-L1 and HeLa cells showed a peptide pair separated by 9 Da corresponding to the mass difference between [$^{12}\text{C}_9$]- and [$^{13}\text{C}_9$]tyrosine isotopes (Fig. 1). Identification of each member of the pair confirmed that the bands were derived from the same protein, transketolase, with the peptide containing a single tyrosine residue explaining the 9-Da mass difference. We analyzed the same peptide at different time points and observed that the ratio of intensity of heavy to light peak steadily increased. After 6 days of adaptation, the entire signal detected corresponded to the heavy peptide, signifying complete incorporation. Therefore, exogenous tyrosine amino acid gets efficiently incorporated into proteins in cell culture and results in an increase in mass of tyrosine-containing peptides that can be easily detected by mass spectrometry analysis.

Use of Antiphosphotyrosine Antibodies to Enrich for Phosphoproteins—In a previous mass spectrometry-based proteomic study to identify substrates of tyrosine kinases in epidermal growth factor signaling pathway, we used antiphosphotyrosine antibodies to “pull down” proteins that were tyrosine-phosphorylated from samples treated with epidermal growth factor (6).

Those bands that were specifically observed in epidermal growth factor-treated samples were analyzed by mass spectrometry. However, we had to confirm phosphorylation by performing a similar experiment where the protein of interest was immunoprecipitated with the molecule-specific antibody to visualize induction of tyrosine phosphorylation using an antiphosphotyrosine antibody.

To circumvent these issues, we have developed a novel approach to identify substrates of tyrosine kinases that combines enrichment using antiphosphotyrosine antibodies with the use of [$^{12}\text{C}_9$]- or [$^{13}\text{C}_9$]tyrosine as a labeling amino acid. This allows one to distinguish between the tyrosine-containing peptides derived from the different experimental cell populations by mass spectrometry. As shown in Fig. 2, different experimental cell populations are labeled by growing them in media containing [$^{12}\text{C}_9$]- or [$^{13}\text{C}_9$]tyrosine. The population of cells labeled with heavy amino acid is treated with the ligand, whereas the light amino acid containing population is left untreated. After mixing, the cell lysates are incubated with antiphosphotyrosine antibodies, and the immunoprecipitated proteins separated by one-dimensional gel electrophoresis. All the gel bands are subsequently excised and digested with trypsin before analysis with reverse liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). The mass spectrum will show different distributions of heavy/light isotopic ratios between the peptide pairs: a ratio greater than 1 will represent peptides derived from tyrosine kinase substrates, as they are enriched in immunoprecipitates from ligand-stimulated cells; a ratio close to 1 will represent peptides with similar intensities indicating that this protein is a contaminant as its tyrosine phosphorylation status is unchanged upon ligand stimulation.

Profiling Tyrosine Kinase Substrates in the Insulin Signaling Pathway—We have applied the above approach to the identification of substrates in the insulin receptor signaling pathway in 3T3-L1 adipocytes which is a well established model system for studying insulin signaling. 3T3-L1 preadipocytes were grown in ^{12}C - or ^{13}C -containing tyrosine and differentiated into adipocytes. On day 8, the population of adipocytes labeled with [$^{13}\text{C}_9$]tyrosine was treated with insulin for 5 min. Lysates from both experimental populations were mixed and immunoprecipitated with antiphosphotyrosine antibodies. Immunoprecipitated proteins were separated by one-dimensional gel electrophoresis and silver-stained. The gel bands were excised, and following trypsin digestion, the different bands were analyzed by LC-MS/MS.

We observed peptide pairs derived from a total of 69 proteins that were separated by 9 Da but with similar intensities of the

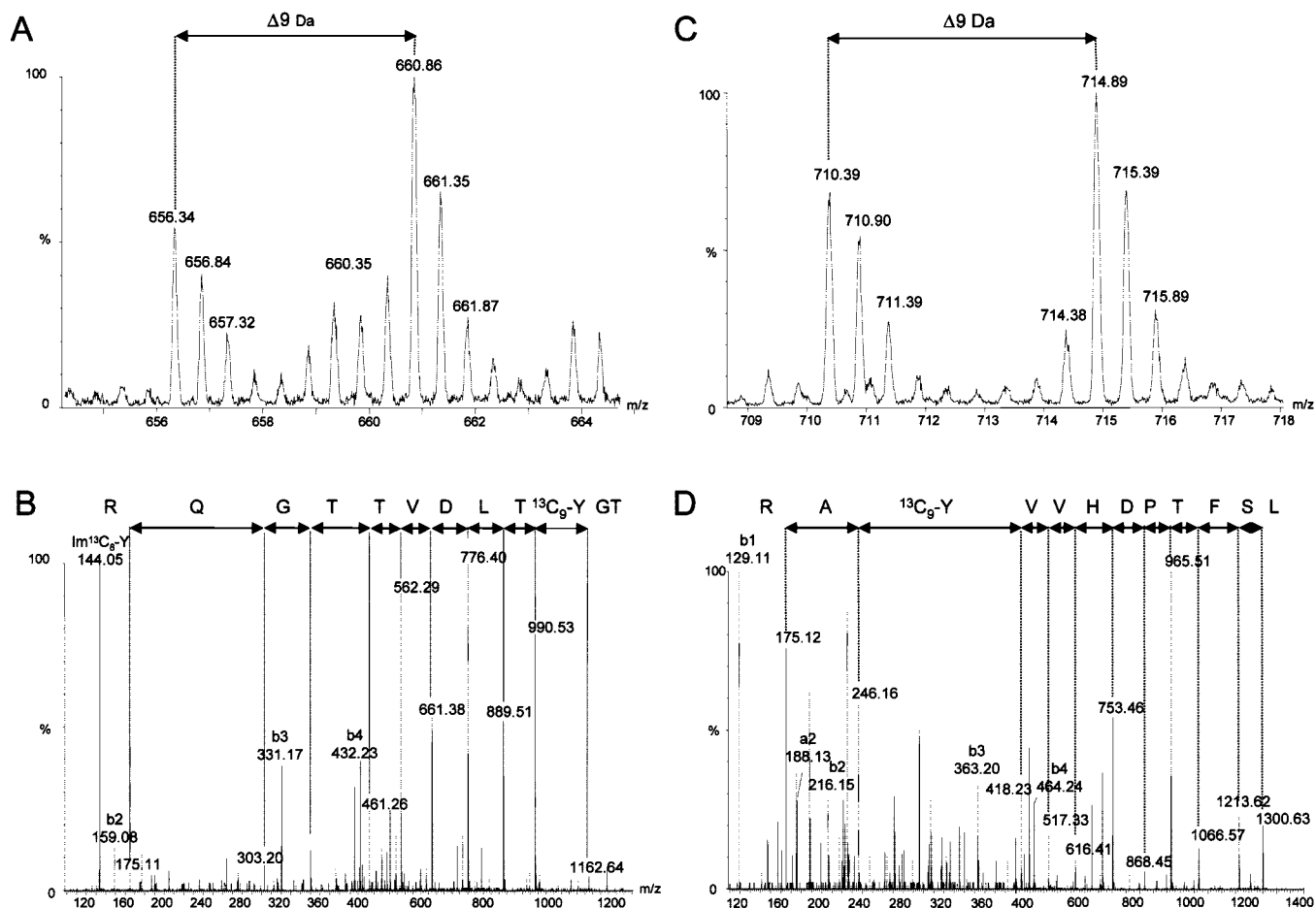


FIG. 4. Identification of substrates of tyrosine kinases in the insulin signaling pathway. A–C, mass spectra of peptide pairs derived from 2 different proteins obtained in the experiment described in Fig. 3. The light and heavy tyrosine-containing peptide pairs from Syncrip (A) and PRTF (C) are separated by a mass of 9 Da as indicated and present a different intensity. The MS/MS spectrum obtained by fragmenting peptides with m/z values of 660.86 and 714.89 is shown in B and D, respectively. The sequences deduced from the y-ion series (marked with vertical lines) are shown on the top of each of the panels.

TABLE III
List of tyrosine phosphorylation sites

Protein name	Peptide sequence	Accession no.	Ratio
ERK1	IADPEHDHTGFLTE _p YVATR	NP_036082	2.9
ERK2	VADPDHDHTGFLTE _p YVATR	NP_036079	2.8
Tnk2	pYATPQVIQAPGPR	NP_058068	0.8
Tweety homolog 2	pYENVPLIGR	NP_444503	1.0

“heavy” and “light” peptide ions. This reflects significant numbers of nonspecific binders or binding of basally phosphorylated proteins in tyrosine. Table I shows the identity of proteins and isotopic ratios of some of these peptide pairs. The mass spectrum of two of such pairs is shown in Fig. 3A, one pair of triply charged peptides (m/z 582.29, 585.32) and one pair of doubly charged peptides (m/z 599.28, 603.79), together with a peptide lacking tyrosine residues (m/z 597.32). MS/MS fragmentation of both peptide pairs revealed that they were derived from methylcrotonoyl-coenzyme A carboxylase 2 (β) protein. The fact that each pair of peptides had the same intensity was also indicative that the labeling of the experimental cell populations had been homogeneous, and equal amounts of protein extracts had been mixed before gel electrophoresis.

To find proteins that become tyrosine-phosphorylated upon activation of insulin receptor, we searched each mass spectrum for the presence of peptides pairs separated by 9 Da and exhibiting an increase in intensity difference of the heavy peptide as compared with the light version. Examination of the mass

spectra corresponding to proteins migrating around 177 kDa showed a pair of peptides (m/z 930.43 and 934.94), which presented an intensity ratio of 3.1 of heavy *versus* light peptides (Fig. 3B). The product ion spectra (MS/MS) of each of the peptides revealed the same sequence, which was assigned to the insulin receptor (Fig. 3C). Several other peptide sequences in the same mass spectrum also confirmed the identity of the insulin receptor.

In the analysis of the remaining bands, we identified a total of nine peptide pairs separated by 9 Da with an isotopic ratio greater than 1.5, indicating that they originated from substrates of tyrosine kinases in the insulin signaling pathway (Table II). As expected, each member of the pair had the same amino acid sequence and contained tyrosine residues. The next best peptide pair after insulin receptor in terms of intensity was derived from insulin receptor substrate 1 (IRS-1) with a ratio of 2.9. IRS-1 is a major substrate insulin receptor and is known to become heavily phosphorylated on tyrosines upon stimulation (20, 21). This phosphorylation ratio matches the

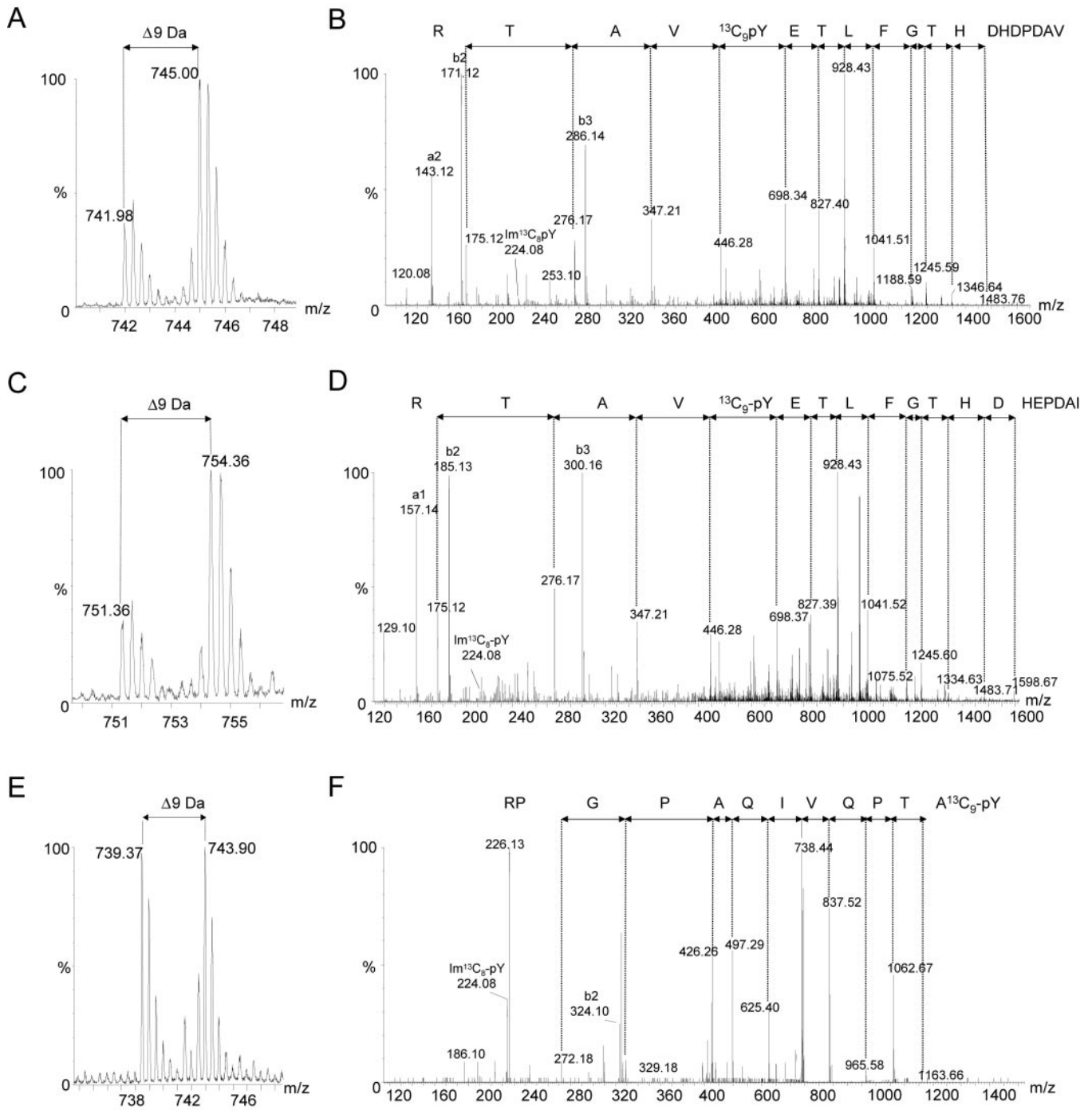


FIG. 5. Mapping of tyrosine-phosphorylated sites by ^{13}C tyrosine labeling. The mass spectrometric analysis of the tyrosine phosphorylation sites in different peptide pairs derived from the experiment described in Fig. 3 is shown. *A* and *C*, mass spectrum of peptide pairs exhibiting different intensities. *B* and *D*, MS/MS of the heavy peptides from *A* and *C*, respectively, that reveals the peptides to be derived from ERK1 and ERK2, respectively. The position of the tyrosine-phosphorylated residue is indicated in the sequence shown on the top of the panels; the immonium ion is indicated as $Im^{13}C_9$ pY. *E*, mass spectrum of a peptide pairs presenting similar intensity corresponding to Tnk2. *F*, the MS/MS spectra of the heavy peptide from *E*, the position of the tyrosine-phosphorylated residue is indicated in the sequence shown on the top of the panel; the immonium ion is indicated as $Im^{13}C_9$ pY.

ratio obtained by densitometric analysis of phosphorylation of IRS-1 in a similar experiment by Wang *et al.* (22). We also identified Gab1 (Grb2-associated Binder-1) as another member of the insulin receptor substrate family that presents a similar domain structure as IRS-1 except that it lacks the phosphotyrosine-binding domain (Table II) (23, 24). This protein contains 17 potential tyrosine phosphorylation sites and has been shown to be phosphorylated *in vitro* and *in vivo* on several of these tyrosines (25, 26). Other peptide pairs presenting high ratios were derived from extracellular signal-regulated kinases,

ERK1 and ERK2, and p38 which are also involved in other pathways besides insulin receptor signaling (27–29). A high ratio was also shown by a pair of peptides that were assigned to the adapter molecules APS, a member of the Lnk family of protein adapters which is known to be phosphorylated by the insulin receptor (30, 31). Syncrin (synaptotagmin-binding, cytoplasmic RNA-interacting protein), a protein highly homologous to heterogeneous nuclear ribonucleoprotein R that interacts with ubiquitous synaptotagmins and has been suggested to be involved in RNA metabolism, was also among the mole-

cles identified (Fig. 4, A and B) (32). This result is consistent with recent reports (33, 34) showing that Syncrin gets tyrosine-phosphorylated upon treatment with insulin which could affect its binding for RNA or its interaction with other RNA-binding proteins.

Interestingly, we found a novel substrate from MS/MS analysis of a peptide pair with a ratio 1.6 that assigned the sequence to "polymerase I and transcript release factor" (PRTF) (Fig. 4, C and D). This molecule was initially described as an interactor with the largest subunit of murine polymerase I and TTF-I (transcription termination factor 1) and is involved in the dissociation of ternary polymerase I transcription complexes (35). It has also been reported that PRTF is phosphorylated at multiple sites, and it is possible that changes in phosphorylation might alter its activity (36). In addition, this protein was cloned as a cofactor that binds to BFCOL1, a transcription factor that represses the mouse type 1 collagen gene promoter (37). It has been shown that overexpression of PRTF inhibits the transcription of the pro- α 2(I) reporter gene in NIH3T3 fibroblasts suggesting an effect through BFCOL1 (38). Thus, it is possible that the regulation of the phosphorylation state of PRTF is part of the mechanism used by insulin or insulin-like growth factor 1 to stimulate collagen formation in lung fibroblasts (39). Our approach therefore allows the unequivocal identification of substrates of tyrosine kinases from a large pool of non-substrate proteins.

Identification of Tyrosine Phosphorylation Sites—Insulin receptor-mediated signal transduction relies on the phosphorylation of tyrosine residues as a mechanism for regulating the protein activity, interactions, localization, and degradation. Modification of the phosphorylation state of specific sites in the same molecule usually results in different variations of the protein activity or function, thus the importance of the identification of the tyrosine-phosphorylated sites involved in each signaling cascade (40). Detection of tyrosine-phosphorylated peptides is cumbersome partly due to the difficulty of identifying tyrosine-containing peptides. Our approach not only facilitates their enrichment but also their identification as all the pairs of peptides separated by 9 Da in the mass spectra contain a single tyrosine residue. Thus, we analyzed the MS/MS spectra of all the peptide pairs differing by 9 Da to identify the subset of tyrosine-containing peptides that were phosphorylated. From this analysis, we could assign the subset of tyrosine-containing peptides that were phosphorylated. Thus, we were able to identify four tyrosine-containing peptides that were phosphorylated (Table III).

Two of the four peptide pairs represented substrates of tyrosine kinases that were induced by insulin signaling. These peptide pairs were derived from ERK1 and ERK2, respectively (Fig. 5, A and C), and revealed phosphorylated tyrosine residues at position 204 in ERK1 and position 185 in ERK2 (Fig. 5, B and D). The phosphorylation of these sites has been reported previously to be necessary for the activation of the kinase activity of ERK1 and ERK2 (41–45). Our results are in agreement with the fact that phosphorylation by mitogen-activated protein kinase/extracellular signal-regulated kinase kinases of ERK1 and ERK2 occurs in an orderly manner with phosphorylation of Tyr-204 occurring first followed by Thr-202 in ERK1 and on Tyr-185 preceding that of Thr-183 in ERK2 (46, 47). Additionally, it has been shown that tyrosines at positions Tyr-204 and Tyr-185 are indeed highly phosphorylated than threonines at position 202 and 183, which might account for the fact that we were only able to observe these singly phosphorylated peptide species (48, 49).

Additionally, we found two tyrosine-phosphorylated residues derived from peptide pairs whose relative intensity ratios were

close to 1. Both of these tyrosine phosphorylation sites are novel. A phosphotyrosine residue was localized at position 842 of Tnk2 (tyrosine kinase, non-receptor, 2), the mouse homolog of human ACK1 (activated p21cdc42Hs kinase) (Fig. 5, E and F). This tyrosine is in the C-terminal region of ACK1 and is conserved from fugu fish to humans. Gene-33, a protein that presents homology with the central region of ACK-1, also presents this tyrosine in a region that is responsible for its interaction with ErbB-2 (50).

A phosphorylated tyrosine residue, Tyr-493, was observed in a peptide derived from Tweety homolog 2, a protein of unknown function that belongs to a novel gene family of putative membrane proteins with five potential transmembrane regions (51, 52). This tyrosine forms part of a putative NPXY motif at the C terminus that is conserved from *Xenopus* to humans. Our identification of a phosphorylation site on tyrosine 493 implicates this protein in tyrosine kinase-mediated pathways. Furthermore, the sequence surrounding this phosphorylated tyrosine resembles a phosphotyrosine-binding domain binding site.

Although our approach simplifies the localization of tyrosine-phosphorylated peptides and substrates of tyrosine kinases in signaling pathways, the reduced number of phosphotyrosine-containing peptides and substrates identified points out the need to further develop methods for enrichment and selection of tyrosine-phosphorylated peptides. Coupling this method with selective enrichment of phosphopeptides by immobilized metal affinity chromatography (53) and/or with specific detection of phosphotyrosine by phosphotyrosine-specific immonium ion scanning method for residues (54) could improve the identification of phosphopeptides.

Conclusions—We have introduced a novel method for easy evaluation of involvement of the immunoprecipitated proteins in kinase-mediated signaling pathways by the use of [13 C]tyrosine labeling of cellular proteins. This method allows easy visualization of non-specifically bound proteins because tyrosine-containing peptides arising from proteins whose phosphorylation status is not affected will exhibit a 1:1 ratio between the normal and [13 C]tyrosine, whereas a protein that has been inducibly phosphorylated will show a ratio greater than 1. In our treatment of adipocyte cells with insulin, a ratio greater than 1.5 was only found in 9 of the 69 proteins identified by LC-MS/MS. This result clearly demonstrates that immunoprecipitation with antiphosphotyrosine antibody in this type of experiment indeed generates a big number of false positives, and that our method should prove to be an invaluable aid to discover true positives and locate peptides that are tyrosine-phosphorylated in high throughput experiments. We have recently demonstrated that the SILAC method can be used for quantitation of the extent of phosphorylation as well (55). Given the simplicity and uniformity of the labeling procedure, the SILAC method should become the preferred method for quantitative and qualitative analysis of phosphoproteome analysis in living cells.

Acknowledgment—We thank members of the Pandey laboratory for fruitful discussions.

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