There is growing evidence that mammalian AMP-activated protein kinase (AMPK) plays a role in protecting cells from stresses that cause ATP depletion by switching off ATP-consuming biosynthetic pathways. The active form of AMPK from rat liver exists as a heterotrimeric complex and we have previously shown that the catalytic subunit is structurally and functionally related to the SNF1 protein kinase from Saccharomyces cerevisiae. Here we describe the isolation and characterization of the two other polypeptides, termed AMPKβ and AMPKγ, that together with the catalytic subunit (AMPKα) form the active kinase complex in mammalian liver. Sequence analysis of cDNA clones encoding these subunits reveals that they are related to yeast proteins that interact with SNF1, providing further evidence that the regulation and function of AMPK and SNF1 have been conserved throughout evolution. The amino acid sequence of the β subunit is most closely related to SIP2 (35% identity), while the amino acid sequence of the γ subunit is 35% identical with SNF4. We show that both AMPKβ and AMPKγ mRNA and protein are expressed widely in rat tissues. We show that AMPKβ interacts with both AMPKα and AMPKγ in vitro, whereas AMPKα does not interact with AMPKγ under the same conditions. These results suggest that AMPKβ mediates the association of the heterotrimeric AMPK complex in vitro, and will facilitate future studies aimed at investigating the regulation of AMPK in vivo.

A number of recent studies have led to the proposal that in mammals an AMP-activated protein kinase (AMPK) plays a major role in the response to metabolic stress (Corton et al., 1994; Hardie, 1994; Hardie et al., 1994). AMPK was first identified through its role in the phosphorylation and inactivation of a number of enzymes involved in lipid metabolism (Carling et al., 1987; Hardie et al., 1989; Hardie, 1992), and subsequently was shown to phosphorylate enzymes in other metabolic pathways (Carling and Hardie, 1989). AMPK has been purified from a number of species, including human, rat, and pig, and in each case is activated allosterically by micromolar concentrations of AMP (Carling et al., 1989; Mitchelhill et al., 1994; Sullivan et al., 1994). The kinase is itself regulated by reversible phosphorylation, being phosphorylated and activated by a distinct AMPK kinase (AMPKK), thereby forming a protein kinase cascade (Carling et al., 1987; Weekes et al., 1994). The phosphorylation and activation of AMPK is markedly stimulated by AMP (Moore et al., 1991; Hawley et al., 1995), making AMPK extremely sensitive to changes in the intracellular concentration of AMP. These findings have led to the proposal that one of the primary roles of AMPK is to conserve ATP during periods of excessive ATP utilization, when AMP levels are elevated (Corton et al., 1994; Hardie et al., 1994).

We recently reported that the deduced amino acid sequence of the catalytic subunit of rat liver AMPK is remarkably similar to the sequence of the yeast protein kinase SNF1 (Carling et al., 1994). In a further study we went on to show that SNF1 is functionally related to mammalian AMPK (Woods et al., 1994). In vitro, SNF1 phosphorylates a specific peptide substrate for AMPK, and there is good evidence that SNF1 phosphorylates and inactivates acetyl-CoA carboxylase in vivo. Furthermore, like AMPK, SNF1 is inactivated by protein phosphatases and can be reactivated by a partially purified preparation of mammalian AMPKK, suggesting functional conservation of the upstream kinases (Woods et al., 1994). The SNF1 protein kinase from Saccharomyces cerevisiae is required for the expression of glucose repressed genes in response to glucose starvation (Celenza and Carlson, 1986; Estruch et al., 1992; Ganoczi, 1992), e.g. the SUC2 gene, which encodes invertase (Carlson and Botstein, 1982). snf1 mutants are unable to utilize a wide range of non-glucose sugars (Carlson et al., 1981; Estruch et al., 1992). In addition, snf1 mutants have been shown to be defective in other aspects of cell growth, e.g. glycerogen synthesis and sensitivity to heat stress (Thompson et al., 1991). SNF1 is physically associated with a 36-kDa polypeptide, termed SNF4 (Celenza et al., 1989), which is itself required for expression of many glucose-repressible genes. SNF4 is thought to function as an activator of SNF1 (Celenza and Carlson, 1989; Celenza et al., 1989), although the mechanism by which SNF4 activates SNF1 is not known.

A number of yeast proteins, which interact with SNF1 in vivo, termed SNF1 interacting proteins or SIPs, have been identified using the two-hybrid system (Yang et al., 1992). Two of these proteins, SIP1 and SIP2, share significant amino acid sequence identity, particularly at their C termini (Yang et al., 1992).
AMPK-Activated Protein Kinase Subunits

AMPK has been purified to apparent homogeneity from both rat and pig liver (Mitchelhill et al., 1994). Two other polypeptides co-purified with the catalytic subunit (molecular mass 63 kDa), and biochemical analysis of the purified kinase complex indicated that AMPK isolated from rat liver exists as a heterotrimer (Davies et al., 1992). Furthermore, the amino acid sequence of SIP2 is 52% identical to GAL83 (Erickson and Johnston, 1993; Yang et al., 1994), GAL83 is involved in the glucose repression of GAL genes, and genetic evidence suggests that GAL83 is involved in the SNF1 pathway (Matsumoto et al., 1993). SIP1, SIP2, and GAL83 have been shown to interact with SNF1, and all three proteins are phosphorylated in an immune complex SNF1 kinase assay (Yang et al., 1994). In the same study it was shown that the C-terminal 80 amino acids of SIP2, termed the ASC domain, were sufficient to mediate interaction with SNF1. The functions of SIP1, SIP2, and GAL83 remain unknown, although it has been proposed that they may act as modulators of SNF1, targeting the kinase to specific intracellular locations and/or substrates (Yang et al., 1994).

Recently, AMPK has been purified to apparent homogeneity from both rat and pig liver (Mitchelhill et al., 1994; Davies et al., 1994). Two other polypeptides co-purified with the catalytic subunit (molecular mass 63 kDa), and biochemical analysis of the purified kinase complex indicated that AMPK isolated from rat liver exists as a heterotrimer (Davies et al., 1994). We subsequently reported that the catalytic subunit of AMPK isolated from rat skeletal muscle did not appear to be associated with any other polypeptides and that this observation might account for the low activity of AMPK detectable in skeletal muscle (Verhoeven et al., 1995). In this paper we report the isolation and cDNA cloning of two AMPK subunits from rat liver which we refer to as AMPKβ and AMPKγ (the catalytic subunit is designated AMPKα) following the terminology of Kemp and colleagues (Stapleton et al., 1994). The β subunit is most closely related to SIP2 and contains a region at its C terminus, which is 50% identical with the ASC domain of SIP1/SIP2/GAL83 (Yang et al., 1994). The γ subunit has a high degree of amino acid sequence identity with SNF4, and this conservation of sequence suggests that, like SNF4, it is necessary for the catalytic activity of AMPK. We show here that AMPKβ interacts with both AMPKα and AMPKγ and that this mediates the assembly of the ternary complex in vitro. The similarity between the mammalian AMPK complex and the SNF1 complex from yeast emphasizes the likelihood that the role of these kinases have been highly conserved throughout evolution.

MATERIALS AND METHODS

Purification and Amino Acid Sequencing of AMPK Subunits—AMPK was partially purified from rat liver up to and including the DEAE-Sepharose ion-exchange step, as described previously (Carling et al., 1989). AMPK was further purified by immunoaffinity chromatography using affinity-purified antibodies raised against a synthetic peptide based on the deduced sequence of AMPKα (Carling et al., 1994). Approximately 200 mg of partially purified AMPK was incubated overnight at 4°C with 5 mg of affinity-purified antibody that had been cross-linked to protein A-Sepharose (Harlow and Lane, 1988). Following extensive washing of the resin with 50 ml Tris/HC1, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM diithiothreitol, 10% (v/v) glycerol (buffer A), protein was eluted with 5 bed volumes of 0.1 M glycine, pH 2.5. The eluate was immediately neutralized by the addition of 0.1 volumes of 1 M Tris/HC1, pH 8, and concentrated in a Centricon-30 microconcentrator (Amicon). Proteins were resolved by SDS-PAGE on a 10% polyacrylamide gel and visualized by staining with Coomassie Blue. The polypeptides migrating at apparent molecular masses of 38 kDa (AMPKβ) and 36 kDa (AMPKγ) were excised from the gel and the proteins cleaved in the gel slice by overnight incubation with CNBr in 90% formic acid at room temperature. The supernatant was removed and dried in a Speed-Vac. The residue was washed twice with water, dried, and resuspended in SDS-gel loading buffer. Peptides were resolved by SDS-PAGE using a Tricine buffer system (Schagger and von Jagow, 1987) and transferred onto a Problot membrane (Applied Biosystems) for sequencing using an Applied Biosystems model 475 sequencer.

Autophosphorylated AMPK was prepared by incubating the immune complex with 0.2 mM [γ-32P]ATP and 0.2 mM AMP at 30°C for 30 min. Unincorporated ATP was removed by extensive washing with buffer A. Protein was eluted from the resin as above. Following SDS-PAGE the gel was dried and subjected to autoradiography at -70°C.

Addition of DNAAs Encoding the β and γ Subunits—Degenerate oligonucleotide primers based on the peptide sequences obtained from the catalytic subunit of AMPKβ or AMPKγ (Fig. 1) were used as follows: β forward primer 1, CGCGGAAARGART; forward primer 2, AARGAARGARTYTGNC; reverse primer 1, ACRTATTITTYT-GRTA; reverse primer 2, TGRTAACGTNASGNGTGNC (primers corresponding to the reverse and complemented sequence of these oligonucleotides were also synthesized but did not yield a distinct product after amplification, indicating that the peptide 38CB1 (GCTAGCA) and 38CB1; γ forward primer 1, GAYTTTAYACTACTAYAYACTYT; forward primer 2, YTNCA(A)CNYTAYAYAA; reverse primer 2, TGYTG- GACRAGA(Y)ATYATCC; reverse primer 2, CCNARNNGCNACRT-ANAC. For both subunits, rat liver cDNA (Clontech) was amplified using forward primer 1 and reverse primer 1 for 30 cycles of 94°C, 1 min; 50°C, 1 min; and 72°C, 1 min. In each case, an aliquot (0.1 μl) of the products of the reaction were used for a second round of amplification using forward primer 2 and reverse primer 2 (same cycles as above). The products from the reaction were purified by agarose gel electrophoresis, cloned into a pGEM-T vector (Promega), and sequenced to confirm their identity.

Isolation of cDNA Clones Encoding the β and γ Subunits—Standard molecular biology techniques were used (Sambrook et al., 1989). The products isolated from amplification of rat liver cDNA were used separately to screen a rat liver cDNA library (λ UniZAP, Stratagene). Hybridization conditions were: 50 ng of probe (~10 ng/μl; 10 ng/μl/ml) in 5 x SSPE, 100 μg/ml sonicated and denatured salmon sperm DNA, 2 x Denhardt's, 0.1% SDS at 65°C for 12 h. Filters were washed with 2 x SSC, 0.1% SDS at room temperature for 1 h, followed by 2 x SSC, 0.1% SDS at 65°C for 30 min and autoradiographed at ~70°C for 18 h, with intensifying screens. Twelve positive clones encoding AMPKβ were isolated from approximately 4 million plaques and one positive clone for AMPKγ was isolated from approximately 1 million plaques. Plasmids were recovered by in vivo excision from the phage vector (Hwang et al., 1988) and the inserts sequenced manually by dyeoxy chain termination (Sanger et al., 1977) using vector and cDNA specific primers.

In order to obtain the 5' end of the cDNA encoding the γ subunit, antisense oligonucleotide primers were synthesized based on the γ cDNA sequence and used to perform 5' RACE-PCR (Frohman et al., 1988). (AMPKγ RACE primer 1, TGGCGTAGTGCAATCGT; RACE primer 2, ATCTAGTGCTCCTCAGAG). Rat liver RACE-ready cDNA (Clontech) was used as a template for amplification using primer 1 and the anchor primer for 30 cycles of 94°C, 1 min; 58°C, 1 min; 72°C, 1 min. A second round of amplification on an aliquot (0.1 μl) of the reaction products was performed using primer 2 and the anchor primer under the same conditions as before. Products from the second round of amplification were isolated by agarose gel electrophoresis, cloned into a pGEM-T vector, and sequenced. In order to construct a cDNA encoding the entire amino acid sequence of the γ subunit oligonucleotide primers spanning the initiating methionine (GCCCAGGTGCACGCGCGGT- GCTAGCATTG) and downstream of the stop codon (GCCCTAGTGC- GACTCCGTTCTCTCAGG) and containing a SalI restriction site (underlined) were synthesized and used to amplify rat liver cDNA. The product (1.1 kb) was cloned into pGEM-T vector to yield pGEM-γ. The inserts from each of three independent clones were sequenced to confirm their authenticity.

Northern Analysis—A rat multiple tissue Northern (Clontech) was probed with either a random primed (Feinberg and Vogelstein, 1983) 1.1 kb cDNA fragment encoding the β subunit or a random primed 1.1 kb cDNA fragment encoding the γ subunit according to the manufacturer's instructions. Following hybridization the blot was washed with 2 x SSC, 0.5% SDS at room temperature for 1 h, followed by 0.2 x SSC, 0.5% SDS at 65°C for 2 x 20 min and autoradiographed at ~70°C for 2-5 days.

Transfection Production—The entire coding sequence of AMPKγ (330 residues) was expressed in Escherichia coli as a fusion protein with glutathione S-transferase. A polypeptide containing the C-terminal 217 residues of the β subunit was expressed in E. coli as a fusion protein with glutathione S-transferase. The fusion proteins were purified on a glutathione-agarose column (Pharmacia) and used to immunize male New Zealand White rabbits. Following three rounds of immunization, antisera was collected and used for Western blot analysis and immunoprecipitations. Antiserum against the catalytic subunit of AMPK was obtained as described previously (Carling et al., 1994).
Western Blotting of Tissue Lysates—Female Wistar rats (250–300 g body weight) were killed by stunning and cervical dislocation, tissues removed, and immediately frozen in liquid nitrogen. Approximately 0.5 g of frozen tissue was ground to a fine powder using a pestle and mortar and homogenized in 10 ml of buffer (50 mM Tris/HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.25 M sucrose, 0.1 M phenylmethylsulfonyl fluoride) using a Polytron homogenizer. After homogenization, SDS was added to a final concentration of 0.5% and the homogenate was boiled for 15 min. The homogenate was centrifuged at 14,000 ∗ g for 15 min and the supernatant removed. The protein concentration of the supernatant was determined using the Lowry assay. 100 μg of protein for each tissue was resolved by SDS-PAGE and transferred to a polyvinylidene membrane. The membrane was blocked by incubation in 10 mM Tris/HCl, pH 7.5, 1 M NaCl, 0.5% Tween, 5% nonfat milk powder (w/v) for 1 h at room temperature. Primary antibody was added to this buffer and the blot incubated for another 2 h. The blot was washed extensively in 10 mM Tris/HCl, pH 7.5, 1 M NaCl, 0.5% Tween and then incubated with an anti-rabbit antibody conjugated to horseradish peroxidase. Antibodies were detected using enhanced chemiluminescence (Boehringer Mannheim).

Interactions Using the Two-hybrid System—Vectors expressing the GAL4 DNA binding domain (pGBT9) and the GAL4 activation domain (pGAD) from the ADH1 promoter (Clontech) were used to construct fusion proteins with each of the AMPK subunits. Plasmids containing the entire coding sequence of the catalytic subunit of rat liver AMPK. Vectors expressing the other constructs contain additional protein sequence encoded by the T7 promoter primer to amplify AMPKβ DNA from a clone contained in pBluescript. The product was digested with EcoRI (underlined sequence in the oligonucleotide) and XhoI (contained within the polylinker of Bluescript) and ligated into either pGEX9 or pGAD that had been digested with EcoRI and SaI. This creates an in-frame fusion between either the GAL4 DNA binding domain or GAL4 activation domain and the C-terminal 201 amino acids of the β subunit. Vectors expressing fusions of the GAL4 activation domain with SNF1, SNF4, SIP1, and SIP2 (Yang et al., 1994) were generously provided by Dr. Marian Carlson, Columbia University.

Table 1

| Peptide | Amino acid sequence |
|---------|---------------------|
| AMPKβ   | 38CB1 VLSAT (H/V) RY (K/Q) KKVYXKL |
|         | 38CB2 VD (S) QKX (S) DV (S) EL (SS) XPGXXYX (E) |
|         | 38CB3 XAPEKEELAQL |
|         | 38CB4 PX (E/H) D (Y) (V/H) (L) X (Q/K) L |
|         | 38CB5 XNH (L/K) YALX1 (K) |
| AMPKγ   | 36CB1 LITITDFIN1IHRKYKSALVQIYELXEHK1XXRXKXVX |
|         | 36CB2 ETEW EY |

Fig. 1. Co-purification of two polypeptides with the catalytic subunit of rat liver AMPK. Partially purified AMPK from rat liver was purified using protein A-Sepharose cross-linked to affinity-purified antibodies raised against AMPKα. Following extensive washing, the antibody-protein A-Sepharose resin was incubated with 0.2 mM [γ-32P]ATP and 0.2 mM AMP at 30 °C for 30 min. Proteins were eluted from the antibody-protein A-Sepharose resin, resolved by SDS-PAGE, and visualized by staining with Coomassie Blue (lane A). An autoradiograph of the same gel is shown in lane B. The migration of molecular mass standards is shown on the right of the figure.
We refer to these polypeptides as the \( \beta \) subunit (38 kDa) and \( \gamma \) subunit (36 kDa) of AMPK, with the catalytic subunit being designated the \( \alpha \) subunit. As can be seen from Fig. 1, both the \( \alpha \) and \( \beta \) subunits undergo autophosphorylation, whereas there is no detectable incorporation of phosphate into the \( \gamma \) subunit. In order to characterize the \( \beta \) and \( \gamma \) subunits further, the purified polypeptides were transferred to a Problot membrane and subjected to N-terminal amino acid sequence analysis. On two separate occasions, no sequence was derived from the N terminus of either AMPK \( \beta \) or AMPK \( \gamma \). However, cleavage of the gel-purified \( \beta \) and \( \gamma \) subunits with cyanogen bromide yielded several peptides from which amino acid sequence was obtained (Table I). Analysis of the Swiss-Prot data base with the peptide sequences from the \( \gamma \) subunit revealed that they were most closely related to sequences within yeast SNF4. No significant identity with any sequences in the data base was found with the peptides from the \( \beta \) subunit.

Isolation of AMPK \( \beta \) cDNA—A partial cDNA sequence encoding AMPK \( \beta \) was obtained by PCR using rat liver cDNA and degenerate oligonucleotide primers corresponding to potential sequences encoding the peptide sequences 38CB2 and 38CB4 shown in Table I (see "Materials and Methods"). A product of approximately 600 bp was isolated and sequenced, revealing that it encoded peptide sequences corresponding to 38CB2 and 38CB4. The residue predicted to be immediately N-terminal to peptide 38CB4 was found to be an aspartic acid, rather than a methionine as expected following CNBr cleavage, indicating that peptide 38CB4 was produced by acid cleavage of an Asp-Pro bond. The PCR product was used to screen a rat liver cDNA library in order to isolate full-length clones. Twelve positive hybridizing clones for AMPK \( \beta \) were isolated from approximately 4 million plaques. Sequence analysis of the longest clones showed that the insert contained an in-frame methionine, which was preceded by a stop codon in the same frame, followed by an open reading frame of 269 amino acids that included all of the peptides obtained by amino acid sequencing of the purified protein from rat liver. The open reading frame was followed by approximately 1 kbp of untranslated sequence, including a polyadenylation site consensus sequence and a poly(A) tail (Fig. 2). The predicted molecular mass of AMPK \( \beta \) from the deduced protein sequence is 30 kDa, which is significantly lower than the apparent mass observed by SDS-PAGE analysis (38 kDa). However, in vitro translation of RNA synthesized from AMPK \( \beta \) cDNA (beginning at the first methionine) produced a protein that exactly co-migrated on SDS-PAGE with the \( \beta \) subunit immunoprecipitated from rat liver (Fig. 3).
result confirms that there is no additional coding sequence upstream of the first methionine in the cDNA we isolated. At present we do not know the reason for the anomalous migration of the β subunit on SDS-PAGE.

Isolation of AMPKγ cDNA—A partial cDNA sequence encoding AMPKγ was obtained by amplifying rat liver cDNA using degenerate oligonucleotide primers corresponding to potential sequences encoding the peptide sequences 36CB1 and 36CB2

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**Fig. 3.** In vitro translation of AMPKβ. [35S]Methionine–labeled AMPKβ was translated in vitro in rabbit reticulocyte lysate programmed with RNA synthesized from AMPKβ cDNA cloned into pET-14b. The lysate was immunoprecipitated using anti-β antibodies attached to protein A–Sepharose and the immunocomplex was analyzed by SDS-PAGE. AMPK was immunoprecipitated from rat liver with anti-β antibodies and resolved by SDS-PAGE on the same gel in order to compare the electrophoretic mobility of the native and recombinant β subunits. Proteins were visualized by staining with Coomassie Blue, and labeled products were detected by fluorography. Lane 1, total lysate in the absence of RNA; lane 2, total lysate programmed with AMPKβ RNA; lane 3, immunoprecipitation of AMPK from rat liver; lane 4, immunoprecipitation of lysate programmed with AMPKβ RNA. Note that in lane 3 all three AMPK subunits are precipitated and that AMPKβ is the upper polypeptide in the 38/36-kDa doublet. The migration of molecular mass markers is shown on the left.

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**Fig. 4.** AMPKβ and AMPKγ are related to yeast proteins that interact with SNF1. A, the deduced amino acid sequences of AMPKβ (top) and AMPKγ (bottom) were aligned using the GAP program in the University of Wisconsin package with a gap weight of 3.0 and a length weight of 0.1. Dots indicate gaps introduced to maximize the alignment. Identities between the two sequences are shown in shaded boxes. B, the deduced amino acid sequences of AMPKγ (top) and SNF4 (bottom) were aligned as above.
either 2 days (AMPK and b) data base with the deduced protein sequence of the SIP2 (Erickson and Johnston, 1993). A search of the SwissProt and GAL83, which have been shown previously to be related to PPILPP to end. A similar, but slightly lower, degree of sequence identity. The amino acid sequences of AMPK g and SNF4 are having the highest degree of identity. The amino acid sequences of AMPK a and SNF4 are 35% identical overall (Fig. 4 a). No other significant similarities were identified with either of the subunits.

Tissue Distribution of AMPK b and AMPK a—Poly(A)-rich RNA isolated from a number of rat tissues was probed with cDNA encoding the b and a subunits. Fig. 5 shows the results of the Northern analysis and compares the expression of the b and a mRNA with the expression of AMPK a, which we have reported previously (Verhoeven et al., 1995). A strongly hybridizing band of approximately 2.4 kb was detected in all of the tissues tested following labeling with a cDNA probe specific for AMPK b. A weakly hybridizing signal at approximately 4.5 kb, present in all tissues, could also be detected with the AMPK b probe. When a a subunit-specific probe was used, a single hybridizing band at approximately 1.8 kb was detected in all tissues, although only a faint signal was detected in testis. A band at approximately 2.4 kb was also detected with mRNA isolated from brain. These results indicate that the messages for both subunits are expressed in a wide number of rat tissues. In contrast to the b and a subunits, the mRNA expression of the a subunit shows marked differences in tissue distribution and is most highly expressed in skeletal and cardiac muscle (see Verhoeven et al. (1995)).

Antibodies raised against fusion proteins of AMPK b or AMPK a with glutathione S-transferase were used to determine the expression of the polypeptides in various rat tissues. We also examined the expression of AMPK a (the catalytic subunit) using antibodies raised against a specific peptides (Carling et al., 1994). Fig. 6 shows the expression of the polypeptides in a number of tissue lysates. All three polypeptides were detected in every tissue tested, although there appeared to be some variation in the relative amounts of the three subunits present in different tissues (for instance compare the expression of the a and b subunits in brain and skeletal muscle). As we have noted previously, there is a small but detectable shift in the mobility of the a subunit between different tissues, which we believe may reflect differences in the phosphorylation state of the enzyme (Verhoeven et al., 1995).

Interaction of the a, b and γ Subunits in the Two-hybrid System—We employed the yeast two-hybrid system (Fields and Song, 1989) in order to examine the interaction of the three AMPK subunits in more detail. The entire coding sequence of the a, b and γ subunits was identified with the yeast protein SNF4 as having the highest degree of identity. The amino acid sequences of AMPK a and SNF4 are 35% identical overall (Fig. 4 b). No other significant similarities were identified with either of the subunits.

Interaction of the a, b and γ subunits remained white in the presence of X-Gal, indicating that the interaction between AMPK a and AMPK b is weaker than the interaction between AMPK a and AMPK γ. This was confirmed by the appearance of blue colonies in the presence of X-Gal (Table II). Similar results with these combinations were obtained when the GBD and GAD fusions were switched. The activity detected in transformants with GBD a and GAD γ or GBD γ and GAD a was very low in comparison with the other combinations, which could indicate that the interaction between AMPK a and AMPK γ is weaker than the other interactions. Very low levels of β-galactosidase activity were detected when yeast were transformed with the same subunit expressed with both GBD and GAD, and these transformants remained white in the presence of X-Gal, indicating that the subunits do not form homodimers.

Since the two-hybrid system is carried out in yeast, it was important to determine any interactions between the mammalian subunits and their yeast counterparts. We therefore extended the study to determine interactions between AMPK subunits and SNF1, SNF4, SIP1, and SIP2. Table III shows the results of the various combinations of rat and yeast proteins in...
the two-hybrid system. We were unable to detect any interaction of AMPKα with any of the yeast proteins. However, AMPKβ gave a signal with both SNF1 and SNF4 and AMPKγ interacted with SNF1, SIP1, and SIP2. Furthermore, AMPKγ interacted with all of the SIP1 and SIP2 fusions tested, including a fusion expressing the C-terminal 120 amino acids of SIP2 (GAD-SIP2 296–415; Yang et al., 1994). Color of transformants was determined by filter assay.

### Table II

**Interaction of AMPK subunits using the two-hybrid system**

| DNA-binding hybrid | Activation hybrid | Color | β-Galactosidase activity |
|--------------------|------------------|-------|-------------------------|
| GAD-α              | GBD-α            | Blue  | 10                      |
| GAD-α              | GBD-β            | Blue  | 3                       |
| GAD-α              | GBD-γ            | Blue  | 34                      |
| GAD-β              | GBD-α            | Blue  | 19                      |
| GAD-β              | GBD-α            | Blue  | <1                      |
| GAD-β              | GBD-γ            | Blue  | 11                      |
| GAD-γ              | GBD-α            | White | <1                      |
| GAD-γ              | GBD-β            | White | <1                      |
| GAD-γ              | GBD-γ            | White | <1                      |

### Table III

**Interaction of AMPK subunits and their yeast counterparts using the two-hybrid system**

GAD-SIP1 201–863, etc., has codon 201 of SIP1 fused to the GAL4 activation domain (Yang et al., 1994). Color of transformants was determined by filter assay.

| Activation hybrid | GBD-α | GBD-β | GBD-γ |
|-------------------|-------|-------|-------|
| GAD-SNF1          | White | Blue  | Blue  |
| GAD-SNF4          | White | White | Blue  |
| GAD-SIP1 201–863  | White | White | Blue  |
| GAD-SIP1 452–863  | White | White | Blue  |
| GAD-SIP2 249–415  | White | White | Blue  |
| GAD-SIP2 282–415  | White | White | Blue  |
| GAD-SIP2 296–415  | White | White | Blue  |

there is no evidence for a stable complex between the α and γ subunits. If the α and γ subunits do interact, then their association must be either transient or weak, or both, and does not survive immunoprecipitation. The translations and immunoprecipitations are carried out in buffers lacking protein phosphatase inhibitors and under conditions that would not be expected to cause activation of endogenous AMPKK, which may be present in the reticulocyte lysate. It is likely that AMPKγ is in the dephosphorylated form following translation, suggesting that phosphorylation by AMPKK is not necessary for formation of the ternary complex. This may also explain why we have been unable to detect AMPK activity in any of the translations.

### DISCUSSION

Full-length cDNA clones encoding AMPKβ were isolated by conventional library screening, and a composite cDNA clone encoding the full-length sequence of AMPKγ was constructed from overlapping clones isolated by a combination of library screening and 5′ RACE. The deduced amino acid sequence of AMPKβ predicts a protein with a molecular mass of 30 kDa. This is considerably lower than the apparent mass of the β subunit isolated from rat liver, as judged by SDS-PAGE. In vitro translation of RNA synthesized from AMPKβ cDNA produced a major product, which exactly co-migrated with rat liver AMPKβ following SDS-PAGE. This finding, coupled with the fact that an in-frame stop codon is present upstream of the first methionine, confirms that the AMPKβ cDNA reported here is full-length. The reason for the anomalous electrophoretic mobility of the β subunit in denaturing gels is unclear, but it is due to post-translational modification of the polypeptide that the reticulocyte lysate system must be competent in carrying out the modification.

The finding that AMPKβ and AMPKγ share sequence identity with yeast proteins that interact with SNF1 strengthens...
the proposal that the functions of the two kinases have been highly conserved throughout evolution (Woods et al., 1994). Although the function of SNF4 is not known, it is necessary for the protein kinase activity of SNF1, and it seems likely that AMPKγ will have a similar role in the activity of AMPK. Despite the obvious similarity between the amino acid sequences of AMPKγ and SNF4, we have not been able to complement snf4 mutants by expression of AMPKγ. In a previous study, we reported that we were unable to complement snf1 mutants by expression of the catalytic subunit of AMPK, which shares 47% amino acid sequence identity with SNF1 (Carling et al., 1994; Woods et al., 1994). Taken together these results indicate that, although the AMPK and SNF1 complexes are highly related, significant differences between the two complexes must exist. One notable difference that is already known is that, while the mammalian kinase is markedly activated by AMP (Carling et al., 1989), no measurable effect of AMP on SNF1 activity has been demonstrated (Mitchelhill et al., 1994; Woods et al., 1994). Whether this difference alone is sufficient to explain the inability of the catalytic subunit to rescue snf1 mutants, and AMPKγ to rescue snf4 mutants, is not yet clear. In order to address this question, a detailed comparison of the structures of the mammalian and yeast kinase complexes, e.g. from crystallographic studies, and the elucidation of the regulation of the kinases are required.

Western blot analysis of rat tissue lysates shows that AMPKβ and AMPKγ subunits are expressed in every tissue examined. Although the blots are not quantitative, there does appear to be some variation in the relative expression of the three subunits in different tissues (Fig. 6). AMPK purified from rat liver appears to exist entirely as a heterotrimeric complex of AMPKα, β, and γ (see Fig. 1 and Davies et al. (1994)). Immunoprecipitation of α from skeletal muscle, however, suggests that it exists predominantly as a monomer, with no kinase activity (Verhoeven et al., 1995), even though AMPKβ and AMPKγ are present in this tissue. These findings raise the interesting possibility that the association of the catalytic subunit with AMPKβ and AMPKγ is regulated and that the structure of the complex may vary between different tissues and/or different conditions. At present the mechanism that leads to the association of the subunits is not known, although our results suggest phosphorylation is not required. Dephosphorylation of the active AMPK complex to an inactive form in vitro does not result in dissociation of the subunits (data not shown), and it is interesting to note that in yeast the association of SIP1 or SIP2 with SNF1 does not require SNF1 protein kinase activity or the presence of SNF4 (Yang et al., 1994).

The results from the two-hybrid experiments indicate that AMPKβ interacts with both AMPKα and AMPKγ, but that the interaction of AMPKα with AMPKγ is very weak. We also tested the interaction of the mammalian subunits with their yeast counterparts in the two-hybrid system. In this case we found evidence for the interaction of AMPKβ with both SNF1 and SNF4 and AMPKγ with SNF1 and SIP1 and SIP2. However, there was no detectable interaction between AMPKα and any of the yeast proteins. These results do not rule out the possibility that the AMPKγ-SNF1 interaction is indirect and could be mediated by an AMPKβ homologue in yeast, e.g. SIP1/SIP2/GAL83, which could act as a bridging protein in a ternary complex. AMPKβ interacts with both AMPKα and AMPKγ forming stable complexes. However, under the same conditions, we could not detect stable complexes between AMPKα and AMPKγ. These results suggest that the formation of the ternary complex between α, β, and γ is mediated by the β subunit. We have not been able to detect AMPK activity in lysates programmed with all three subunits, or in immune complexes from these lysates. This may be due to the dephosphorylated form of the kinase and/or the lack of sufficient protein in the translation system to allow detection of kinase activity.

Two different models for the association of the three subunits can be predicted based on the results of this study. The first model is one in which the β subunit links the α and γ subunits (Fig. 8A). The second model involves a conformational change in either the α or γ subunit, or both, upon binding of the β subunit, which would then allow direct interaction between the α and γ subunits (Fig. 8B). It is interesting to note the similarities between the AMPK complex and the heterotrimeric complex formed between CDK-activating kinase, cyclin H, and p36/MAT1 (RING finger protein) (Fisher et al., 1995; Devault et al., 1995). Although there is no significant amino acid sequence identity between the AMPK subunits and the CDK-activating kinase subunits, the regulation and association of the two kinase complexes bear some obvious similarities.

Although the functions of AMPKβ and AMPKγ remain unclear, a possible insight can be gained by comparison with a proposed model for the SNF1 complex in yeast. SNF4 is necessary for SNF1 kinase activity in vitro (Woods et al., 1994) and may therefore fulfill a similar function to cyclins in the activation of cyclin dependent kinases (Jeffrey et al., 1995). AMPKγ by analogy would play a similar role in the activation of AMPK. Biochemical evidence suggests that SNF1 forms a relatively stable complex with SNF4, and that this complex has protein kinase activity in vitro (Mitchelhill et al., 1994). The SNF1-SNF4 complex interacts with one of a number of related proteins, which include SIP1, SIP2, and GAL83 (Yang et al., 1994). It has been proposed that these proteins act as adaptors or targeting subunits, directing the kinase to specific intracellular substrates (Yang et al., 1994). In this model, the formation of different SNF1-SNF4-adapter complexes would allow selective phosphorylation of the downstream targets of SNF1, if the adapter proteins recognize different substrates. It is not clear whether the assembly of the SNF1-SNF4-adapter complex

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is regulated, or how the adaptor proteins act to promote phosphorylation of target substrates. However, there is clear evidence from other systems that one mechanism for regulating the phosphorylation of a protein is to regulate the distribution of the kinase, or phosphatase, acting on that protein via specific targeting subunits (Hubbard and Cohen, 1993; Coghlan et al., 1995). Could AMPK β act as a targeting subunit for the AMPK complex? It seems unlikely that the function of AMPK β is merely to bring together the α and γ subunits, especially given the fact that in yeast there appears to be a family of proteins related to AMPK β. It will be interesting to determine whether or not there is a family of proteins related to AMPK β in mammalian cells, and whether these proteins act as adaptors for AMPK.

Finally, it is interesting to note that, although the interaction of SNF1 and SNF4 is often used as a model for the two-hybrid system, our results imply that this interaction could in fact be indirect. Given the similarities between the mammalian AMPK and yeast SNF1 complexes it is possible that the interaction between SNF1 and SNF4 is mediated by a member of the SLP1/SIP2/GAL83 family of proteins. In addition to SNF4, a number of other polypeptides were found to co-purify with SNF1 (Stapleton et al., 1994). Although SLP1 was not identified, it is possible that some of these co-purifying polypeptides are members of the SLP1/SIP2/GAL83 family, which could mediate the association of SNF1 and SNF4 in a ternary complex, analogous to AMPK β in the mammalian complex.

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