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Molecular cloning, expression and chromosomal localisation of human AMP-activated protein kinase

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Abstract A cDNA encoding rat liver AMP-activated protein kinase (AMPK) was used to isolate human skeletal muscle AMPK cDNA clones. Human AMPK cDNA is more than 90% homologous to the rat sequence and predicts a protein of molecular mass 62.3 kDa, which closely agrees with the mass observed in Western blots of human tissues. AMPK antibodies were also shown to immunoprecipitate AMPK from human liver extracts. A cDNA probe was used to identify a 9.5kb transcript in several human tissues and to isolate human genomic clones. PCR mapping of rodent/human hybrid cell lines localised the human AMPK gene to chromosome 1, and fluorescent in situ hybridisation with a human genomic clone was used to sub-localise the human AMPK gene to 1p31.

Key words: AMP-activated protein kinase; Molecular cloning; Tissue expression; Chromosomal localisation

1. Introduction

AMP-activated protein kinase (AMPK) is a recently defined multisubstrate enzyme that plays a key role in the regulation of fatty acid and cholesterol metabolism [1,2]. Previous studies with rat liver AMPK have shown that in vitro it phosphorylates and inactivates 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and acetyl-CoA carboxylase [3], key enzymes regulating de novo biosynthesis of cholesterol and fatty acids, respectively [1,2]. AMPK has also been shown to phosphorylate hormone-sensitive lipase in vitro, thereby preventing activation by cAMP-dependent protein kinase [4]. There is evidence that these enzymes are phosphorylated in vivo, suggesting a role for AMPK in the co-ordinate regulation of cholesterol and fatty acid biosynthesis and the release of these esterified lipids from intracellular stores [5–9]. Although detailed biochemical studies have been carried out with rat liver AMPK, there is little information regarding the properties of other mammalian AMPKs. However, using the SAMS peptide phosphorylation assay [10] we have recently characterised a partially purified preparation of human liver AMPK and have shown that it shares similarities with the rat enzyme [11].

Although rat liver AMPK was purified over 3000-fold several years ago [12], little progress had been made towards the cloning and molecular characterisation of this important regulatory protein kinase. However, we recently reported the isolation of cDNA clones encoding rat liver AMPK which show homology to yeast and plant protein kinases involved in the regulation of carbon metabolism [13]. As the first step towards a more detailed characterisation of human AMPK, we describe here the isolation of a cDNA encoding human skeletal muscle AMPK and identify the chromosomal localisation of the gene. We also show that AMPK antibodies can specifically immunoprecipitate AMPK activity from human liver extracts.

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2. Materials and methods

2.1. Isolation of human AMPK cDNAs and genomic clones

A radiolabelled cDNA (1.8kb) encoding rat AMPK [13] was used to screen a human skeletal muscle λgt10 cDNA library (Clontech) according to the manufacturer's instructions. Positive clones were plaque purified and insert sizes verified by PCR using vector primers. The *Eco*R1 inserts were subcloned into pBluescript (Stratagene) and cDNA sequences determined by dideoxy chain termination [14] using vector and AMPK-specific oligonucleotides. Sequence analysis was carried using the University of Wisconsin Genetics Computer Group software package [15].

To isolate human genomic clones, rat AMPK cDNA was radiolabelled and used to screen an EMBL3 human placenta genomic library (Clontech) using the procedures described above. Three clones (EMBL1–3) were plaque purified and the DNA isolated from plate lysates of clone EMBL1 was sequenced with several AMPK-specific oligonucleotides to verify clone identity.

2.2. Cloning the 5′ end of human AMPK cDNA

To clone the 5′ end of the human AMPK cDNA, 1 μg human skeletal muscle poly(A)$^+$ RNA was converted to single-stranded cDNA using a pre-amplification kit (Gibco/BRL). A sense primer (Pex 211) derived from the untranslated region of rat AMPK (5′-GGGCCCCTCCGGCGCGCGGCTT-3′) was used in combination with an antisense primer (Apex21) derived from the cloned human sequence (5′-CAACAACATCTAAACTGCGAAT-3′) to amplify the intervening region in a 50 μl reaction containing: reaction buffer (Promega), 50 pmol of each primer, 200 μmol of each dNTP and 1 unit of *Taq* DNA polymerase (Promega). The predicted PCR product of 200 base pairs was gel-purified and direct sequenced with several nested antisense primers using the Circumvent thermal cycle kit (NEB).

2.3. Northern and Southern blotting

A human multiple tissue Northen blot (Clontech) was probed with ^{32}P-labelled rat AMPK cDNA according to the instructions supplied. A blot containing human, hamster and mouse genomic DNAs digested with a number of restriction enzymes (Bios Laboratories) was probed with radiolabelled human AMPK cDNA according to the manufacturer's instructions.

2.4. Immunoblotting and immunoprecipitation

Antibody was raised against the amino acid sequence of rat AMPK for residues 361–374 (PGLKPHPERMPPLI) as described previously [13]. Immunoblotting was performed on a multiple human tissue Western blot (Clontech) of proteins isolated from tissue homogenates. The

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filter was probed with the AMPK-peptide antiserum described above and a goat anti-rabbit horseradish peroxidase-labelled secondary antibody. Visualisation was carried out using an Enhanced Chemiluminescence (ECL) detection kit (Amersham International).

For immunoprecipitations, AMPK was purified from human liver up to the DEAE-Sepharose step [12]. The indicated volume of AMPK antiserum was added to the kinase preparation and incubated at 4°C for 2 h. The volume of antiserum added was kept constant by the addition of rabbit immune serum raised against an unrelated peptide. A 50% (v/v) slurry of protein A-Sepharose was added and the incubation continued for a further 2 h at 4°C. The mixture was centrifuged at 14,000 × *g* for 5 min and the supernatant was assayed for AMPK activity by phosphorylation of the synthetic SAMS peptide substrate as previously described [11].

2.5. Somatic cell hybrid DNA amplification

The human/rodent somatic cell panel of PCRable DNAs were purchased from Bios Corp. (New Haven, CT). A sense primer (5′-TTTTGATTCCACAACTGCAGAGAG-3′) derived from the 3′ end of the human AMPK coding sequence was used in combination with an antisense primer (5′-TAGTGCAATAACAGAAAGAAACTA-3′) to amplify the intervening region as described above. PCR was performed with genomic DNA from human, hamster or mouse (controls) and with DNA from the somatic cell hybrids.

2.6. Fluorescent in situ hybridisation (FISH)

DNA isolated from the human genomic clone EMBL1 was labelled by nick translation with biotin 14-dATP for 90 min at 15°C using the BioNick Labelling system and according to the manufacturer's instructions (Gibco/BRL). Metaphase chromosome slide preparations were made in the conventional way using human blood lymphocytes, with BrdU incorporation during late S-phase cell division. FISH mapping of metaphase chromosome preparations was carried out using the protocol of Lichter et al. [16] as modified by Takahashi [17]. The slides were washed in PBS before being counter stained with a 1 μg/ml propidium iodide and 1 μg/ml DAPI mixture in an antifade solution (Vector Labs). A confocal laser scanning microscope was used to detect the fluorescence signals.

3. Results

3.1 Isolation and analysis of human AMPK cDNA

A partial rat AMPK cDNA probe was used to screen a human skeletal muscle cDNA library and isolate 34 positive clones. Flanking λgt10 primers were used to determine the cDNA insert sizes from six plaque purified clones, and a 2.2 kb cDNA insert from clone HS27 was subcloned into pBluescript and sequenced. Since the deduced open reading frame did not contain an in-frame initiating methionine residue, the sense primer Pex211 from the untranslated region of the rat cDNA sequence [13] and the antisense primer Apex 21 from the cloned

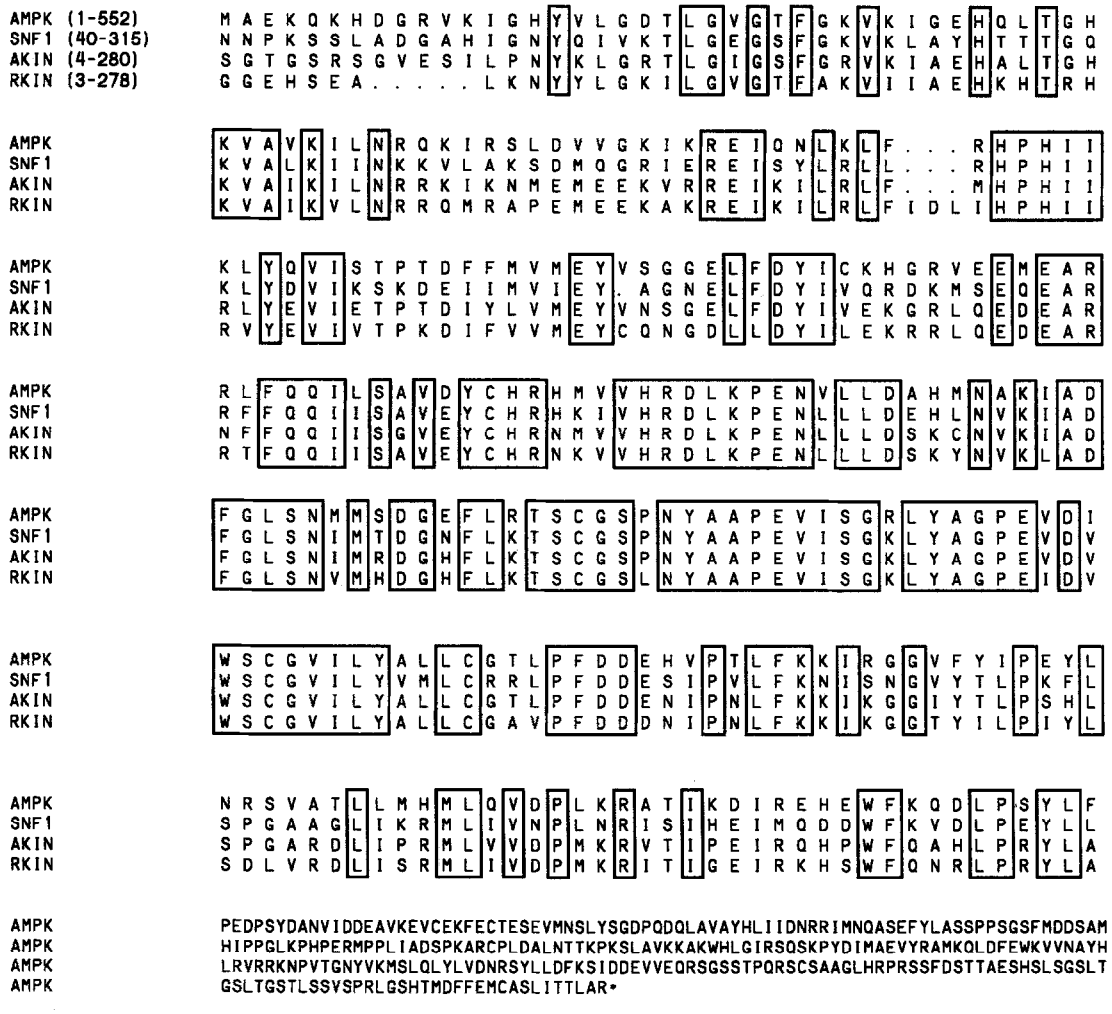

Fig. 1. The deduced amino acid sequence of human AMPK and comparison with yeast SNF1 and plant protein kinases RKIN1 and AKIN10. The sequences were aligned using the PILEUP programme on the University of Wisconsin package. Dots indicate gaps introduced to maximise the alignment. Amino acids identical between the four protein kinases are boxed.

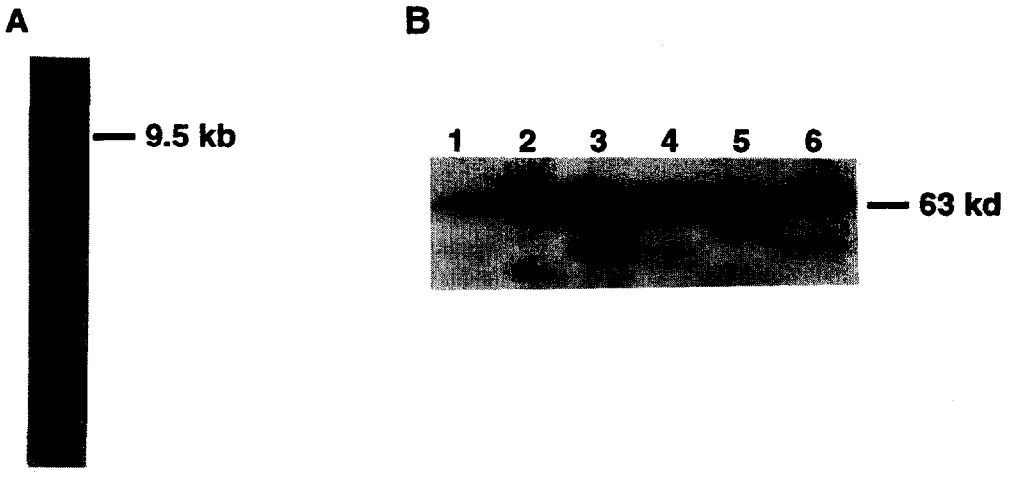

Fig. 2. Northern and Western blot analysis of human AMPK. (A) Human skeletal muscle mRNA was hybridised to radiolabelled rat AMPK cDNA. The position of the 9.5 kb AMPK transcript is marked. (B) A multiple tissue Western blot of proteins (~75 μg total protein per lane) isolated from whole tissue homogenates of brain (lane 1), heart (lane 2), kidney (lane 3), lung (lane 4), skeletal muscle (lane 5), and liver (lane 6) was probed with AMPK-specific antisera as described.

human cDNA sequence were used to amplify and subsequently sequence the N-terminal region from human skeletal muscle cDNA.

The deduced primary structure of human AMPK is shown in Fig. 1. The predicted molecular mass of human AMPK based on an open reading frame (ORF) of 1656 nucleotides is 62,327 Da. The cloned sequence contains all the invariant residues characteristic of serine/threonine protein kinases, with the sequences DLKPEN (residues 139–144) and GSPNYAAPE (residues 175–183) being particularly indicative of serine/threonine specificity [18]. Comparison of human AMPK cDNA with the recently cloned rat AMPK [13] reveals a high degree of homology, with 90% and 98% identity at the nucleotide and amino acid levels, respectively. As shown in Fig. 1, human AMPK shares a high degree of homology with yeast and plant protein kinases involved in the regulation of carbon metabolism. The overall identity at the amino acid level is 48% (SNF1), 47% (RKin1) and 46% (Akin10).

3.2. *Northern and Western blot analysis*

In order to determine the size and tissue distribution of the human AMPK transcript, a Clontech blot was subjected to Northern analysis with a rat AMPK cDNA probe. A single mRNA species of 9.5 kb was detected in skeletal muscle (Fig. 2A) and also found in human heart and kidney upon prolonged exposure (data not shown). Analysis of a Clontech protein blot using an anti-peptide antibody to rat AMPK showed that the apparent molecular mass of the enzyme from a variety of human tissues and the hepatic cell line HepG2 (not shown) is 63 kDa (Fig. 2B).

3.3. *Immunoprecipitation of AMPK*

To demonstrate that we had cloned human AMPK, lysates from human liver were partially purified using DEAE-Sepharose chromatography and incubated in the presence of increasing concentrations of the antipeptide antibody Ab 361/374 [13]. As shown in Fig. 3, there was a loss of AMPK activity (~70%) in the liver lysate following immunoprecipation of the immune complex. By contrast, no loss in activity was observed following immunoprecipitation with control antiserum (data not shown).

3.4. *Chromosomal localisation of human AMPK*

Detection of a simple restriction pattern with human genomic DNA probed with radiolabelled human AMPK cDNA suggests that AMPK exists as a single-copy gene (Fig. 4A). To localise the human AMPK gene, primers derived from the coding region of human AMPK cDNA were used in a PCR reaction with DNA from the somatic cell hybrids. The appearance of the expected product (~190 nt) from 4 of 20 cell line hybrids containing chromosome 1 was 100% concordant with the gene being located on chromosome 1 (data not shown).

To confirm this result and sublocalise the gene, fluorescent in situ hybridisation was carried out on normal metaphase chromosomes using human genomic clone EMBL1. As shown in Fig. 4B, clear signals were localised to the short arm of chromosome 1 (1p31).

4. Discussion

Rat AMPK has been purified and well-characterised biochemically [12], and we have reported the cloning of cDNAs

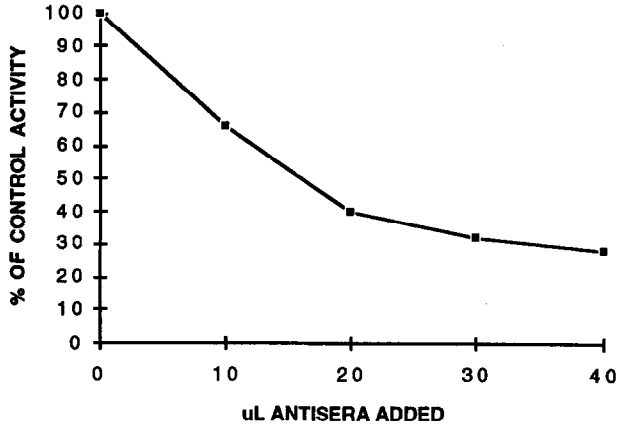

Fig. 3. Immunoprecipitation of AMPK. AMPK was purified from human liver up to the DEAE step and incubated with control serum and increasing amounts of AMPK-specific antiserum for 2 h at 4°C. Activities are plotted as the percentage of the activity in the control supernatant.

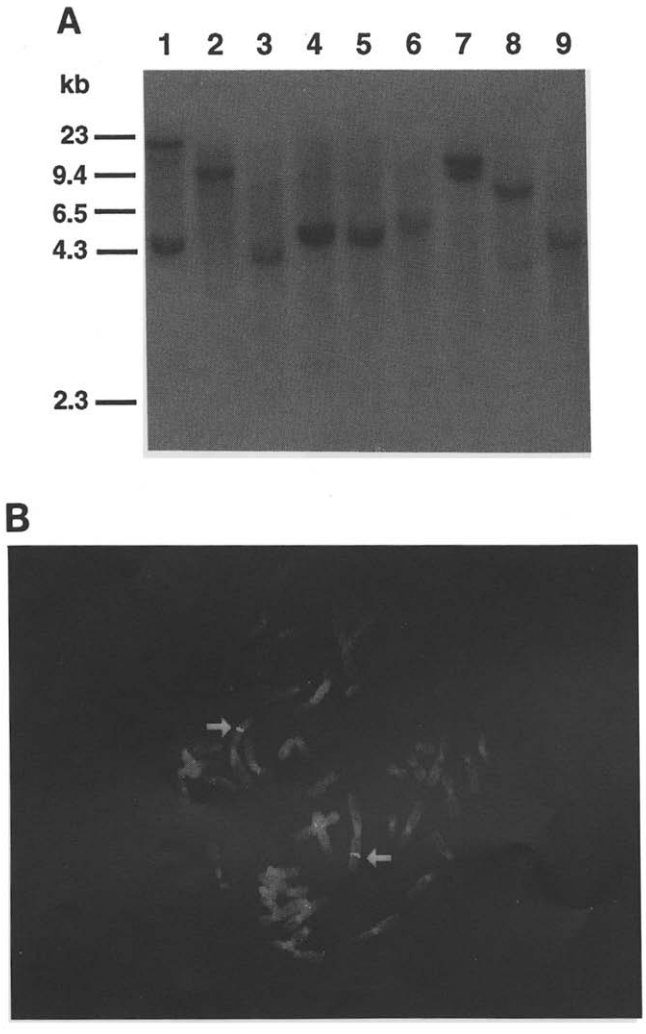

Fig. 4. Southern analysis and chromosomal localisation of the human AMPK gene. (A) Samples of human, hamster and mouse DNA digested with *Eco*RI (lanes 1,2,3, respectively), *Hind*III (lanes 4,5,6) and *Pst*I (lanes 7,8,9) were hybridised to radiolabelled human AMPK cDNA. (B) FISH of human metaphase chromosomes with the human AMPK genomic clone, EMBL1. The arrows indicate the positive fluorescence signals observed on the short arms of chromosome 1.

encoding the catalytic subunit of this protein kinase [13]. As the first step towards a more detailed understanding of human AMPK, we have recently described biochemical properties of partially purified human liver AMPK [11]. Here we report the isolation and sequence of the catalytic subunit of human skeletal muscle AMPK. Since none of the six clones analysed contained an initiating methionine residue, we used sequence information derived from the non-coding region of rat AMPK [13] to clone a cDNA containing the entire coding region of the catalytic subunit of human AMPK. Based on the observation of several well-conserved sequence motifs, the cloned human AMPK cDNA is classified as a member of the serine/threonine protein kinase family [18].

Homology of human and rat AMPK cDNA to other protein kinases ranging from *Saccharomyces cerevisiae* [19] to higher plants [20,21] suggests that these proteins form part of a larger family which may also be functionally related. The high degree of homology of AMPK with the yeast protein kinase SNF1 [19] suggests that AMPK and SNF1 may have similar intracellular roles. Although we have not attempted to rescue the *snf*1 phenotype by complementation with human AMPK, previous work has shown that rat AMPK cDNA is unable to complement the *snf*1 mutation [22]. It has recently been shown that unlike wild-type yeast, *snf*1 mutants lack SAMS peptide kinase activity [22]. Furthermore, the SNF1 protein kinase, like AMPK, is itself regulated by reversible phosphorylation and phosphorylates and inactivates acetyl-CoA carboxylase in vivo [22]. These observations imply that AMPK and SNF1 are functionally, as well as structurally, related.

Southern analysis of human genomic DNA with an AMPK cDNA probe suggests that a single gene is present in the human genome. Strong hybridisation to genomic digests of hamster and mouse DNA indicates that AMPK has been highly conserved and this observation should facilitate the cloning of AMPK from other species. Using somatic cell hybrid DNA with AMPK-specific primers it has been possible to localise the gene to chromosome 1. Furthermore, using FISH, the human AMPK gene was sublocalised to chromosome 1p31. The human AMPK transcript is expressed in a number of tissues and its expression has also been demonstrated in HepG2 and the Hela cell line by PCR using AMPK-specific primers (data not shown). The predicted molecular mass of human AMPK based on the cloned cDNA sequence is approximately 63 kDa, which is similar to the molecular mass seen in human, rat [13] and HepG2 Western blots.

We have not attempted to express recombinant human AMPK cDNA as proof of its functionality since recent work with rat AMPK failed to demonstrate expression of active protein in *E. coli*, baculovirus or mammalian cells [13]. However, using previously described anti-peptide antisera [13], we have shown that AMPK activity can be immunoprecipitated from human liver extracts. In addition, the activity in the immune complex, like the native enzyme, can be stimulated by micromolar concentrations of 5′-AMP. Although the reasons why recombinant rat AMPK is not active are not known, recent evidence suggests that AMPK isolated from liver may exist as a heterotrimeric complex ([23,24] and our unpublished results) with the additional subunits being required for catalytic activity.

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