Mammalian 5'-AMP-activated Protein Kinase Non-catalytic Subunits Are Homologs of Proteins That Interact with Yeast Snf1 Protein Kinase*

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The 5'-AMP-activated protein kinase is responsible for the regulation of fatty acid synthesis by phosphorylation and inactivation of acetyl-CoA carboxylase. The porcine liver 5'-AMP-activated protein kinase 63-kDa catalytic subunit copurifies 14,000-fold with a 38- and 40-kDa protein (Mitchelhill, K. I. et al. (1994) J. Biol. Chem. 269, 2961–2964). The 63-kDa subunit is homologous to the Saccharomyces cerevisiae Snfl protein kinase, which regulates gene expression during glucose derepression. Peptide amino acid and polymerase chain reaction-derived partial cDNA sequences of both the pig and rat liver enzymes show that the 38-kDa protein is homologous to Snf4p (CAT3) and that the 40-kDa protein is homologous to the Siplp/Spm/GAL83 family of Snflp interacting proteins. Sucrose density gradient and cross-linking experiments with purified 5'-AMP-activated protein kinase suggest that both the 38- and 40-kDa proteins associate tightly with the 63-kDa catalytic polypeptide in either a heterotrimeric complex or in dimeric complexes. The 40-kDa subunit is autophosphorylated within the 63-kDa subunit complex. The sequence relationships between the mammalian 5'-AMP-activated protein kinase and yeast Snf1p extend to the subunit proteins consistent with conservation of the functional roles of these polypeptides in cellular regulation by this family of metabolite-sensing protein kinases.

The 5'-AMP-activated protein kinase phosphorylates and inhibits the rate-limiting enzymes in the fatty acid and sterol synthesis pathways (acetyl-CoA carboxylase and HMG-CoA reductase, respectively). This regulation, as well as the observation that hormone-sensitive lipase is also a substrate for the kinase, has led to the concept that this kinase plays a coordinating role in the control of lipid metabolism (reviewed in Ref. 1). The regulation of lipid metabolism by the 5'-AMP-activated protein kinase is most significant under conditions of cellular stress, such as metabolic fuel limitation, ATP depletion, and heat shock (2–6). The phosphorylation of hydroxymethylglutaryl-CoA reductase and acetyl-CoA carboxylase has been recognized for several years (1, 7, 8). Recently, sufficient quantities of purified porcine 5'-AMP-activated protein kinase were obtained to provide partial amino acid sequence information (9) that revealed a striking amino acid sequence homology between the 63-kDa subunit of the porcine 5'-AMP-activated protein kinase and the yeast Snf1 protein kinase subfamily (9). The complete cDNA sequence of a rat homolog of the porcine 5'-AMP-activated protein kinase, which also is homologous to Snf1p, has recently been reported (10). The 5'-AMP-activated protein kinase and Snf1 protein kinase share functional properties; both kinases phosphorylated and inactivated yeast acetyl-CoA carboxylase (9). Additional support for this relationship has now been provided by Wood et al. (11), who found that glucose limitation in Saccharomyces cerevisiae was accompanied by parallel activation of Snf1p and inactivation of yeast acetyl-CoA carboxylase. This finding in yeast also parallels previous observations in mammalian cells, where glucose depletion leads to apparent activation of the 5'-AMP-activated protein kinase (5).

Snf1 protein kinase was initially identified because it was required for the expression of glucose derepressible genes (12) and interacts with a number of proteins. Snf4p (CAT3) is physically associated with Snf1p (9, 13–15) and co-purifies with it (see below). Using the two hybrid system, Carlson and her colleagues (16, 17) have identified other Snflp interacting proteins including Siplp, Sip2p (also referred to as Spm2p) and sip3 (16, 17). Sip1p is a substrate for the Snf1 protein kinase in vitro (16).

In view of the close structural and functional relationship between the 5'-AMP-activated protein kinase and Snf1 protein kinase, it was of interest to investigate whether the 38- and 40-kDa proteins that copurified with the 63-kDa catalytic subunit of the 5'-AMP-activated protein kinase were related to these Snf1p-interacting proteins. In the present study, we report that, based on both peptide and PCR2-derived cDNA sequences of pig and rat liver enzymes, the 38-kDa protein is a mammalian homolog of Snf4p (CAT3) and the 40-kDa subunit is a homolog of Sip1p.

**EXPERIMENTAL PROCEDURES**

Enzyme Purification—The 5'-AMP-activated protein kinase was purified from porcine liver and rat liver as described previously (9).

Peptide Sequencing—The 5'-AMP-activated protein kinase preparations were run on SDS-PAGE (7.5–16% gradient gel) that had been polymerized for at least 8 h. Gels were stained with Coomassie Blue, destained in acetic acid/methanol/water (7/12.5/81.5, v/v), and then washed extensively in water. Bands for sequencing were excised from the gel and processed as described previously (9). Either Asp-N endoproteinase or trypsin peptides were purified with a Brownlee RP-300 C8
reversed phase column (2.1 x 250 mm) using a Hewlett-Packard 1090 high pressure liquid chromatograph and developed with a standard 0.1% trifluoroacetic acid (v/v) to 60% CH$_3$CN (v/v) in 0.085% trifluoroacetic acid gradient at 100 µl/min over 60 min. Peaks were monitored at 214 nm and collected manually. Each peak was rechromatographed on a Hypersil ODS column (5 µm, 2.0 x 100 mm), and the peaks were N-terminally sequenced on either an Applied Biosystems 471A protein sequencer or a Hewlett-Packard G1000A protein sequencer.

PCR-derived cDNAs for 38- and 40-kDa Subunits—Peptide sequences for the 38- and 40-kDa proteins (see Figs. 2a and 3) were used to generate a series of degenerate oligonucleotides for reverse transcriptase-PCR. In brief, cDNA was synthesized from either pig or rat liver RNA using either oligo(dT)$_6$-dT$_12$ or peptide-derived degenerate primers and reverse transcriptase. The cDNA was then amplified with selected degenerate primers (and nested primers), chosen by preliminary partial alignment of peptide sequences with the homologous yeast proteins (see “Results”). The major products were separated on ethidium bromide-agarose gels, the bands cut out, glass wool purified, and cloned using either TA cloning (Novagen) or PCR-Script (Stratagene). Plasmid DNA was prepared from positive clones and sequenced using dideoxynucleotides and Sequenase (U. S. Biochemical Corp.) as recommended by the manufacturer.

Sucrose Density Gradients—Five samples were analyzed on sucrose density gradients: catalase (500 µg), bovine serum albumin (500 µg), recombinant α-casein kinase II subunit, casein kinase II (α$_B$,β), and purified 5'-AMP-activated protein kinase with or without inclusion of 100 µM 5'-AMP in the gradient. Each sample was made to 100 µl and applied to a 10-ml 5–20% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and 300 mM NaCl, the latter to prevent casein kinase II from aggregating. Centrifugation was performed (100,000 × g, 19 h, 4 °C) in a Beckman SW 28 rotor. Fractions (400 µl) were collected and assayed appropriately. Catalase and bovine serum albumin were measured with the Coomassie Blue dye binding assay (18), and casein kinase II α-subunit, casein kinase II holoenzyme, and 5'-AMP-activated protein kinase were assayed in the presence of their appropriate peptide substrates (9, 19).

Protein Cross-linking—Purified 5'-AMP-activated protein kinase (1.8 µg) was incubated with increasing concentrations of the cross-linker bis(sulfosuccinimidyl)suberate (BS$_3$) made in H$_2$O, in a final volume of 750 µl at room temperature for 30 min. The reactions were quenched with 100 µl of 1 x glycine. Each sample was trichloroacetic acid-precipitated (6% v/v) and run on SDS-PAGE (7.5–17% gradient gel). Protein bands were visualized by Coomassie Blue staining.

Kinase Autophosphorylation—Prior to autophosphorylation, 14 µg of pure 5'-AMP-activated protein kinase was desalted on a PD-10 gel filtration column. We observed that the phosphorylation of the 40-kDa subunit was sensitive to the salt concentration and therefore necessitated a desalting step. Varying concentrations of 5'-AMP-activated protein kinase were incubated in a final volume of 1.4 ml in the presence of 20 µM (γ-32P)ATP (1,500 cpm/pmol) for 30 min at 37 °C. Reactions were stopped with trichloroacetic acid precipitation, and the resuspended pellet was run on SDS-PAGE (7.5–17% gradient gel). The gel was stained with Coomassie Blue, dried, autoradiographed, and quantitated by liquid scintillation counting. Results were plotted as activity per mass of 5'-AMP-activated kinase protein as a function of the kinase concentration (M, 100,000).

RESULTS AND DISCUSSION

Porcine liver 5'-AMP-activated protein kinase was purified 14,000-fold using three chromatographic steps that included a peptide substrate affinity column (9). The 63-kDa catalytic subunit, which is related to the yeast Snfl protein kinase, co-purified with two other proteins of 38 and 40 kDa as assessed by SDS-PAGE (Fig. 1a). Tryptic peptide maps of each protein were different from the 63-kDa subunit, indicating that they were not partial proteolysis products of it (results not shown). Snfl protein kinase interacts with a variety of other proteins. When Snflp is purified from bakers' yeast, it co-purifies with Snf4p (CAT3) (Fig. 1a) (9) and can be immunoprecipitated with both Snf4p (CAT3) and Sip1p from yeast extracts (14, 17). We found that Sip1p (M, 96,000) did not copurify with Snfl protein kinase from bakers' yeast using nickel-agarose chromatography (Fig. 1a) (9).

The rat and porcine 38- and 40-kDa subunits isolated by SDS-PAGE were digested with trypsin or Asp-N endoproteinase and sequenced on either an Applied Biosystems 471A protein sequencer or a Hewlett-Packard G1000A protein sequencer.
GAL83, Sip2p, and Sip1p that are not conserved in the 40-kDa subunit. It seems probable that the 90-residue region containing the invariant residues will represent a conserved structural motif common to this family of proteins. The PCR-derived cDNA sequence permitted us to align the peptide amino acid sequences that covered essentially its entire length and provided compelling evidence that the cDNA sequence is correct and that of an isoform or other member of the GAL83 family (Fig. 2a). The cDNA-derived sequences of the pig and rat cDNA and/or peptide sequences. a, comparison of 40-kDa subunit, GAL83, Sip2p, and Sip1p. Shown is the alignment of the rat 40-kDa protein to Sip2p (123-2491), GAL83 (120-2471), and Sip1p (446-622). The sequences, aligned with the Filep program (CCG, University of Wisconsin), were formatted by an Excel macro with the residues identical to the 40-kDa protein sequence shaded (22).

Fig. 2. a, comparison of rat and pig liver 40-kDa subunit sequences. Peptide sequences were obtained as described under "Experimental Procedures." The peptide sequences were aligned according to the cDNA-derived sequence. Peptide amino acid (aa) sequences are underlined. Phenylthiohydantoin-amino acids with equimolar identification are notated X. The shaded residues in the partial cDNAs are identical between pig and rat cDNA and/or peptide sequences. b, comparison of 40-kDa subunit, GAL83, Sip2p, and Sip1p. The shaded residues in the partial cDNA sequences are identical between pig and rat cDNA and/or peptide sequences. a, comparison of 40-kDa subunit, GAL83, Sip2p, and Sip1p. Shown is the alignment of the rat 40-kDa protein to Sip2p (123-2491), GAL83 (120-2471), and Sip1p (446-622). The sequences, aligned with the Filep program (CCG, University of Wisconsin), were formatted by an Excel macro with the residues identical to the 40-kDa protein sequence shaded (22).

100 ± 20 kDa reported for the rat enzyme using gel chromatography on Superose-12 (21). Addition of the masses of the three 5'-AMP-activated protein kinase subunits determined by SDS-PAGE in the presence of reducing agent gives a total of 141 kDa, substantially higher than the value for a complex obtained from sucrose density gradient centrifugation. The apparent mass of the 40-kDa protein obtained from SDS-PAGE is 37 kDa when run in the absence of reducing agent (data not shown); there is still not good agreement between the two methods of estimation of the complex mass. If the values obtained by SDS-PAGE are overestimates, then there is a heterotrimeric kinase complex consisting of the 63-, 40-, and 38-kDa proteins. Alternatively, the 5'-AMP-activated protein kinase complex could consist of a nearly equal mixture of dimers of 63- and 40-kDa subunits together with dimers of 63- and 38-kDa subunits, but we consider this less likely.

Further evidence in favor of the concept that all three proteins are part of a complex (or complexes) was obtained by cross-linking experiments, where it was found that increasing concentration of cross-linking agent BS² caused the disappearance of all three proteins and the appearance of an approximate 200-kDa species (Fig. 4b). At intermediate concentrations of cross-linker, a 120-kDa species was apparent but disappeared at higher concentrations of the cross-linker. These cross-linking experiments were done at low protein concentrations to favor intramolecular cross-linking. We were unable to use sedimentation equilibrium analysis because attempts to concentrate the purified 5'-AMP-activated protein kinase (in 2 M NaCl, 30% ethylene glycol-containing buffer) and transfer it into a suitable buffer were not successful with the amounts of enzyme available. Whether heterotrimer or mixtures of heterodimers, it seems reasonable to conclude that the 40- and 38-kDa proteins can be regarded as kinase subunits. Recently the rat liver enzyme has been purified by ATP-γ-Sepharose chromatography (22), and the 63-kDa catalytic subunit was reported to copurify with 38- and 35-kDa proteins consistent with earlier observations with purified porcine liver 5'-AMP-activated protein kinase (9). Using glycerol density gradients the mass of the rat liver enzyme was estimated to be 190 ± 10 kDa (22). While somewhat higher than their earlier estimate of 100 kDa (21) it is nevertheless apparent that the enzyme most likely exists as a heterotrimer although a mixture of heterodimers cannot be unequivocally excluded. As a point of nomenclature, we propose that the catalytic subunit (63 kDa) be designated the α subunit, the 40-kDa protein the β subunit, and the 38-kDa protein the γ subunit of the mammalian 5'-AMP-activated protein kinase.
the alignment of Snf4p and the rat partial cDNA-derived 38-kDa subunit sequence with the aligned peptide amino acid sequence from both pig and rat enzymes. Residues identical to the 38-kDa cDNA-derived sequence are shaded. Homology between the 38-kDa Drotein and Snf4 protein extends over the entire length of Snf4p.

SIP2 null mutations or any pairwise combinations do not affect regulation of the GAL1 gene, it has been suggested that the three genes are redundant and that additional members of the family remain to be identified (20). It seems reasonable that there will be corresponding multiple counterparts in mammalian cells, but they may not necessarily all associate with protein kinases. Active Snf1p/Snf4p protein kinase complex isolated from baker's yeast does not contain detectable amounts of a 96-kDa protein (Fig. 1a) corresponding to Sip1p, indicating that Sip1p is not essential for enzyme activity (9), nor does the Snf1p/Snf4p complex co-purify with any other proteins in stoichiometric amounts. However, our purification strategy for Snf1p/Snf4p relies on nickel-agarose binding to the polyhistidine tail of Snf1p, so that any proteins associating with Snf1p via this region may not be recovered. It seems reasonable that the 40-kDa subunit of the 5'-AMP-activated protein kinase may not be essential for enzyme activity, although it is clearly tightly bound. Although the 40-kDa subunit is a substrate for the 63-kDa catalytic subunit of the 5'-AMP-activated protein kinase complex it can nevertheless be purified by the peptide substrate affinity column in the presence of 500 mM NaCl (9).

In summary, this study has shown that mammalian liver cells contain a family of proteins involved in the regulation of lipid metabolism that are homologous to the Snf1p, Snf4p (CAT3), and GAL83 yeast proteins. This opens up a wide vista of opportunities for studying their structure/function relationships, physiological roles, and their genes.

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