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Adipocytes contain a novel complex similar to the tuberous sclerosis complex

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Abstract

Recently we identified a novel 250 kDa protein in adipocytes that is a substrate for the insulin-activated protein kinase Akt. We refer to this protein as AS250 for Akt substrate of 250 kDa. AS250 has a predicted GTPase activating protein (GAP) domain at its carboxy terminus. This domain shows some homology to the GAP domains for Rheb at the carboxy terminus of the protein tuberin and for Rap1 in the protein Rap1 GAP. The present study further characterizes AS250. The cDNA sequence for human AS250 is reported, and the sites that undergo phosphorylation upon insulin treatment of adipocytes have been identified by tandem mass spectrometry. We have found that in adipocytes AS250 exists as a complex with a novel protein of 1484 amino acids known as KIAA1219. The complex of AS250 with KIAA1219 is notably similar to the important regulatory complex of the protein tuberin with hamartin (the tuberous sclerosis complex), in the size of its subunits, the location of the GAP domain, and its phosphorylation by Akt. In an effort to detect the cellular role of the AS250/KIAA1219 complex, we generated 3T3-L1 adipocytes that largely lack AS250 by shRNA knockdown and examined several insulin-dependent effects. The knockdown of AS250 had no effect on insulin activation of the kinases, Akt, 70 kDa S6 kinase, or ERK1/2, or on insulin-stimulated actin bundling, and it had only a slight effect on insulin-stimulated GLUT4 translocation.

Keywords: Adipocyte; Akt; GTPase activating protein; Insulin; KIAA1219; Tuberous sclerosis complex

1. Introduction

A key signaling pathway from the insulin receptor results in the activation of the protein kinase Akt. Akt occupies a central position in insulin action. It phosphorylates and thereby modulates the activity of a number of proteins that control major metabolic effects of insulin, including the stimulation of glucose transport and glycogen and protein synthesis [1]. In order to understand insulin signaling and action more completely, we have searched for novel substrates of Akt in adipocytes, a cell type that is highly insulin-responsive [2]. By immunoisolation with an antibody against the phosphomotif generated by Akt phosphorylation, we have recently identified and preliminarily characterized four new Akt substrates [3]. One of these substrates is a protein of approximately 250 kDa, which in this study is designated AS250 for Akt substrate of 250 kDa.

Human AS250, the complete sequence of which is described here, is a protein of 1873 amino acids. As described in [3], the only distinctive domain in AS250 is a predicted GTPase activating protein (GAP) domain at its carboxy terminus. This predicted GAP domain in AS250 is most similar to the GAP domain in the protein tuberin (also known as TSC2), which is specific for the small G protein Rheb. By blast analysis, the AS250 GAP domain is 33% identical/53% similar to the GAP region of tuberin. Thus, the sequence similarity between the two GAP domains is considerable, but it does not approach identity. The GAP domain of AS250 is also similar to the GAP domain in the protein Rap1 GAP, which acts on the small G protein Rap1. In this case, the GAP domains are 29% identical/49% similar.

Tuberin is associated with the protein hamartin (also known as TSC1) in a complex called the tuberous sclerosis complex

Abbreviations: EST, expressed sequence tag; GAP, GTPase activating protein; MS, mass spectrometry; TSC, tuberous sclerosis complex.

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(TSC). TSC plays a key role in the regulation of cell growth [4,5]. Similarly to AS250, tuberin is phosphorylated by Akt. There is considerable evidence that this phosphorylation inhibits its GAP activity toward Rheb, and thereby leads to the elevation of the GTP form of Rheb. Rheb in its GTP form activates the kinase mTor, which in turn stimulates protein synthesis by several mechanisms.

Thus, AS250 and tuberin resemble each other in that both are substrates for Akt and contain a GAP domain. Moreover, the architecture of the two proteins is similar. Tuberin is also a large protein (1807 amino acids), and its GAP domain is also located at its carboxy terminus. In the present study, we report that AS250 exists in a complex with a novel protein known as KIAA1219. KIAA1219 is a protein of unknown function in the same size range as hamartin. Thus, the AS250/KIAA1219 complex is a novel complex that resembles TSC. In addition, we describe the effects of insulin on the phosphorylation of specific sites on AS250 and report on efforts to identify the function of AS250, including a survey of the effects of its knockdown in 3T3-L1 adipocytes.

2. Materials and methods

2.1. Antibodies

The antibody against AS250 was the previously described affinity-purified rabbit one against a peptide in mouse AS250 corresponding to amino acids 451–467 of the human protein [3]. The antibody against KIAA1219 was an affinity-purified rabbit antibody against the peptide corresponding to amino acids 1435–1453, prepared in the same way as anti-peptide antibody against AS250. Antibodies against the following were purchased from Cell Signaling Technology (listed as antigen (catalog number)): phosphotyrosine (9411); Akt pThr 308 (9275); Akt pSer 473 (9271); S6 kinase pThr389 (9205); ERK1/2, pThr202/pTyr204 (9101).

2.2. Cloning and plasmids

The cDNA sequence for full-length human AS250 was determined for us by Cytomyx, Cambridge, United Kingdom, as follows. Initially we deduced the likely full-length cDNA sequence from the overlapping sequences of partial cDNA's and ESTs in the database. The 5′ fragment of the cDNA was then obtained by reverse transcription PCR with human liver mRNA as template. The 3′ fragment was obtained by PCR from a human lung cDNA library. Both the 3′ and 5′ fragments overlapped substantially with a large middle fragment present in the partial cDNA KIAA1272 (gi6331238), which was obtained from Kazusa DNA Research Institute. The complete cDNA sequence was assembled from the sequences of these three fragments. It was verified by sequencing both strands corresponding to the entire coding region. The designated start Met in the sequence is supported by the fact that this Met codon is the first Met codon following a 5′ upstream in-frame stop codon in an EST (BC1200_F03 OriGene 5′ read for XM_046600). The following plasmids were generous gifts: p3XFLAG-Rap1 GAP plasmid and the pGEX plasmids for GST fusion proteins of Rap2B, M-Ras, R-Ras, and TC21, from Dr. Lawrence Quilliam at Indiana University School of Medicine; pGEX plasmids for GST-Rap1A and GST-Rheb, Dr. Ariel Castro at Indiana University School of Medicine; pGEX for GST-RalA, Dr. Phillip Robinson, Children's Medical Research Institute, Australia.

2.3. Cell culture

3T3-L1 fibroblasts from the American Type Culture Collection were carried as fibroblasts and differentiated into adipocytes as described in [3]. Adipocytes were used at days 7 to 14 after differentiation. Cells were serum-starved for 2 h, and then treated with 100 nM insulin or left unstimulated for 10 min.

2.4. Immunoprecipitation, tandem mass spectrometry, and immunoblotting

For the immunoprecipitation of AS250, each 10 cm plate of 3T3-L1 adipocytes was lysed in 1 ml of 3% nonaethyleneglycol dodecyl ether, 40 mM Hepes, 150 mM NaCl, pH 7.5, with phosphatase and protease inhibitors (10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 10 mM β-glycerophosphate, 10 μg per ml aprotinin, 10 μM leupeptin, 10 μM EP475, and 1 μM pepstatin). The lysate was cleared of nuclei by centrifugation at 27,000×*g* for 20 min. The cleared lysate was incubated with 10 μg antibody per ml for 2 h, and then the immune complexes were collected by mixing with 10 μl per ml protein A-sepharose beads for 2 h. The beads were washed with 0.5% nonaethyleneglycol dodecyl ether, 40 mM Hepes, 150 mM NaCl, pH 7.5, containing phosphatase inhibitors (see above). The immune complexes were then released with SDS sample buffer containing 10 mM dithiothreitol at 100° for 5 min.

For MS, the polypeptides in the SDS samples of the immune complexes were separated by SDS PAGE and stained with colloidal coomassie blue. The bands of interest were cut from the gel, and then digested in gel with trypsin. The yield of AS250 from one 10 cm plate was about 250 ng, as assessed by coomassie blue staining and visual comparison with known amounts of standard proteins.

For the protein identification, the tryptic peptides were sequenced by microcapillary liquid chromatography MS/MS on an ion trap mass spectrometer, as described in [3]. For analysis of the effect of insulin on phosphorylation at specific sites on AS250, AS250 was isolated by immunoprecipitation from basal and insulin-treated adipocytes as described above. Then targeted ion MS/MS was conducted for each phosphopeptide and the corresponding nonphosphorylated form for ratio analyzed quantitation, as described previously [6].

Immunoblotting was performed as in [3]. In brief, polypeptides were separated by SDS PAGE and transferred to Immobilon-P membrane, and the membrane was treated with primary antibody followed by horseradish peroxidase-conjugated secondary antibody and then chemiluminescence reagent.

2.5. AS250 knockdown

The procedure for the knockdown of AS250 was one in which a shRNA corresponding to a sequence in mouse AS250 was continually expressed in 3T3-L1 fibroblasts and adipocytes through retroviral infection and selection, as described previously [7]. The sequence for the AS250 shRNA was GATGTGAAGGAATCAGACT; that for the control shRNA, which does not correspond to any known mouse cDNA, was CAGTCGCGTTTGCGACTGG.

2.6. Recombinant proteins and GAP assay

Recombinant GST fusion proteins of the small G proteins, Rheb, Rap1A, Rap2B, M-Ras, R-Ras, RalA, and TC21 were generated in *Escherichia coli* and purified on glutathione-sepharose, as described in [8]. Flag-tagged Rap1 GAP was expressed in HEK293 cells, and isolated from a nonionic detergent lysate by immunoadsorption on beads with immobilized anti-Flag, as described in [8]. The yield of Flag-Rap1 GAP was approximately 30 μg per 10-cm plate. As a negative control for this preparation, a nonionic detergent lysate of nontransfected HEK293 cells was adsorbed with anti-Flag beads.

The GAP assay was carried out as described previously [8]. In brief, the small G protein was loaded with α^{32}P GTP and then incubated with the GAP protein or the negative control. At various times samples were taken, and α^{32}P GDP was separated from α^{32}P GTP by thin layer chromatography, and the radioactivity in each was quantitated by phosphorimaging. The GAP assay was carried out with the AS250/KIAA1219 complex that had been isolated by immunoprecipitation from a nonionic detergent lysate of basal 3T3-L1 adipocytes and was on beads; an immunoprecipitate with irrelevant rabbit immunoglobulin served as the negative control. As a positive control for this assay, we measured the activity of Flag-tagged Rap1 GAP on anti-Flag beads and the control anti-Flag beads, prepared as described above.