Mammalian AMP-activated Protein Kinase Subfamily*

(Received for publication, October 23, 1995)

David Stapleton, Ken I. Mitchelhill, Guang Gao†, Jane Widmer†, Belinda J. Micheli, Trazel Teh, Colin M. House, C. Shamala Fernandez, Timothy Cox§, Lee A. Witters§, and Bruce E. Kemp†

From St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia and the Endocrine Metabolism Division, Dartmouth Medical School, Hanover, New Hampshire 03755-3833

The mammalian 5'-AMP-activated protein kinase (AMPK) is related to a growing family of protein kinases in yeast and plants that are regulated by nutritional stress. We find the most prominent expressed form of the hepatic AMPK catalytic subunit (α) is distinct from the previously cloned kinase subunit (α2). The α1 (548 residues) and α2 (552 residues) isoforms have 90% amino acid sequence identity within the catalytic core but only 61% identity elsewhere. The tissue distribution of the AMPK activity most closely parallels the low abundance 6-kilobase α1 mRNA distribution and the α1 immunoreactivity rather than α2, with substantial amounts in kidney, liver, lung, heart, and brain. Both α1 and α2 isoforms are stimulated by AMP and contain noncatalytic β and γ subunits. The liver α1 isoform accounts for approximately 94% of the enzyme activity measured using the SAMS peptide substrate. The tissue distribution of the α2 immunoreactivity parallels the α1 8.5-kilobase mRNA and is most prominent in skeletal muscle, heart, and liver. Isoforms of the β and γ subunits present in the human genome sequence reveal that the AMPK consists of a family of isoenzymes.

The 5'-AMP-activated protein kinase (AMPK) was initially identified as a protein kinase regulating hydroxymethylglutaryl-CoA reductase (1). Subsequently, the AMPK was shown to phosphorylate hepatic acetyl-CoA carboxylase (2) and adi-pose hormone-sensitive lipase (3). The AMPK appears to act as a metabolic stress-sensing protein kinase switching off biosynthetic pathways when cellular ATP levels are depleted and when 5'-AMP rises in response to fuel limitation and/or hypoxia (4). Partial amino acid sequencing of hepatic AMPK (5, 6) revealed that it consists of 3 subunits, the catalytic subunit (63 kDa), and two noncatalytic subunits, β (40 kDa) and γ (38 kDa).

The AMPK is a member of the yeast SNF1 protein kinase subfamily that includes protein kinases present in plants, nematodes, and humans (5–9). The AMPK catalytic subunit, α, has strong sequence identity to the catalytic domain of the yeast protein kinase SNF1, which is involved in the induction of invertase (SU2) under conditions of nutritional stress due to glucose starvation (10). Both Snf1p and the AMPK require phosphorylation by an activating protein kinase for full activity (11). The sequence relationship between Snf1p and AMPK led to the finding that these enzymes share functional similarities, both phosphorylating and inactivating yeast acetyl-CoA carboxylase (5, 11, 12). Nevertheless, the AMPK does not complement SNF1 in yeast (11), indicating that their full range of functions are not identical. The noncatalytic β and γ subunits of AMPK are also related to proteins that interact with Snf1p; the β subunit is related to the SIF1/SIF2/GAL83 family of transcription regulators and the γ subunit to SNF4 (also called CAT3) (6, 13).

Experimental Procedures

Peptide Sequencing—Peptides were derived from rat and porcine α1 subunit of the AMPK, by in situ proteolysis (5), and sequenced on either an Applied Biosystems 471A Protein Sequencer or a Hewlett Packard G1000A Protein Sequencer.

Tissue Distribution—Activity Studies—A 35% saturated ammonium sulfate fraction was prepared for each tissue, following homogenization in AMPK homogenization buffer (HB, 50 mM Tris-HCl, pH 8.5, 250 mM sucrose, 5 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM EGTA, 1 mM EDTA, 1 mM diithothreitol, 1 mM benzamidine, 1 μM pyrabine trypan inhibitor, and 0.2 mM phenylmethylsulfonyl fluoride). The resultant pellet was resuspended in 5 ml of HB and assayed for protein concentration (14). The AMPK was assayed as described previously (5) with the following modifications: a final reaction volume of 120 μl was used, enzyme aliquots (30 μl) containing 1 μg of protein predicted in 50 μl Tris-HCl, pH 7.5, and 0.05% (v/v) Triton X-100 were used to initiate the reaction. Three aliquots (30 μl) were removed after 2, 4, and 6 min. Reactions were performed in duplicate ± 5'-AMP (200 μM), with a minus peptide substrate control. The specific activity of the enzyme was determined using linear rates of phosphorylation with the specific synthetic peptide substrate SAMS (15). The AMPK was purified from rat or primate liver as described previously using substrate affinity chromatography (5).

Isolation of AMPK cDNA—A radiolabeled cDNA (774 base pairs) encoding porcine AMPK α1 was used to screen a rat hypothalamus Zap II cDNA library (Strategen) according to the manufacturer’s instruc- tions. Positives were plaque-purified on subsequent rounds of screening, and phagemid from positive clones were rescued with helper phage (Stratagene). Screening of 7 × 104 plaques yielded three unique clones, the largest consisting of an open reading frame, corresponding to AMPK α1 (2–549). The AMPK α1 5′ end was isolated using a 5′-rapid amplification cDNA ends kit (Life Technologies, Inc.) with an α1-specific primer to residues 41–48 and rat liver cDNA.2 Human AMPK α1 (14–270) was isolated from fetal human liver cDNA primed with sense and antisense partially degenerate oligonucleotides to α1, peptide sequence by reverse transcription-polymerase chain reaction.3 Human AMPK α1, 2 D. Stapleton, K. I. Mitchelhill, G. Gao, J. Widmer, B. J. Micheli, T. Teh, C. M. House, C. S. Fernandez, T. Cox, L. A. Witters, and B. E. Kemp, manuscript in preparation.

3 D. Stapleton, T. Teh, T. Cox, and B. E. Kemp, unpublished data.
residues 291–448, is a partial length human liver cDNA clone obtained from the Lawrence Livermore National Laboratory (clone 78297, accession number T50799).

Northern Blotting—A rat multiple tissue Northern (MTN) blot (Clontech) containing 2 μg of poly(A)^+ RNA of individual tissues was probed with ^32P-labeled rat AMPKα1 and α2 cDNAs according to the instructions supplied.

Production of Anti-AMPK Antibodies—Polyclonal antibodies to AMPKα1 and α2 were prepared as follows. Peptides based on the predicted amino acid sequences of AMPKα1 for residues 339–358 (DFYLATSPPDSFLDDHHLTR) and AMPKα2 for residues 352–366 (MDDSAMHIPPGLKPH) were synthesized and coupled to keyhole limpet hemocyanin (Sigma, H-2133) via a cysteine residue added to the N terminus of the peptide using the heterobifunctional reagent, N-succinimidyl-3-(2-pyridyldithio)propionate (Pharmacia, Uppsala, Sweden). New Zealand White rabbits were immunized with 2 mg of peptide conjugate initially in 50% (v/v) Freund's complete adjuvant and in 50% (v/v) Freund's incomplete adjuvant for subsequently immunizations. Rabbits were boosted fortnightly with 2 mg of peptide conjugate and bled 7 days after booster injections. Anti-AMPKα1 and α2 peptide antibodies were purified by peptide affinity chromatography.

Western Blotting—Multiple rat tissue Western blots were prepared as follows. Rat tissues were homogenized in AMPK HB (see above), and a 2.5–7% polyethylene glycol 6000 fraction was prepared. The resultant pellet was resuspended in 5 ml of HB and assayed for protein concentration (14). 100 μg of each tissue fraction was analyzed by SDS-PAGE (13% acrylamide gels) (16), transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany), and probed with ^32P-labeled rat AMPKα1 and α2 antibodies, respectively. Primary antibody was detected using anti-rabbit IgG antibody conjugated to horseradish peroxidase (DAKO, Carpenteria, CA) and 0.032% 3,3'-diaminobenzidine (D-5637, Sigma) together with 0.064% H2O2.

Purification of AMPKα2—Affinity-purified AMPKα2 antibody (2 mg) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The unbound fraction from the substrate affinity column was applied directly to the AMPKα1 antibody column, washed with 5 volumes of phosphate-buff- ered saline, and eluted with 200 mM glycine buffer, pH 2.5, and immediately neutralized.

RESULTS

In the course of sequencing the porcine AMPKα, we found that the amino acid sequence of some peptides derived from the pig liver AMPKα subunit did not match those deduced from the rat liver cDNA sequence (7, 8). Therefore, the rat liver AMPK catalytic subunit, α1, was purified, and peptides accounting for 40% of the protein were sequenced (222/548 residues, Fig. 1). Eight of the sixteen peptides contained mismatched residues with the reported AMPK cDNA sequence, but did match the pig liver enzyme sequence. Using reverse transcription-polymerase chain reaction and cDNA library screening, we obtained cDNA sequence of the rat hypothalamus enzyme that accounted for all of the peptide sequences of the purified rat liver AMPK catalytic subunit containing mismatches (Fig. 1). The cDNA sequence of this AMPK catalytic subunit has been named α2, since it corresponds to the purified enzyme and is clearly derived from a gene different from the previously cloned α1 sequence (now referred to as α1). The α1 isoform of the AMPK catalytic subunit accounts for approximately 94% or more of the SAMS peptide phosphotransferase activity of rat liver and is therefore the predominant expressed hepatic isoform. Despite sequencing multiple preparations of the AMPK catalytic subunit from both pig and rat liver, no peptides were obtained that matched the α2 isoform sequence.

Within the catalytic cores of the α1 and α2 isoforms, there is 90% amino acid identity but only 61% identity outside the catalytic core (Fig. 1). Strong homology between the α1 and α2 sequences in the vicinity of the substrate binding groove, inferred from the protein kinase crystal structure for positions P–5 to P–1 (17), suggest that the substrate specificities will be related. The substrate anchoring loop (also called the lip or activation loop) contains an insert Phe-Leu170 for α1, since it corresponds to the purified enzyme and is clearly derived from a gene different from the previously cloned α1 sequence (now referred to as α1). The α1 isoform of the AMPK catalytic subunit accounts for approximately 94% or more of the SAMS peptide phosphotransferase activity of rat liver and is therefore the predominant expressed hepatic isoform. Despite sequencing multiple preparations of the AMPK catalytic subunit from both pig and rat liver, no peptides were obtained that matched the α2 isoform sequence.
The α2 8.5-kb mRNA is most abundant in skeletal muscle with lower levels in liver, heart, and kidney as reported recently (8, 18). In contrast, there were very low levels of the α1 6-kb mRNA in all tissues examined except testis, where a low level of 2.4-kb mRNA was observed (Fig. 2A). A testis-specific kinase related to Snf1p has been reported (19), but the corresponding transcript is 1.6 kb and may not be related to the 2.4-kb transcript seen here. The low levels of α1 mRNA explains why α1 was more difficult to clone than the α2 isoform (Fig. 2B). Northern blot analysis of the β and γ subunits revealed a complex pattern of expression. The β subunit mRNA was least abundant with similar levels across a range of tissues except brain, whereas the γ subunit mRNA was abundant in heart, lung, skeletal muscle, liver, and kidney. An earlier report on the tissue distribution of the AMPK activity had claimed that it was predominantly a liver enzyme (15). In view of the mRNA distribution of the α1 and β subunits, we reassessed the tissue distribution of the AMPK activity. The kidney contained the highest specific activity with similar levels in the liver, lung, and heart (Fig. 3) and little, if any, activity in skeletal muscle. It is clear that the AMPK activity has a wider tissue distribution than appreciated heretofore (15), and this closely parallels the distribution of α1 mRNA and not that of α2 mRNA. Using peptide-specific antisera to α1 (residues 339–358) and α2 (residues 352–366), we found that the α2 immunoreactivity was predominant in the heart, liver, and skeletal muscle (Fig. 2E) where there is also the highest concentrations of α2 mRNA. In contrast, the α1 immunoreactivity is widely distributed (Fig. 2D) as is the less abundant α1 mRNA. The antibody to α2 recognized a minor component in the purified α1 preparation (Fig. 2E, lane 1), but sufficient amounts of this have not been obtained to determine whether it represents weak cross-reactivity with a form of α1, an additional isoform of the AMPK or a low level contaminant of the α2 preparation by the α2 isoform. The antibody to α2 does not immunoprecipitate α1 activity from affinity-purified α1 AMPK. Both α1 and α2 migrate on SDS-PAGE at approximately 63 kDa (Fig. 2D and E). Unexpectedly, we found that the liver α1 immunoreactivity was not bound by the peptide substrate affinity column. This column specifically binds the α1 isoform. Using immune precipitation of the effluent from the peptide substrate affinity column with α2 specific antibody, we found that the α2 isoenzyme contained β and γ subunits (Fig. 4) and catalyzed the phosphorylation of the SAMS peptide. Immune precipitates of α1 and α2 showed variable activation by 5′-AMP ranging from 2–3- and 3–4-fold, respectively. There was also an approximate 60-kDa band recognized by the α1-specific antibody in tissue extracts from heart and lung (Fig. 2D). This band is not present in the purified liver enzyme, and its relationship to the α1 isoform is not yet known.

The proportion of SAMS peptide phosphotransferase activity bound to the peptide affinity column with a single pass varied (ranged 90–92%, n = 7, and 74–86%, n = 6 rat liver preparations). With recycling, approximately 94% of the activity was bound to the column. The residual activity was attributable to the α2 isoform activity based on immunoprecipitation with the α2-specific antibody. However, the amount of protein immunoprecipitated based on Coomasie Blue staining (Fig. 4) indicated that there was substantially more α2 protein than was expected from only 6% of the total SAMS peptide activity. The specific activity of the α2 isoform is not yet known in the absence of bound antibody. Based on the α2 cDNA sequence, Carling et al. (7) reported that a peptide specific antibody immunoprecipitated virtually all of the partially purified AMPK activity from liver. The peptide used in their experiments, PGLKPHERMPLI, contains 8/15 residues that are identical (underlined) between α1 and α2 so it seems reasonable that their polyclonal antiserum may recognize both isoforms. In this event, their immunoprecipitation data are consistent with our results.

The present work makes plain that there is an isoenzyme family of AMPK catalytic subunits, increasing the complexity of activity analysis. This also raises the question of what function the α2 isoform has and whether α2 associates with a specific subset of β and γ subunits. A significant fraction of the α2 isoform mRNA has a 142-base pair out-of-frame deletion within its catalytic domain that would encode a truncated, nonfunctional protein (8, 18). The close sequence relationship between the α1 isoforms from pig, rat, and human (Fig. 1)
means that there is strong conservation across species. Previously, it was reported that human liver does not contain AMPK mRNA (20); however, it is now clear that the α3 isoform of the enzyme eluted from the substrate affinity column (flow-through), enzyme eluted from the α2 specific antibody column (AMPK α2) and enzyme eluted from the α3 specific antibody column (AMPK α3) as described under "Experimental Procedures." Molecular masses of the AMPK subunits are shown in kDa.

Recent genome sequencing has revealed multiple isoforms of the noncatalytic γ and β subunits of the AMPK. There appear to be at least three isoforms of the γ subunit in brain with γ2 and γ3 present, distinct from the rat liver γ1 isoform. Human brain also contains multiple β subunit isoforms distinct from the rat liver β1 isoform. The accession numbers for putative AMPK β and γ subunit isoforms are: γ2, M78939; γ3, R35524; β2, R20494; β3, R14746. Thus, a potentially large subfamily of heterotrimeric AMPKs, based on various combinations of all three AMPK subunits, may be present.

The structural relationships between the AMPK and SNF1 kinase, as well as the presence of multiple isoforms, brings into focus a vista of questions concerning the diverse physiological roles of this new subfamily of protein kinases. Whereas the AMPK regulates lipid metabolism in hepatocytes under conditions of metabolic stress, its role in other tissues, including the heart and kidney, are unknown. Recent studies by Kudo et al. (22) have shown that the AMPK is activated during cardiac ischemia, and the activation persists during reperfusion, possibly contributing to the ischemia-driven decoupling of metabolism and cardiac mechanical function. Regulation of cardiac acetyl-CoA carboxylase by AMPK plays an important role in the switching of cardiac metabolism between the use of glucose and fatty acids as oxidative fuel (23). In the β cell of the pancreas, where AMPK subunits are highly expressed in islet cells, glucose availability rapidly regulates acetyl-CoA carboxylase through changes in AMPK-directed phosphorylation, suggesting strongly a role for AMPK in stimulus-secretion coupling for insulin release (24). In addition to these metabolic roles, members of the SNF1 protein kinase subfamily appear to play important roles in development, with the par-1 gene of Caenorhabditis elegans playing an essential role in embryogenesis (25).

Acknowledgments—We are grateful to Frosa Katsis for the preparation of synthetic peptides.

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