A Method to Identify Serine Kinase Substrates

Akt PHOSPHORYLATES A NOVEL ADIPOCYTE PROTEIN WITH A Rab GTPASE-ACTIVATING PROTEIN (GAP) DOMAIN

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This study describes a method for the identification of the substrates of specific serine kinases. An antibody specific for the phosphomotif generated by the kinase is used to isolate phosphorylated substrates by immunoprecipitation, and the isolated proteins are identified by tandem mass spectrometry of peptides. This method was applied to the identification of substrates for the protein kinase Akt, which specifically phosphorylates the RXXXS/T motif. 3T3-L1 adipocytes were treated with insulin to activate Akt, and the putative Akt substrate proteins were isolated by immunoprecipitation with an antibody against the phospho form of this motif. This led to the identification of a novel 160-kDa substrate for Akt. The 160-kDa substrate for Akt, which was designated AS160, has a Rab GAP domain. Recombinant AS160 was shown to be a substrate for Akt, and two sites of phosphorylation, both in RXXXS/T motifs, were identified by mass spectrometry and mutation. Insulin treatment of adipocytes caused AS160 to redistribute from the low density microsomes to the cytosol.

Protein phosphorylation is a key cellular regulatory mechanism. The human genome contains ~1000 kinases. A major issue is to identify the protein substrates for each kinase. A number of different approaches have been developed (reviewed in Ref. 1). These include: in vitro phosphorylation of cell homogenates with recombinant kinases, screening of expression libraries with recombinant kinases, searching for kinase interacting proteins by the yeast two-hybrid screen, and the generation of mutated kinases that can function only with an ATP derivative. Each of these methods has its advantages and limitations, and additional methods are needed.

Each serine kinase typically phosphorylates Ser/Thr within a particular motif, and for many kinases the motif has been defined through identification of the sites of phosphorylation on substrate proteins and on peptide libraries (2). Phosphospecific antibodies against the phosphorylated form of these motifs are available or can be generated. Thus, immunoprecipitation with these antibodies, when combined with tandem mass spectrometry of tryptic peptides, offers an approach for the isolation and identification of substrates for specific serine kinases. In the past this approach has been used for the identification of substrates for tyrosine kinases through the use of antibodies against phosphoryrosine, but to our knowledge it has not previously been used with serine kinases.

In the present study, we applied this method to find substrates for the protein kinase Akt (also known as protein kinase B), which specifically phosphorylates Ser or Thr in the motif RXXXS/T (3). We have employed 3T3-L1 adipocytes, a cell type in which Akt is rapidly activated by insulin treatment (4). This method has led to the isolation of a novel target for Akt that contains a Rab GAP domain and two PTB domains.

EXPERIMENTAL PROCEDURES

Antibodies—A key reagent for this study is the antibody for the Akt phosphomotif RXXXS/T, where X is any amino acid. This antibody, which is referred as the PAS antibody (phospho Akt substrate), was purchased from Cell Signaling Technology, Beverly, MA (catalog number 9611). It is an affinity-purified rabbit antibody that has been shown to react specifically with the Akt phosphomotif in 12 known Akt substrates (literature from Cell Signaling Technology). The method used to generate the antibody is not described, but presumably an antiseraum with this specificity could be generated by immunization with a mixture of phosphopeptides in which all amino acids except Cys were put into the X positions, and the antibody could be affinity-purified on the immobilized mixture of phosphopeptides. An affinity-purified antibody against the carboxyl terminal 12 amino acids of mouse AS160 (PTND-KAKAGNKP) was generated as described in Ref. 5. Antibody against the FLAG tag (catalog number F-3165) was purchased from Sigma.

Plasmids—The cDNA encoding AS160 (6) in pBluescript SK vector was a gift from Dr. Paul A. Fuhrman (16). Flag-AS160 plasmid was kindly provided by the Kazusa DNA Research Institute. It was amplified by PCR with a 5’ primer containing a NotI site and a 3’ primer with a BamHI site and then spliced into the p3XFLAG-CMV-10 vector (Sigma). This yielded AS160 with a triple FLAG tag at the amino terminus. Mutants of AS160 in the p3XFLAG-CMV-10 vector were generated by PCR using the QuikChange XL site-directed mutagenesis kit from Stratagene, and the mutations were verified by DNA sequencing.

Cell Culture—3T3-L1 adipocytes were carried as fibroblasts and differentiated as described (7). Cells were placed in serum-free Dulbecco’s modified Eagle’s medium for 2 h before use and were then treated with 160 nM insulin for 10 min, unless stated otherwise. COS7L cells, purchased from Invitrogen, were transfected with 10 μg of plasmid DNA/10-cm plate with the LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Immunoprecipitation and Immunoblotting—For isolation of the proteins with the PAS antibody, 10-cm plates of adipocytes were each treated with insulin or not, washed with phosphate-buffered saline, and solubilized in 0.6 mL of 4% SDS, 10 mM dithiothreitol, 300 mM NaCl, 100 mM Hepes, pH 7.5, with protease inhibitors (10 μg/L concentration each of pepstatin, leupeptin, and EP475) at 100 °C for 5 min. The SDS lysate
was treated with 25 nm N-ethylmaleimide to cap the thiol groups and diluted with 5 ml of 1.7% thesit, 150 mM NaCl, 50 mM Hepes, pH 7.5.

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20, 150 mM NaCl, 20 mM Tris-Cl, pH 7.5, treated with primary antibody, were transferred to electrophoretically to Immobilon-P (Millipore). The membranes were blocked with 10 mg/ml bovine serum albumin, 0.3% Tween 20, and the immunoprecipitates were immunoblotted with this antibody. The 1x load is derived from 15% of a 10-cm plate. Repetitions of both experiments gave similar results.

was treated with 25 nm N-ethylmaleimide to cap the thiol groups and diluted with 5 ml of 1.7% thesit, 150 mM NaCl, 50 mM Hepes, pH 7.5. The mixture was centrifuged at 20,000 rpm for 30 min, and the infranatant was passed through a 0.45-micron filter to remove residual triglyceride droplets. PAS antibody (7 μg per plate) was added, and after 2 h the immunoprecipitate was collected on protein A-Sepharose (10 μl per plate) for 2 h. The beads were washed four times with 0.5% thesit, 150 mM NaCl, 50 mM Hepes, pH 7.5, and the immunoprecipitates were solubilized in SDS sample buffer at 100 °C for 5 min.

For immunoblotting proteins were separated by SDS-PAGE and then transferred to electrothermally to Immobilon-P (Millipore). The membranes were blocked with 10 mg/ml bovine serum albumin, 0.3% Tween 20, 150 mM NaCl, 20 mM Tris-Cl, pH 7.5, treated with primary antibody in the same solution, washed, treated with horseradish peroxidase-conjugated secondary antibody, and developed with the chemiluminescence reagent Supersignal (Pierce).

Tandem Mass Spectrometry—Proteins were separated by SDS-PAGE and the gel was stained with the colloidal Coomassie Blue reagent (Invitrogen). Proteins of interest were digested in gel with trypsin. The tryptic peptides were sequenced by microcapillary liquid chromatography MS/MS on an ion trap mass spectrometer (Thermo-Finnigan LCQ DECA XP, as described (8)). To detect a specific site of phosphorylation, a targeted ion MS/MS experiment was conducted for each putative phosphopeptide. In these experiments the predicted precursor mass-to-charge ratio of the phosphopeptide was subjected to MS/MS for the entire chromatographic run. This procedure significantly increases detection sensitivity by capturing only the peptide of interest at the moment of its chromatographic elution while excluding peptic background.

RESULTS

Isolation of Insulin-elicited Phosphoproteins with the PAS Antibody—3T3-L1 adipocytes were untreated or treated with rapamycin, wortmannin, or LY294002 and then subsequently exposed to insulin or not. Rapamycin inhibits insulin activation of the 70-kDa S6 kinase but not of Akt, whereas wortmannin and LY294002 inhibit activation of PI 3-kinase and thereby of Akt (9, 10). SDS samples of the cells were solubilized with the PAS antibody (Fig. 1A). Seven insulin-elicited phosphoproteins (pp250, pp160, pp105, pp75, pp47, pp43, and pp32) were detected. Wortmannin and LY294002 blocked the phosphorylation of all of these except possibly pp75, but rapamycin blocked only the phosphorylation of pp32. These results thus suggested that pp250, pp160, pp105, pp47, and pp43 are Akt substrates, whereas pp32 may be an S6 kinase substrate. Insulin also caused a decrease in the phosphorylation of a 60-kDa protein, which was blocked with wortmannin and LY294004, but not by rapamycin. In Fig. 1A insulin treatment was for 10 min. Examination of the time course for changes in phosphorylation detected with the PAS antibody showed that for each phosphoprotein the change was approximately maximal after 10 min of insulin treatment (data not shown).

To isolate the proteins that underwent changes in phosphorylation, we carried out immunoprecipitation with the PAS antibody. Cells were first lysed in SDS to denature the proteins and thereby expose the phosphopeptide motifs, and then an excess of nonionic detergent was added and the immunoprecipitation performed. By this approach it was possible to isolate pp250, pp160, pp105, pp75, and pp60 (Fig. 1B). No pp32 was detected in the immunoprecipitate, and the strong signal from the antibody heavy chain precluded determining whether pp47 and pp43 were present. The immunoprecipitate derived from insulin-treated cells showed slightly more of the pp250, pp160, pp105, and pp75 than did that derived from untreated cells. However, the difference was not as large as was observed with blotting of the cell lysates (Fig. 1A). A possible explanation is that the PAS antibody, which is a mixture, contains only a limited amount of antibody against any specific XXXXX/T sequence, and thus the percent yield upon immunoprecipitation was antibody-limited and so less for the insulin sample. Considerably more pp60 was present in the immunoprecipitate derived from untreated adipocytes. Subsequently we found that pp32 could be isolated by immunoprecipitation from a nonionic detergent lysate of adipocytes (see Fig. 3A).

Identification of the Insulin-elicited Phosphoproteins—Immunoprecipitations of the phosphoproteins from SDS/nonionic detergent lysates of untreated and insulin-adipocytes were performed on a large scale (six 10-cm plates). The pp250, pp160, pp105, pp75, and pp60 bands were separated by SDS-PAGE, subjected to tryptic digestion, and the peptides sequenced by MS/MS. In a separate isolation pp32 was identified in this way after isolation from a nonionic detergent lysate of the LDM fraction of adipocytes, where it was most abundant (data not shown).

Initially, we have focused on pp160. MS/MS sequence analysis of the sample from insulin-treated cells yielded 15 peptides identical to those in the human protein gi7662198 and thus indicated that pp160 is the mouse version of this protein. gi7662198 is a protein of 1299 amino acids whose function is unknown. Its cDNA was previously cloned as part of a large scale cDNA cloning project (6). The protein is predicted to contain two PTB domains and a Rab GAP domain (Fig. 2). It has two sites, Ser-588 and Thr-642, that are predicted to lie in excellent motifs for Akt phosphorylation (2).

To establish the identity of pp160 definitively, we raised an antibody against the carboxyl terminal sequence of the mouse protein and used it, together with the PAS antibody, in reciprocal immunoprecipitations and immunoblotting with nonionic detergent lysates of basal and insulin-treated adipocytes. The results exhibited the pattern expected (Fig. 3). Immunoprecipitation from the insulin lysate with the antibody against pp160 (hereafter referred to as AS160 for Akt substrate of 160 kDa) yielded a 160-kDa protein detected by the PAS antibody (lane 4), and immunoprecipitation from the insulin lysate with
treated 10-cm plates of adipocytes were lysed in 1 ml of 3% thesit, 150 mM NaCl, 40 mM Hepes, pH 7.5, with phosphatase inhibitors (10 mM sodium pyrophosphate, 10 mM NaF, 2 mM EDTA, 10 mM sodium vanadate) and protease inhibitors. The lysates were cleared by centrifugation, the supernatants were immunoprecipitated with the PAS antibody or the antibody against AS160, and the immunoprecipitates were immunoblotted with both antibodies. The load on each lane was derived from 20% of a 10-cm plate. A repetition of this experiment gave similar results.

The PAS antibody yielded a 160-kDa protein detected by the AS160 antibody (lane 6). Phosphorylation of AS160 was accompanied by a slight decrease in electrophoretic mobility (compare lanes 7 and 8).

MS/MS sequencing of the pp32 band from insulin-treated adipocytes identified 14 peptides from the 29-kDa ribosomal protein S6 together with smaller numbers of peptides from the similarly sized ribosomal S2, S3, and S3A proteins. Possibly these immunoprecipitated as a complex. It is likely that the PAS antibody reacted with the S6 protein in this mixture. The S6 protein is known to be phosphorylated by the 70-kDa S6 kinase on the Ser-236, which is an RXRXXS sequence (RRRLSS236L) (11).

The pp60 band, obtained from the lysate, of untreated cells contained the mouse version of the well characterized rat adipocyte protein perilipin. Perilipin is a 56-kDa protein that is associated with the lipid droplets; it is constitutively phosphorylated, is heavily phosphorylated in response to agents that activate cAMP-dependent protein kinase, and undergoes dephosphorylation in response to insulin (12). The sites of perilipin phosphorylation have not been identified, but presumably the PAS antibody reacts with one of these. Perilipin contains only one Ser/Thr that is in a motif likely to react with this antibody, Ser-385 (KGRAMS385L).

The pp250-, 105-, and 75-kDa bands each contained peptides from several proteins. We are currently generating antibodies against the candidate in each band most likely to be the one reacting with the PAS antibody, to identify it. None of the proteins present in these bands are on the list of known Akt substrates (3).

Sites of Phosphorylation in AS160 and Phosphorylation by Akt —To determine whether AS160 was phosphorylated on one or both of the predicted Akt sites (Fig. 2), the two predicted tryptic phosphopeptides were targeted for MS/MS in the tryptic digest of mouse AS160 isolated from insulin-treated adipocytes. The expressed sequence tag data base contains portions of the sequence of mouse AS160, and from these the sequences of the two mouse tryptic peptides corresponding to human tryptic peptides with Ser-588 and Thr-642 were obtained. The mouse and human sequences are: LGS588M(T)DSFER and AHT642FSHPPS(3)/K(R), where each amino acid in parentheses is for the human sequence. The two tryptic phosphopeptides were detected by targeted MS/MS, and the resulting fragmentation spectra established that the sites of phosphorylation were Ser-588 and Thr-642.

To determine directly whether AS160 was a substrate for Akt, we examined the phosphorylation of recombinant AS160, as well as mutant forms with Ser-588, Thr-642, or both converted to Ala, by recombinant Akt 1. Phosphorylation was detected by immunoblotting with the PAS antibody. The data in Fig. 4 shows that Akt phosphorylation of AS160 and its S588A mutant yielded AS160 that was detected well by the PAS antibody. In contrast, Akt phosphorylation of the T642A mutant yielded an AS160 that reacted very weakly with the PAS antibody (see the legend to Fig. 4), and phosphorylation of the double mutant resulted in an AS160 that showed no detectable reaction with the PAS antibody. Thus, Akt phosphorylated AS160 on Thr-642 and probably on Ser-588. The PAS antibody probably binds more strongly to the phosphopeptide sequence containing Thr-642 than to that containing Ser-588.

We have also examined the phosphorylation of AS160 and the T642A/S588A mutant after isolation by immunoprecipitation with anti-FLAG from SDS/thesit lysates of basal and insulin-treated transfected COS7 cells. In this in vitro system insulin stimulated phosphorylation of AS160 3-fold, and the T642A/S588A mutant showed almost no phosphorylation, as assessed by blotting with the PAS antibody (data not shown).
Subcellular Distribution of AS160—In untreated adipocytes AS160 was concentrated in the LDM fraction (Fig. 5A). Treatment with insulin caused a marked redistribution of AS160 from the LDM to the cytosol. As is the case for AS160 phosphorylation, treatment with wortmannin or LY294002 inhibited the insulin-elicited redistribution (Fig. 5B). One of the components of the LDM is vesicles containing the glucose transporter GLUT4 (13). In response to insulin these move to and fuse with the plasma membrane (reviewed in Ref. 14). We isolated GLUT4 vesicles from the LDM by immunoadsorption (13) and found by immunoblotting that AS160 was not located in these vesicles (data not shown).

Tissue Distribution of AS160—SDS lysates of mouse tissues were immunoblotted for AS160 with the AS160 antibody. All the tissues examined (brain, testes, spleen, kidney, pancreas, lung, thymus, liver, heart, quadriceps, and brown and white fat) showed a band at ~160 kDa, with the strongest signals in the brain and pancreas (data not shown). Thus, these results indicate that AS160 has a widespread tissue distribution. Previously the mRNA for AS160 was shown to be widely expressed in human tissues (6).

DISCUSSION

This study illustrates a method for the isolation of substrates for serine kinases by means of phosphomotif-specific antibodies, which are now becoming commercially available. Application of the method requires that the kinase have a specificity for Ser/Thr within a fairly well defined motif and that the antibody against the phosphomotif functions in immunoprecipitation. The method is best applied in cell types where the kinase can be activated by some agent so that the phospho-proteins reacting with the phosphomotif-specific antibody after activation can be compared with those seen before activation. Using this approach, we have identified one novel substrate for Akt, AS160, and expect to identify at least two more, pp250 and pp105.

One complication of the method is that in a number of instances several kinases have very similar specificity with respect to the motif in which the Ser/Thr lies, and consequently the definitive identification of the activated kinase responsible for phosphorylation requires information from other types of experiments. Our results illustrate this situation. The insulin-elicited pp32 proved to be ribosomal S6 protein that was phosphorylated by S6 kinase rather than Akt. In addition, the serum- and glucocorticoid-induced protein kinase has a specificity that is very similar to that of Akt and is activated by insulin (15). Consequently, it is possible that some of the substrates detected with the PAS antibody are targets of this kinase, rather than or in addition to Akt.

The AS160 protein is a previously undescribed Akt substrate. Our results show that insulin treatment of adipocytes causes its subcellular redistribution and are consistent with the proposal that this effect is due to its phosphorylation. In its overall structure AS160 partially resembles a recently characterized GAP for Rab6, which has a single PTB domain in its NH₂-terminus and a Rab GAP domain 34% identical to the one in AS160 (16). Since the Rabs are key proteins in membrane trafficking (17), phosphorylation of AS160 by Akt may function to connect insulin signaling to membrane trafficking through an effect on its GAP activity toward a particular Rab. Insulin-stimulated trafficking of GLUT4 to the plasma membrane requires activation of PI 3-kinase, and there is evidence that Akt is a downstream kinase required for this process (14, 18). Thus, despite the fact that AS160 is not located in GLUT4 vesicles, it remains a candidate to be a component of the signaling pathway from the insulin receptor to GLUT4 trafficking. Efforts are under way to test this possibility and to identify the Rab for which AS160 is a GAP.

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