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Induction of adipose conversion in 3T3-L1 cells is associated with an early phosphorylation of a protein partly homologous with mouse vimentin

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Induction of adipose conversion in 3T3-L1 by bezafibrate has been previously shown to be enhanced by dibutyryl cAMP and to be associated with an early phosphorylation of a 60 kDa acidic protein [Biochim. Biophys. Acta 1054 (1990) 219–224; FEBS Lett. 285 (1991) 63–65]. We describe here the isolation and sequencing of two peptides of the protein concerned. Both appear to be homologous with two respective amino acid sequences of the mouse intermediate filament protein vimentin.

Adipocyte differentiation; 3T3-L1; Bezafibrate; Protein phosphorylation; Vimentin

1. INTRODUCTION

Hypolipidemic aryloxyalkanoic acids of varying hydrophobic backbones (i.e. clofibric acid, bezafibrate) have been reported to induce adipose conversion in cultured 3T3-L1 cells as well as in primary rat epididymal preadipocytes similar in nature and extent to that induced by IBMX [1,2]. The induction of adipose conversion in 3T3-L1 cells by bezafibrate was found to be synergistic with but_2-cAMP, forskolin or theophylline. The synergistic effect required the simultaneous presence of the fibrate inducer and but_2-cAMP in the culture medium during the induction phase (usually 48 h), after which both could be replaced by an insulin-containing medium, allowing for the differentiated cells to develop gradually into mature adipocytes [3]. The earliest change observed during bezafibrate/but_2-cAMP- or IBMX-induced adipose conversion consisted of a phosphorylation of a 60 kDa acidic protein precipitating with the nuclear fraction [4]. The correlation observed between induction of adipose conversion and phosphorylation of a discrete protein by the concerned effectors might indicate that phosphorylation of the 60 kDa protein could be intrinsic to the inductive sequel of adipose conversion. The concerned protein has now been isolated and sequenced and found to be partly homologous with mouse vimentin.

2. MATERIALS AND METHODS

3T3-L1 cells were grown to confluence in 35 or 60 mm plates in DMEM supplemented with 10% FCS as previously described [2] and subsequently incubated for 2 h with [^{32}P]H_3PO_4 in a phosphate-free Tyrode solution supplemented with 0.4% fatty acid-free bovine serum albumin. Following equilibration of the intracellular pool of ATP with ^{32}P, the ^{32}P-labelled cells were incubated for an additional 2 h with 1 μM dexamethasone and either 300 μM bezafibrate, 50 μM but_2-cAMP, both or with 500 μM IBMX. The medium was then removed and the cells were washed with saline (4 × 1.0 ml). A nuclei-rich fraction was prepared by lysing the cultured cells in 1.5 ml/plate of a solution composed of 10 mM Tris-HCl buffer, pH 7.5, 5 mM $MgCl_2$, 25 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.5 mM Nonidet P-40, followed by centrifuging the cell lysate for 5 min at 500 × g as described by Zechner et al. [5] and Doglio et al. [6].

The nuclei-rich fraction derived from a 6 cm dish was either dissolved in 100 μl of 2D-PAGE loading buffer, composed of 9.3 M urea, 2% Nonidet P-40, 5% ampholines (pH 3–10) and 5% mercaptoethanol, and subjected to 2-dimensional gel electrophoresis, or was extracted at 0°C by 0.5 ml of an ice-cold extraction medium prepared according to Dignam [7] (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) or Nervi [8] (10 mM Tris-HCl, pH 8.5, 1.5 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 10% glycerol and 0.8 M KCl). The suspended precipitate was forced 10 times up and down a pipetor's plastic tip, vortexed for 10 min and left on ice for 30 min. The non-extractable residue was precipitated by a 5 min centrifugation at 4°C, 25,000 × g. The extracts were dialyzed at 0°C, against 2 × 1 l of H_2O, lyophilized and dissolved in the 2D-PAGE loading buffer. The non-extractable residue was similarly dissolved in the same buffer.

2D gel electrophoresis was carried out as described by O'Farrel [9], using molecular weight markers from an Amersham 'Rainbow' kit and pH markers from a Pharmacia 'Carbamylate' kit. For autoradiography, the gels were fixed in 10% (v/v), acetic acid/25% methanol (v/v) in water, dried and exposed to X-ray film at −70°C.

For sequencing, the 60 kDa protein was electroblotted for 20 h from the 2D gel to a PVDF membrane at a current density of 2 mA/cm^2 in 0.005% SDS and 10% methanol in × 0.5 Towbin buffer [10]. Enzymatic cleavage of the 60 kDa protein was carried out as described by Cleveland et al. [11] by applying the SDS-gel piece containing the protein onto another 15% SDS gel together with the SDS-resistant V8 protease. Following cleavage on the gel the resulting peptides were separated electrophoretically on the 15% SDS gel, and electroblotted to a PVDF membrane as described above but in the absence of added SDS.

Electroblotted proteins and peptides were identified on PVDF

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membranes by both autoradiography and Coomassie blue staining (5 min incubation with freshly prepared 0.025% Coomassie blue R250 in 40% methanol, followed by destaining in 50% methanol and finally in H_2O) and were semi-quantitated by comparing the color intensity with that of molecular weight markers. For microsequencing, the relevant PVDF pieces were cut out and pooled. Microsequencing was performed on a 475A protein sequencing system consisting of Model 470A gas-phase microsequencer equipped with an on-line phenylthiohydantoin analyzer, Model 120A and 900A data system (Applied Biosystems, Foster City, CA). The protein samples on PVDF were subjected to Edman degradation in a modified reaction cycle [12]. Conversion of the thiazolinone derivatives to phenylthiohydantoin-amino acids was carried out with 25% TFA. Phenylthiohydantoin-amino acid derivatives were separated by reversed-phase HPLC on a PTH C_{18} column (2.1 × 220 mm; Applied Biosystems) using sodium acetate buffer/tetrahydrofuran/acetonitrile gradient [13]. A computer search was carried out in 'Swiss-Prot' and 'PIR-Protein' data bases, using GCG package, version 7, of Genetic Computer Group, Madison, Wisconsin.

For immunoblotting, the 60 kDa protein was similarly electroblotted from the 2D gel to a nitrocellulose membrane in 20% methanol. Immunoblot assays were carried out essentially as described by Maniatis [22] using either polyclonal goat anti human vimentin antibodies (Sigma) or monoclonal mouse anti human vimentin antibodies (Vim-13.2, Sigma). The respective first antibody was detected with an anti-goat or an antimouse polyvalent antibody conjugated to alkaline phosphatase (Sigma). 3% BSA was used for blocking.

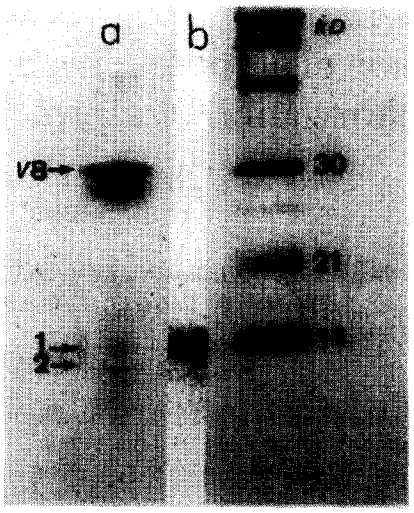

Fig. 1. V8 cleavage of the 60 kDa ^{32}P-labelled protein. The 60 kDa ^{32}P-labelled protein cleaved by V8 protease and electrophoresed on a 15% SDS gel as described in Section 2. Peptides were detected by Coomassie blue staining (a) as well as by radioactivity (b) monitored by phosphorimaging (Fuji). Peptides 1 and 2 are denoted by arrows.

3. RESULTS

The 60 kDa protein to be analyzed was identified by 2D SDS-PAGE of the nuclei rich fraction of $^{32}P_i$-labelled bezafibrate/bu_2-cAMP- or IMBX-induced 3T3-L1 cells as previously described (Fig. 2, ref. 4). The 60 kDa protein could not be extracted from the nuclei-rich fraction by standard procedures for preparing nuclear extracts using extraction media described by Dignam et al. or Nervi et al. (containing 0.42 or 0.80 M NaCl, respectively) [7,8]. It was however fully recovered in the non-extractable nuclear residue. For sequencing, 50–100 pmol of the 60 kDa protein were electroblotted from the 2D SDS-PAGE to a PVDF membrane and subjected to automated Edman degradation. No signal was however generated after the first 10 cycles, indicating that the amino end of the isolated protein was presumably blocked. To overcome this difficulty, the protein was enzymatically cleaved into peptides separated by 15% SDS-PAGE prior to microsequencing, as described in Section 2. Two resulting peptides were subjected to microsequencing, a radioactive one of 14 kDa (peptide 1) and a shorter one (peptide 2) (Fig. 1). Amino acid sequences found at the NH_2 termini of peptides 1 and 2, respectively, were as follows:

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                  ↓   ↓ ↓
Peptide 1:  L-Q-E-L-N-D-A-F-M-R-Y-I-D-K-V-R-F
Peptide 2:  N-F-A-L-E-A-A-N-Y-Q-D-T
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Peptide 1 sequence was best matched with the stretch of amino acids Nos. 106–123 of mouse vimentin (mismatches are marked by arrows). Peptide 2 sequence was similarly found to fully match the stretch of amino acids 348–360 of mouse vimentin. Both vimentin sequences were found to be preceded by glutamic acid as expected from the specificity of protein cleavage by V8. Peptide 1 was also found to be partly homologous with a sequence (a.a. Nos. 35–50) of mouse lamin and the 'QELND' motif could be identified within several other structural proteins, such as desmin and periferin. The sequence of peptide 2, however, could be identified within vimentin only but not within lamin or any of the other proteins.

The homology of the 60 kDa protein with vimentin was further confirmed by reacting the blotted 2D SDS gel with either polyclonal or monoclonal anti-vimentin antibodies (Fig. 2). Both were found to specifically react with the concerned ^{32}P-labelled protein.

4. DISCUSSION

In addition to our sequencing and immunoblotting results, some other known properties of vimentin agree with the characteristics of the 60 kDa protein: (i) Vimentin is a cytoskeleton protein, being non-extractable by salt or detergent, which precipitates with the nuclear fraction under conditions of cell fractionation as employed here [14]; (ii). Its reported molecular weight (54–58 kDa) and PI (5.2) [14,20] agree with the respective characteristics of the 60 kDa protein [4]; (iii) vimentin's reported susceptibility to N-acetylation [15] is in line with the blocked N-terminal end of the 60 kDa protein as reported here.

Phosphorylation and dephosphorylation of vimentin have been well documented and suggested to be involved in cytoskeleton reorganization associated with processes of cell division and differentiation [15–18].

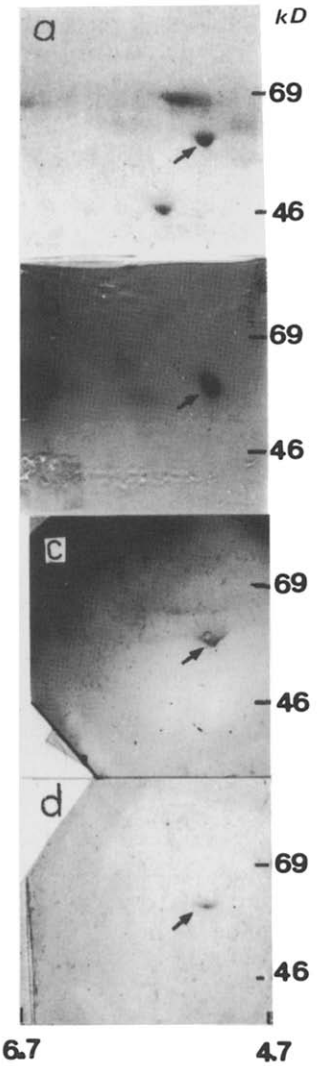

Fig. 2. Immunoblotting the 60 kDa ^{32}P-labelled protein with antivimentin. Nuclear proteins from ^{32}P-labelled bezafibrate/But$_2$-cAMP-induced cells were electrophoresed by 2D SDS-PAGE, as described in Section 2. (A) Coomassie staining; (B) autoradiogram; (C) immunoblot with polyclonal antivimentin antibodies; (D) Immunoblot with monoclonal antivimentin antibodies. The arrows point to the relevant protein.

Phosphorylation of vimentin in the course of adipocyte maturation could thus initiate its rearrangement in cells induced for lipid accumulation. It has indeed been reported by Franke et al. [19] that rearrangement of intermediate filament proteins, including vimentin, is involved in the formation of the fat droplet protein membrane during adipose conversion of 3T3-L1 cells. Furthermore, phosphorylation and dephosphorylation of protein components of the 'lipid droplet cage', including vimentin and perilipin, have recently been suggested by Londos et al. [20] to be involved in regulation of lipolysis in mature adipocytes. It is noteworthy that a protein encoded by pOb24 previously reported as an early marker of adipose converion, has recently been identified to be homologous with the α chain of collagen type VI [21], thus indicating that the expression, modification and rearrangement of structural proteins may comprise some of the earliest events in the course of adipose conversion.

In spite of the apparent ^{32}P-labelling of peptide 1 (Fig. 2), the site of bezafibrate/cAMP-induced phosphorylation of 3T3-L1 vimentin may not be inferred from studies reported here. An RKXXS protein kinase A motif which could serve as a potential site for phosphorylation [23] may indeed be identified within the 14 kDa peptide 1 (a.a. 221–225). It should be noted however, that all previously reported protein kinase A-phosphorylation sites of vimentin are rather located within the stretch of the first 70 amino acids starting at the blocked NH_2-terminus while the RKXXS site at a.a. 221–225 has not been previously reported to be phosphorylated by protein kinase A in vitro [24] or in vivo [25]. Alternatively, the radioactivity co-electrophoresed with peptide 1 following V8 cleavage could perhaps be accounted for by an additional 14 kDa peptide derived from the first ~ 140 a.a. of vimentin and which co-electrophoresed with a non-labelled 14 kDa peptide 1. Being blocked in its NH_2-terminus, this additional peptide could not interfere with the sequencing of peptide 1 but still may account for ^{32}P-labelling of vimentin induced by bezafibrate/cAMP.

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