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Proteomic analysis of cAMP-mediated signaling during differentiation of 3 T3-L1 preadipocytes

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ABSTRACT

Initiation of adipocyte differentiation is promoted by the synergistic action of insulin/insulin-like growth factor, glucocorticoids, and agents activating cAMP-dependent signaling. The action of cAMP is mediated via PKA and Epac, where at least part of the PKA function relates to strong repression of Rho kinase activity, whereas Epac counteracts the reduction in insulin/insulin-like growth factor signaling associated with complete repression of Rho kinase activity. However, detailed knowledge of the Epac-dependent branch and the interplay with PKA is still limited. In the present study, we present a comprehensive evaluation of Epac-mediated processes and their interplay with PKA during the initiation of 3 T3-L1 preadipocyte differentiation using a combination of proteomics, molecular approaches, and bioinformatics. Proteomic analyses revealed 7 proteins specifically regulated in response to Epac activation, 4 in response to PKA activation, and 11 in response to the combined activation of Epac and PKA during the initial phase of differentiation. Network analyses indicated that the identified proteins are involved in pathways of importance for glucose metabolism, inositol metabolism, and calcium-dependent signaling thereby adding a novel facet to our understanding of cAMP-mediated potentiation of adipocyte differentiation.

1. Introduction

The development of obesity is related not only to increased fat cell mass but also to increased fat cell number as the result of fat cell differentiation [1]. Much data on preadipocyte differentiation has been acquired from cell culture studies, where 3 T3-L1 and 3 T3-F442A mouse fibroblasts as well as mouse embryo fibroblasts have been used as models. Treatment of mouse fibroblasts with insulin/insulin-like growth factor and glucocorticoids like dexamethasone is sufficient for the induction of adipocyte differentiation [2,3]. However, inclusion of agents that elevate cellular concentrations of cAMP, such as isobutylmethylxanthine (IBMX) or forskolin, during the initial phase of differentiation, accelerates the differentiation process [2,3]. In the cytoplasm, cAMP activates protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). PKA attenuates RhoA GTP loading and Rho kinase activation, and this action has previously been shown to be crucial for adipocyte differentiation [3,4]. Rho kinase regulates insulin/insulin like growth factor receptor (IGFR) signaling pathway via phosphorylation of insulin receptor substrate (IRS). When Rho kinase is highly active, it phosphorylates serine residue 612 of IRS-1, which causes inhibition of IGFR signaling. On the other hand, at low activity, Rho kinase enhances IGFR signaling by phosphorylation of IRS-1 serine residues 632 and 635 [5,6]. Epac activates the G proteins Rap1 and Rap2, which induces important changes in cytoskeleton organization and cell adhesion [7]. We reported that activation of both PKA and Epac is required for differentiation of 3 T3-L1 preadipocytes and mouse embryo fibroblasts by employing the two cAMP analogues 8-pCPT-2′-O-Me-cAMP (007) and N6-monobutyryl cAMP (MB), which selectively activate Epac and PKA, respectively [3]. Similarly, we showed that activation of both PKA and Epac is also required for adipocyte differentiation of human multipotent adipose-derived stem cells [8].

Abbreviations: HMG-CoA synthase, hydroxymethylglutaryl-CoA synthase; IBMX, isobutylmethylxanthine; IGFR, insulin-like growth factor receptor; IPS 1, inositol-3-phosphate synthase 1; IRS, insulin receptor substrate; myo-inositol, 1D-myo-inositol 3-phosphate; PCB, pyruvate carboxylase; PGAM-B/M, phosphoglycerate mutase 1/2; PTAC97, importin subunit beta 1

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The aim of the present study was to examine the role of cAMP-mediated signaling via Epac and PKA in potentiation of differentiation of 3 T3-L1 preadipocytes using proteomics in combination with molecular approaches and network analyses. We show that expression of a limited number of proteins selectively is regulated in response to activation of PKA, Epac, or both during the initial stage of differentiation. Using a systems biology approach, we show that these proteins are involved in pathways regulating glucose metabolism, inositol metabolism, and calcium-dependent signaling adding novel information on cAMP-regulated processes accentuating initiation of adipocyte differentiation.

2. Materials and methods

2.1. Cell culture and differentiation

3 T3-L1 mouse fibroblasts were cultured up to the third passage before the start of differentiation. Cells were maintained and induced for differentiation as described [3]. In short, 3 T3-L1 cells were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM, Invitrogen™: Cat. No. 41966029) supplemented with 10% calf serum. Two-days postconfluent (designated as day 0) cells were induced to differentiate with DMEM supplemented with 10% fetal bovine serum (FBS), 1 μM dexamethasone (Sigma), 1 μg/ml insulin (Sigma), and 0.5 mM IBMX (Sigma). For analyses of the roles of PKA and Epac, IBMX was replaced with 200 μM of 8-pCPT-2′-O-Me-cAMP (Biolog) (007 treatment), 100 μM of N6-monobutyryl-cAMP (Biolog) (MB treatment), or 200 μM of 8-pCPT-2′-O-Me-cAMP + 100 μM of N6-monobutyryl-cAMP (007 + MB treatment). After 48 h, media were replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. Cells were subsequently refed every 48 h with fresh DMEM supplemented with 10% FBS. Cells were cultured up to day 8 of differentiation. At day 8, triglycerides present in differentiated cells were visualized by Oil-Red-O-staining [9].

2.2. Sample collection for two-dimensional gel analysis

Cells were washed three times with warm (37 °C) HANK's buffer and collected by scraping. After collection, cells were immediately frozen in liquid nitrogen. The cell lysates were prepared by the addition of lysis buffer (7 M urea, 2 M thiourea, 2% Chaps, 0.4% DTT, 0.5% pharmalyte 3–10, 0.5% IPG 6–11 buffer) and shaking overnight. Each sample was centrifuged at 1500 rcf for 15 min at 10 °C and the supernatants were transferred to new tubes. Protein concentration was determined by the Bradford method [10]. For the 48 h time point samples, cells from three independent experiments were analyzed. Results shown as supplementary data represent the results of one biological replicate.

2.3. Two-dimensional gel electrophoresis

To analyze changes in protein expression and/or modification during differentiation, samples collected at day 0, 8 h, 24 h, 48 h, and day 8 of differentiation were analyzed by two-dimensional (2D) gel electrophoresis.

2D gel electrophoresis was carried out as described [11]. Briefly, the first dimension 18 cm IPG strips, covering a pH gradient from 4 to 7 (IPG 4–7) (GE Healthcare: Cat. No. 17-1233-01) were rehydrated in two steps. First, strips were rehydrated overnight in 200 μl of sample diluted in lysis buffer (300 μg of protein per gel were loaded). Next, strips were rehydrated for 6 h in 100 μl of lysis buffer. Isoelectric focusing was performed using a linearly increasing profile: 0 V to 600 V for 2:15 h, 600 V to 3500 V for 8 h, and 3500 V for 9:25 h. After focusing, the strips were incubated in equilibration buffer (6 M urea, 2% SDS, 30% glycerol, 50 mM Tris–HCl pH 8.8, 1% DTT) for 15 min and then frozen at −80 °C. After thawing, gels were incubated in equilibration buffer again for 15 min. The second dimension SDS-PAGE was performed using the vertical electrophoresis system Protean II™ (BioRad) and 12.5% (w/v) laboratory-made acrylamide gels (acrylamide/*N,N′*-ethylene-bis-acrylamide ratio, 200:1). Gels were run overnight at 20 °C; 6 mA per gel were applied for the first 2 h of electrophoresis, then 12 mA per gel. The running buffer contained 0.67% Tris-Base, 1.44% of glycine, and 1% SDS. Additionally, buffer recirculation was applied. The gels were stained with Sypro Ruby (Invitrogen™: Cat. No. S11900) and scanned on a Typhoon scanner with an excitation wave length of 488 nm and emission filter at 610 nm. Gel images were analyzed using the DECODON Delta 2D software, version 3.6 (Decodon GmbH). In total, 1512 reproducible spots were detected on experimental gels. All detected spots were matched. The average standard deviation of 2D gels within the groups was smaller than 20%. All identifications were tested by Mascot scoring algorithm, where both MS and MS/MS results were used for score calculation. Only identification with $p < 0.05$ was considered significant. In cases when more than one protein reached the significance threshold, both were reported in the identification table. Additionally, theoretical p*I* and mass (according to Swiss-Prot database) of identified proteins have been compared to spots' position on the gel. Two diagrams were created: protein p*I* to *x* position on the gel (*x* position on the gel represents separation according to p*I* value) and protein mass to *y* position on the gel (*y* position on the gel represents separation according to protein mass). The trend line has been drawn on both diagrams. Spots, for which the position on the diagrams did not fit to the general trend, have been excluded from the results.

2.4. Mass spectrometry

2.4.1. In-gel digestion

Spots exhibiting significant changes were cut out from the gel. The gel plugs were washed with 100 μl of water for 5 min. Water was removed and gel plugs were washed with 100 μl of 100% acetonitrile (ACN) for 20 min. ACN was removed and gel plugs were dried in a Speed Vac. Dry gel plugs were rehydrated with 10 μl of trypsin solution (10 μg/ml, dissolved in 50 mM NH_4HCO_3; pH 7.8) for 20 min on ice. Unabsorbed trypsin was removed and the gel plugs were covered with 20 μl of 50 mM NH_4HCO_3 (pH 7.8). Digestion was carried out overnight at 37 °C.

2.4.2. Target loading

Desalting and concentration of peptides mixtures were done as described before [12]. In-house made microcolumns, prepared from GE Loader micropipette tips, packed with POROS R2 (Applied Biosystems, USA) were used. The columns were pre-washed with 10 μl of 0.1% trifluoroacetic acid (TFA). 10 μl of peptides mixture from the digested protein were loaded onto microcolumns. The columns were then washed with 10 μl of 0.1% TFA. Peptides were released from the column with 3 μl of 5 μg/μl α-cyano-4-hydroxycinnamic acid diluted in 70% ACN/0.1% TFA and eluted directly onto the MALDI plate. Trypsin digested β-galactosidase was used as a standard for instrument calibration and was placed on every third spot on the MALDI plate.

2.4.3. Sample analysis

A 4800 MALDI TOF/TOF Analyzer from Applied Biosystems was used for recording positive ion MS and MS/MS spectra. For MS analysis, the mass range from 700 to 3500 Da was selected. Total laser shots number was set to 800 and a fixed laser intensity of 3100 was used. For MS/MS analysis, the total number of shots was set to 1280. For each MS spectrum, at least three of the most intensive peaks were selected for MS/MS analysis. MS and associated MS/MS data were combined into a single mass list in the mgf. file format using an in-house script developed by Jakob Bunkenborg, University of Southern Denmark. Mass lists were searched in Swiss-Prot 57.7 (497,293 sequences; 175,274,722 residues) database using an in-house MASCOT server (Matrix Science Ltd., London, UK). The MASCOT server settings were: database, SwissProt; enzyme, trypsin; maximal number of missed cleavages, 1; significant threshold, $p < 0.05$; MS mass accuracy, 50 ppm; MS/MS, 0.6 Da; partial

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