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Identification and characterization of D-AKAP1 as a major adipocyte PKA and PP1 binding protein

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Abstract

Protein kinase A (PKA) plays an important role in the regulation of lipid metabolism in adipocytes. The activity of PKA is known to be modulated by its specific location in the cell, a process mediated by A-kinase anchoring proteins (AKAPs). In order to examine the subcellular localization of PKA in this tissue we performed a search for AKAP proteins in adipocytes. We purified a 120 kDa protein which can bind both the regulatory subunit of PKA as well as the catalytic subunit of protein phosphatase 1 (PP1). This protein was found to be enriched in the lipid droplet fraction of primary adipocytes and was identified as D-AKAP1. This protein may play an important role in the regulation of PKA in adipocytes.
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Initial biochemical studies using homogenized cell extracts defined much of the metabolic and signalling pathways that have become classic examples of biological regulation. The application of cell biology techniques and the recognition of distinct protein: protein interaction domains revealed that proteins are specifically targeted to precise locations in the cell. In the case of signalling, these proteins are often co-localized to close proximity of their substrates thereby increasing specificity and control of signalling pathways. The selective localization of these proteins can be the result of post-translational modification of the protein (for example lipid modification), structural changes (for example exposure of a nuclear localization sequence) or interaction with specific targeting proteins.

In adipocytes, like all eukaryotic cells, there are a number of distinct subcellular organelles. One major element of adipocytes is the lipid droplet. These droplets consist of a neutral lipid core surrounded by a membrane monolayer [1,2]. Although lipid droplets are present in a number of other cell types, they are especially dominant in adipose tissue. A number of proteins have been identified as lipid droplet associated proteins. These proteins may be constitutively localized to lipid droplets or may transit between lipid droplets and another location in response to a signalling event.

The primary role of adipose tissue is control of the storage and release of lipids in response to nutritional and hormonal signals. A number of proteins involved in lipid metabolism have been shown to be lipid droplet localized including hormone-sensitive lipase (HSL), perilipin, and acyl-coA synthase [3]. These proteins are all known to be modulated by protein phosphorylation. Specific co-localization of protein kinases or phosphatases to these substrates is likely to play a key role in their regulation. If the former hypothesis is true, this would require that signalling proteins must be at some point in their lifetime present at the lipid droplet. Some proteins, for example perilipin, are known to be localized to the lipid droplet exclusively. This situation strongly supports the possibility

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that signalling proteins may also be localized to the lipid droplet.

The major protein kinase involved in the stimulation of lipolysis is thought to be protein kinase A (PKA). PKA phosphorylates a number of proteins involved in lipid metabolism, including HSL and perilipin, which functions to increase the rate of breakdown of triglycerides into free fatty acids and glycerol. PKA is known to be anchored to a number of different locations in the cell by specific targeting proteins known as A-kinase anchoring proteins (AKAPs; reviewed in [4–6]). In addition to the PKA anchoring function, many AKAPs also bind other signalling enzymes including a variety of other protein kinases [7–14], protein phosphatases [10,15–18], and cyclic nucleotide phosphodiesterases [19,20] as well as a wide variety of other proteins [6,21,22]. Recent work in adipocytes has shown that inhibition of PKA anchoring in these cells blocks nearly all isoproterenol-stimulated phosphorylation events [23].

This work describes experiments which involve the examination of the subcellular localization of PKA in adipocytes and the identification and purification of a specific AKAP which may anchor both PKA and PP1 to the lipid droplet of adipocytes.

Materials and methods

Materials. Male Sprague–Dawley rats were maintained in the University of Calgary Animal Health Unit. Antibodies raised against PKA catalytic subunit and RII were generated as described in [24]. Antibodies raised against PP1γ1 were described in [25] and against PP2A were described in [26]. Antibodies raised against RI were generously donated by Dr. John D. Scott (Oregon Health Sciences University, Portland, OR). Antibodies raised against PP6 were described in [27]. PKA and RII were purified from bovine heart according to [28] and PP1 was purified as described [26]. Digoxigenin-NHS and anti-digoxigenin antibodies were purchased from Roche. Protein kinase A [29] and Phosphorylase phosphatase [30] assays were performed as previously described. PKI was a gift from Dr. Michael D. Walsh (University of Calgary).

Purification of adipocyte lipid fraction. Isolated rat epididymal adipose tissue was washed into cold homogenization buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 250 mM Sucrose, 5 mM EDTA, 5 mM Sodium pyrophosphate, 25 mM NaF, 1 mM Benzamidine, 0.2 mM PMSF, and 0.1% β-Mercaptoethanol). Cells were lysed using a Dounce homogenizer with 10 strokes. The lysate was clarified by centrifugation at 20,000 rpm in a SS-34 rotor. The floating fat sample was removed and washed twice with cold Wash Buffer (Homogenization buffer without Sucrose) before use.

Preparation of DIG-probes and far Western overlays. Proteins were dialysed into PBS, pH 7.4, prior to coupling. DIG (~1 mg) was dissolved in 20 μL DMSO and 2 μL of this solution was added to 25 μg protein in 350 μL PBS, pH 8.5. This was allowed to react for 10 min at room temperature and was quenched by the addition of 100 μL of 1 M Tris, pH 8.0. The labelled protein was dialysed for 1 h against 1 L 25 mM Tris, pH 8.0, then 4 h against 4 L of PBS, pH 7.4, followed by 4 L PBS, pH 7.4, overnight. Protein was diluted into 25 mL of Western blot wash buffer with 1 mg/mL BSA. Proteins for overlays and Western blots were immobilized on nitrocellulose membranes using standard protocols and blocked overnight with 5% (w/v) skim milk powder. Blots were incubated with DIG-probes for 4 h or with primary antibodies for 1 h at room temperature with gentle shaking, washed extensively, and were visualized using ECL.

Purification of D-AKAP1 from adipose tissue. Adipose AKAPs were purified by immobilized RII affinity chromatography. RII- and Blank-Sepharose columns were prepared as described in manufacturer's instructions with 1 mg RII coupled per milliliter CH-Sepharose. Blank matrix was prepared by coupling Tris to the beads. Adipose tissue was dissected from fat pads and chopped into small pieces. Cells were lysed in homogenization buffer supplemented with 3% (v/v) Triton X-100. The lysate was clarified by centrifugation at 20,000 rpm for 25 min in a SS-34 rotor at 4 °C. The lysate was split in half and loaded onto the RII- or Blank-Sepharose beads for 2 h at 4 °C. The beads were poured into disposable columns and washed with 150 mM of wash buffer then 50 mL of wash buffer with 1 M NaCl. Proteins were eluted at room temperature with 1% (w/v) SDS solution after incubation for 30 min. Purified proteins were concentrated using a 10 kDa molecular weight cut-off Centricon (Amicon) and stored at −80 °C.

Identification of D-AKAP1 by mass spectrometry. Protein bands were cut from the SDS–PAGE gels and in-gel digested with trypsin [31]. Extracted tryptic peptides were purified with Poros R2 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions (http://protana.com). The peptides were concentrated in a nano-electrospray capillary and placed in the source head of a QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems) to derive de novo peptide sequences. Peptide sequences were searched against the NCBI non-redundant sequence database using the FASTS algorithm [32].

Results

PKA and PP1 are present in the lipid droplet enriched fraction of adipocyte extracts

In order to examine the localization of PKA and various protein phosphatases we used polyclonal antibodies raised against the subunits of PKA (PKAcs, RI, and RII), and PP1, PP2A, and PP6. We used these antibodies to probe adipocyte extracts from primary adipose tissue. Under low speed centrifugation, adipose tissue separates into a soluble fraction and a floating lipid fraction. Fig. 1A shows

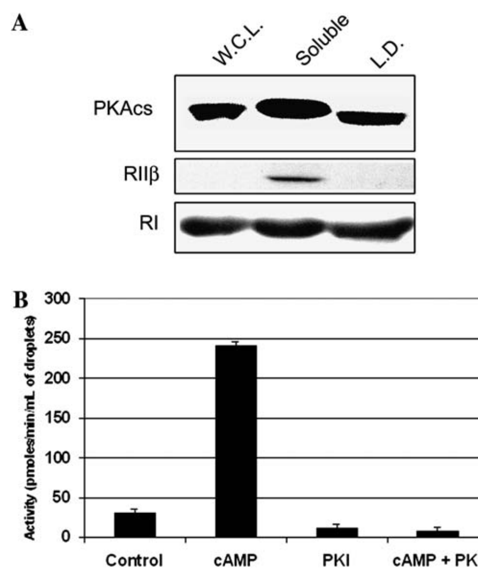

Fig. 1. PKA is present in the lipid droplet fraction of adipocytes. (A) Western blotting of whole cell lysate (WCL), soluble, and lipid droplet (LD) fractions from rat adipose tissue probed with antibodies raised against the catalytic (PKAcs), RIIβ, and RI subunits of PKA. (B) PKA assays of purified lipid droplets using kemptide as a substrate. Activity is measured in the presence of cAMP or PKI. Results are the average of three experiments.

that both the RII and PKA catalytic subunits are present in both the soluble and lipid fractions, whereas the RI subunit is present only in the soluble fraction. Control proteins specific for each fraction (perilipin for the lipid droplet and talin for the soluble fraction) indicated minimal cross-contamination between samples. Protein kinase assays were then performed on purified lipid droplets and cAMP-dependent protein kinase activity towards a well established PKA substrate could be detected biochemically (Fig. 1B). This activity was nearly completely abolished by the specific PKA inhibitor PKI indicating that the protein kinase activity is PKA and that it can be activated from this fraction by cAMP.

PPP family protein phosphatases have been implicated in several of the key dephosphorylation events in adipocytes relating to triglyceride metabolism [33–43]. We used antibodies specific for the catalytic subunits PP1, PP2A, and PP6 to examine their distribution in adipose tissue. Both PP1 and PP2A were detected in both the soluble and lipid fraction while PP6 was detected only in the soluble fraction (Fig. 2A). The A-subunit (PR65) of the PP2A trimer was also detected with a similar distribution to the PP2A catalytic subunit. Protein phosphatase assays were performed on adipocyte lipid fractions and protein phosphatase activity was detected. Treatment with the specific PP1 inhibitor I-2 showed only partial inhibition of phosphatase activity consistent with what would be expected by both PP2A and PP1 being present and active on the lipid droplet (Fig. 2B).

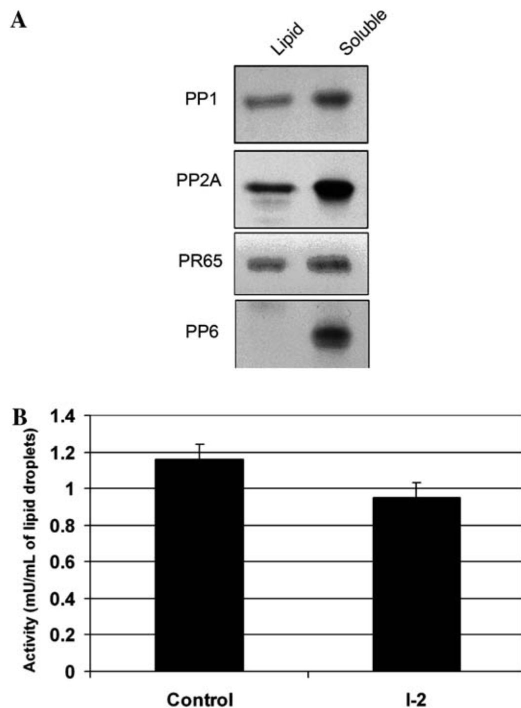

Fig. 2. Protein phosphatases are present in the lipid fraction of primary adipocytes. (A) Soluble and lipid fractions from rat adipose tissue were run on SDS–PAGE, transferred to nitrocellulose and probed with antibodies raised against the catalytic subunits of PP1, PP2A, and PP6 and the A-subunit of PP2A (PR65). (B) Phosphorylase phosphatase assays were performed on purified lipid fractions in the absence or presence of the PP1 inhibitor I-2. Results are the average of three experiments.

PKA and PP1 both bind to a 120 kDa protein enriched in the lipid droplet fraction of adipocytes

Since, there was no reason to believe that either the PKA heterotetramer or the PP1 or PP2A catalytic subunit would interact with the lipid droplet alone, we next searched for binding proteins which could mediate the enzyme-lipid droplet interaction. Both PP1 and PKA-RII are known to have several regulatory subunits which can localize the catalytic subunit to a variety of subcellular locations. We used a digoxygenin based overlay technique in order to examine the lipid droplet fraction for *in vitro* PP1 and RII binding proteins (Fig. 3).

We detected a 120 kDa band in the lipid droplet enriched fraction which interacts with two isoforms of PP1 (γ and α) that precisely co-migrated with a band detected by an overlay with labelled RII. This band was the major detected binding partner of both RII and PP1 in the lipid droplet fraction. The RII-p120 interaction in the overlay assay was completely blocked by the peptide Ht31 but not by a mutated peptide (Ht31-Proline; data not shown). This peptide interacts with the dimerization/docking region of RII and competitively inhibits the interaction with all known AKAPs [7,21,44–57].

Purification and identification of D-AKAP1 as a major adipocyte AKAP

In order to identify this major PP1 and PKA binding protein from adipocytes, we solubilized lipid droplet

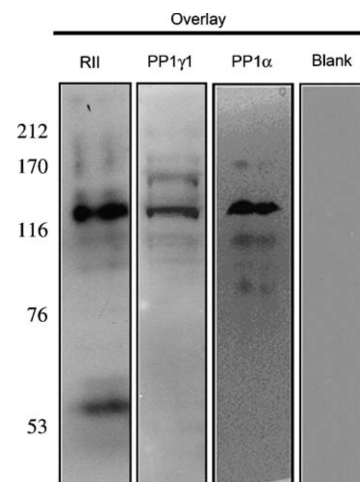

Fig. 3. A lipid droplet enriched protein interacts with RII and PP1 *in vitro*. Proteins enriched on lipid droplets were run on SDS–PAGE and transferred to nitrocellulose. Digoxygenin-labelled PP1γ1, PP1α, or RII were used to probe lipid droplet fractions from rat adipose tissue. Blank indicates a lipid droplet fraction blot probed with secondary antibody only. Molecular weight markers are shown on the left (kDa).

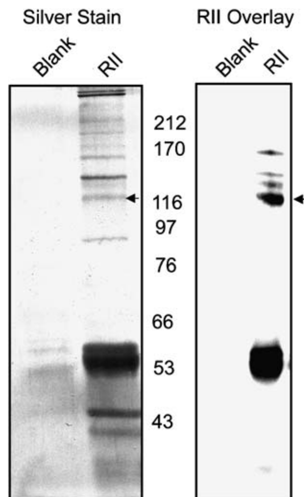

Fig. 4. Purification of AKAPs from rat adipose tissue by RII-affinity chromatography. Triton-solubilized rat adipose extracts were incubated with RII immobilized or control (blank) matrix. After extensive washing, proteins were eluted and visualized via silver staining (left) or RII overlay (right). The major RII binding protein band (120 kDa and indicated with an arrowhead) was excised and identified as D-AKAP1. Molecular weight markers are shown in the center (kDa).

proteins by adding 3% (w/v) Triton X-100 to the homogenization buffer to enhance the solubility of the p120 AKAP. This extract was applied to either RII immobilized onto Sepharose beads, or onto control (blank) beads with no protein coupled. After stringent washing, proteins were eluted with 1% SDS.

Fig. 4 shows that a number of proteins were specifically purified by the RII but not the blank beads. Overlay analysis of these fractions allowed for the major AKAP (p120) to be identified and excised for analysis by mass spectrometry. All peptides from the p120 protein were identified by tandem mass spectrometry to be D-AKAP1 (GenBank Accession No. NP44617). Mass spectrometry identified 2 peptides by MS–MS (220–234: SEFPILAPGGGGGEK and 237–247: SGPPQVDELLK) and a third peptide by MALDI-TOF (734–749: IEQTAFQIISQVILEEATEEIR). The position of the 734–749 peptide conclusively identified the longer 121 kDa splice variant rather than the shorter 84 kDa variant of this protein. The purified protein was the same size as the major protein detected by overlay analysis of fat droplet proteins using RII and PP1. These data suggest that AKAP84/121 is the major PKA and PP1 anchoring protein in adipocytes and that it is present in the lipid droplet enriched fraction of an adipocyte lysate.

Discussion

Lipid droplets are a unique and relatively poorly studied organelle. They exist in a number of tissues but are especially predominant in adipocytes. Several enzymes of triglyceride metabolism are present on the surface of the lipid droplet including perilipin, hormone-sensitive lipase, acyl-CoA synthase, and others [3]. We used a simple homogenization and centrifugation technique to separate the lipid fraction from the soluble fraction. We took advantage of the generalization that the floating lipid fraction contains the intracellular lipid droplets and their associated proteins, while the soluble fraction contains the cytosolic proteins. This technique has been used by a number of groups in order to examine the subcellular localization of a variety of adipocyte proteins [3,58–67]. It should be noted however that fractionation data themself do not indicate the presence of lipid droplet associated proteins, and other experiments will be needed to confirm the localization of these proteins.

In the case of PKA, the RIIβ subunit is present in both the lipid and soluble fraction. This distribution suggest that there are at least two populations of RIIβ-PKA in adipocytes, including a lipid associated subset and a soluble subset. This is consistent with the presence of a lipid droplet associated AKAP in adipocytes. In addition to the subcellular fractionation data, PKA localization is also supported by biochemical assays of lipid droplets. In the case of protein phosphatases, we found evidence of both PP1 and PP2A, as well as corresponding phosphorylase phosphatase activity in lipid droplet fractions. Since many protein phosphatases do not have activation mechanisms like protein kinases, the localization of these phosphatases is very important to the regulation of phosphatase activity. The localization of phosphatases is thought to be controlled by regulatory subunits. Our data therefore suggests that there may be lipid droplet localized PP1 and PP2A regulatory subunits. Since there are a number of phosphorylation targets on the lipid droplet, it is possible that both PP2A and PP1 could both be present physiologically.

The presence of RII in a lipid droplet fraction prompted us to examine the AKAPs present in adipose tissue. The most abundant of these proteins was a 120 kDa AKAP, which also co-migrated with an apparent PP1 binding protein on SDS–PAGE. Proteins were solubilized from lipid droplets by homogenization of cells in detergent and after purification of this 120 kDa protein and identification by mass spectrometry, the protein was identified as D-AKAP1. Based on the purification and on overlay analysis, this protein seems to be the major AKAP in adipocytes, so would be expected to play a major role in the regulation of PKA in this tissue.

The PKA regulatory subunit binding site is located at residues 303–322 of the rat AKAP121 sequence [68–70]. The AKAP identified in adipose tissue is able to bind RII *in vitro*. Furthermore, we showed that this protein was also able to bind PP1 *in vitro*. Human D-AKAP1 (known as AKAP149) binds PP1 via a K-G-V-L-F motif in the human isoforms [16,71]. The regulation of PKA in adipocytes by AKAP84/121 is important for a number of reasons. The co-localization of PP1 with PKA ensures site-specific tight regulation of PKA mediated phosphorylation events in adipose tissue. The discovery of AKAP84/121 as the major PKA and PP1 binding protein in adipocytes indicates that it likely plays a key role in the regulation of adipocyte

physiology. Our data also imply that D-AKAP1 may anchor PKA and PP1 to the surface of lipid droplets. The specific subcellular target of AKAP84/121 is unknown at this time. Since, the majority of isoproterenol stimulated phosphorylation events can be inhibited with Ht31 peptide [23], this suggests that most PKA events in adipocytes are AKAP directed. Our data show that AKAP84/121 is the major AKAP in adipocyte cells and is likely of key importance to the regulation of the activity of PKA in these cells.

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