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Protein Expression and Purification 30 (2003) 230–237

Protein Expression & Purification

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Mammalian AMP-activated protein kinase: functional, heterotrimeric complexes by co-expression of subunits in *Escherichia coli*

Dietbert Neumann,[a] Angela Woods,[b] David Carling,[b] Theo Wallimann,[a] and Uwe Schlattner[a,*]

[a] *Institute of Cell Biology, Swiss Federal Institute of Technology, ETH-Hönggerberg, CH-8093 Zürich, Switzerland*
[b] *MRC Clinical Sciences Centre Cellular Stress Group, Imperial College, Du Cane Road, London W12 0NN, United Kingdom*

Received 4 February 2003, and in revised form 30 March 2003

Abstract

The 5′-AMP-activated protein kinase (AMPK) plays a critical role in the regulation of cellular energy homeostasis. AMPK is a heterotrimer composed of a catalytic subunit (α) and two regulatory subunits (β and γ). To date, purified AMPK has only been obtained in small, microgram quantities from tissues. Here, we describe an expression and purification system for production of functional AMPK in *Escherichia coli.* A plasmid carrying all three subunits of AMPK (α1, β1, and γ1) for T7 RNA polymerase-driven transcription of a single tricistronic messenger was constructed, allowing spontaneous formation of the heterotrimeric complex in the bacterial cytosol. AMPK was purified from the bacterial lysates by single-step nickel-ion chromatography, utilizing a poly-histidine tag fused to the N-terminus of the α-subunit. The recombinant AMPK complex was monodisperse, as shown by gel filtration chromatography with elution of a single peak at a Stokes radius of 52 Å. Bacterially expressed AMPK was entirely inactive, yet it could be activated by upstream kinase in the presence of AMP. Sufficient quantities of purified functional AMPK should prove to be an invaluable tool to solve many of the pertinent questions about its molecular structure and function, in particular facilitating protein crystallization for X-ray structure analysis.

Keywords: Bacterial expression; Polycistronic; Protein complex

Mammalian 5′-AMP-activated protein kinase (AMPK)[1] belongs to a highly conserved protein kinase subfamily, which probably occurs in every eukaryotic cell and plays a unique and central role in maintaining cellular energy homeostasis [1–3]. AMPK is activated by various forms of stress including heat shock, hypoxia and ischemia, as well as by muscle exercise and other stimuli that compromise cellular ATP levels [4,5].

The multiple functions of AMPK in the regulatory network of cellular energy homeostasis are reflected by its complex structure. Native AMPK is a heterotrimeric protein consisting of catalytic α subunits (63 kDa) and regulatory β (30 kDa) and γ subunits (37–63 kDa) [6]. Assembly of the heterotrimeric complex is essential for enzyme activity. So far, two α, two β, and three γ isoforms have been identified, with the β-subunit myristoylated, and the γ-subunit N-acetylated [7,8]. The γ-subunits largely differ in size due to an N-terminal extension found in γ2 and γ3. The regulatory mechanisms involved in activation of AMPK are complex and far from fully understood. AMPK, as its name implies, is activated allosterically by AMP as the cellular AMP:ATP ratio rises. A decreasing phosphocreatine/creatine (PCr/Cr) ratio [9], another sensitive parameter for the cellular energy state [10], also activates AMPK. In addition, the kinase can be activated by unknown AMP-independent pathways [11–13]. Besides allosteric

* Corresponding author. Fax: +41-1-633-10-69.
E-mail address: uwe.schlattner@cell.biol.ethz.ch (U. Schlattner).

[1] *Abbreviations used:* AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; SAMS, the synthetic peptide HMRSAMSGLHLVKRR; T172, threonine 172 of the α-subunit of AMPK.

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doi:10.1016/S1046-5928(03)00126-8

mechanisms, phosphorylation of AMPK by upstream kinase(s) is essential for activation. Threonine-172 (T172) of the α-subunit is the major site of phosphorylation [14] and mutation of T172 in the α-subunit to alanine renders the enzyme inactive [15]. However, there are at least two additional sites in the α-subunit and other putative sites in the β-subunit [7,15].

Once activated, AMPK acts to conserve ATP levels through direct regulation of key metabolic enzymes and the transcriptional control of specific genes [2,3]. Growing evidence supports the involvement of AMPK in human health and disease. Through cellular regulation of insulin-independent glucose uptake [16,17] and glycogen metabolism, AMPK plays a role in exercise-induced adaptation of skeletal muscle, type II diabetes, obesity, and the metabolic syndrome [5,18,19]. Mutations in the γ3-unit lead to glycogen storage disease in pigs [20] and homologous mutations in the γ2-subunit of AMPK have been linked to hypertrophic cardiomyopathy [21,22].

Despite the growing significance of AMPK, research is hampered by the limited availability of purified protein. Small, microgram amounts of AMPK, consisting of non-uniform subunit isoform composition, may be purified from liver [23,24] or from cells transiently expressing the heterotrimer [15]. To date, no expression system has been developed to generate larger quantities of the functional trimeric enzyme. Expression of single subunits in *Escherichia coli* did not yield soluble proteins and attempts to co-renaturate singly expressed subunits were unsuccessful (D. Neumann and D. Carling, unpublished data). In this study, we report a new polycistronic bacterial expression system yielding milligram amounts of heterotrimeric and functional AMPK.

Materials and methods

Subcloning AMPK genes into monocistronic vectors

The multiple cloning sites of commercial pET-vectors (Novagen, Madison, Wisconsin) were modified by standard techniques [25] to contain unique *Xba*I, *Spe*I, and *Blp*I restriction sites (5′–3′, see Fig. 1a). Synthetic linkers were obtained by hybridization of the oligonucleotide pairs TATGGCTAGCATGACTAGTG/GATCCACTAGTCATGCTAGCCA or CATGGCTAGCATGACTAGTG/GATCCACTAGTCATGCTAGC (recognition sequences of restriction enzymes underlined) and inserted into pET-3a (*Nde*I/*Bam*HI digested) or pET-3d (*Nco*I/*Bam*HI digested), respectively, to yield the modified vectors pET-3ax and pET-3dx. To construct the pET14dx-vector encoding an N-terminal polyhistidine-tag, we inserted another synthetic linker (CATGAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCTCCATGGGACACCACA

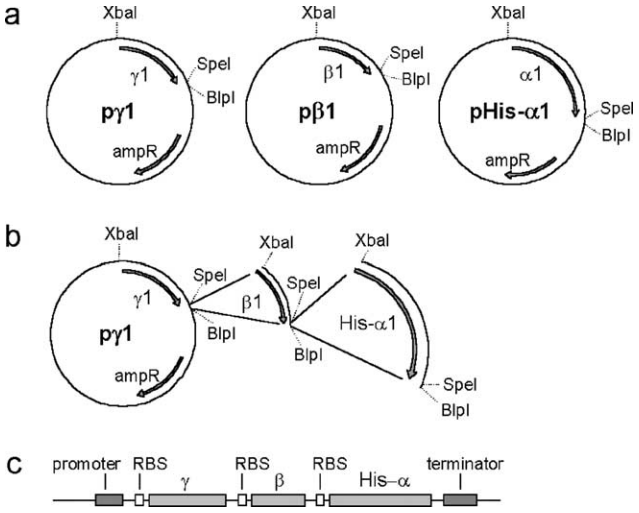

Fig. 1. Cloning scheme for the construction of tricistronic vectors. (a) The cDNA sequences encoding the subunits of AMPK were subcloned into individual pET-plasmids, harboring an ampicillin resistance gene (ampR). (b) Successive restriction and ligation of individual cistrons to the acceptor vector pγ1 lead to a polycistronic gene (c) consisting of a promoter, ribosome-binding sites (RBS), the sequences of γ-, β-, and His-tagged α-subunits of AMPK, as well as a transcriptional terminator. The three restriction sites necessary for cloning are indicated.

CCA hybridized to CTAGTGGTGGTGTC CCATGG AGCCGCGCGGCACCAGGCCGCTGCTGTGATGA TGATGATGATGGCT) into pET-3dx (*Nco*I/*Spe*I digested).

The cDNAs encoding AMPK subunits (GenBank Accession Nos. X95578, X95577, and U40819) were subcloned by PCR and inserted into the *Nde*I/*Spe*I sites of pET3-ax (γ1-subunit, resulting in pγ1), the *Nco*I/*Spe*I sites of pET-3dx (β1-subunit, resulting in pβ1), and the *Nco*I/*Spe*I sites of pET14dx (α1-subunit, resulting in pHis-α1r). The following primers were utilized for subcloning: 5′γ1 AATTTCTACATATGGAGTCGGTTG, 3′γ1 AATTCGACTAGTGCTCAGGGCTTC; 5′β1 TACTTCGCCATGGGCAATACGAGCAG, 3′β1 TTATTGACTAGTCCTCTCATATGGG; and 5′α1 TATTCCATGGCCGAGAAGCAGAAG, 3′α1 TAATATGCACTAGTTACTGTGCAAG. Since the open reading frame of α1 contained a cluster of rare codons resembling a bacterial ribosome-binding site around nucleotides 330, the sequence was modified by silent mutagenesis using the primers TCGCCGCATTATGAACGAAGCCAAAG and TTGTCAATAATCAGGTGGTAGGCAACTG (mismatches underlined), resulting in pHis-α1. Open reading frames of all constructs were sequenced utilizing the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Rotkreuz) in an ABI Prism 310 Sequencer according to manufacturer's instructions. The vectors pHis-α1, pβ1, and pγ1 were used for construction of the polycistronic expression vector (see results section and Fig. 1). Table 1 lists all constructs used throughout this study.

Table 1
Overview of plasmid constructions used throughout this study

Constructs	Insert/Modification
pET3ax	Introduction of *Spe*I site to pET3a
pET3dx	Introduction of *Spe*I site to pET3d
pET14bx	Introduction of *Spe*I site to pET14b
pγ1	γ1 subcloned into the *Nde*I/*Spe*I sites of pET3ax
pβ1	β1 subcloned into the *Nco*I/*Spe*I sites of pET3dx
pHis-α1	α1 subcloned into the *Nde*I/*Spe*I sites of pET14bx
pγ1β1	pγ1 digested with *Spe*I/*Blp*I and ligated to *Xba*I/*Blp*I-fragment of pβ1
pγ1β1His-α1	pγ1β1 digested with *Spe*I/*Blp*I and ligated to *Xba*I/*Blp*I-fragment of pHis-α1

Expression and purification of recombinant AMPK

The tricistronic plasmid pγ1β1His-α1 was transformed into competent host cells (*E. coli* BL21-CodonPlus (DE3)-RIL, Stratagene, Basel) and incubated overnight at 37 °C on LB agar containing 150 μg/ml ampicillin and 25 μg/ml chloramphenicol. Resuspended cells were used to inoculate LB medium containing appropriate antibiotics. Cultures were grown in a shaker incubator at 37 °C. Protein expression was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside final concentration at an O.D. (600 nm) of 0.6 and cultures were grown for additional 4 h. Cells were harvested, washed twice with 0.9% NaCl, resuspended in lysis buffer (15% sucrose (w/v), 50 mM Na-phosphate, pH 8.0, 50 mM NaCl, 10 mM imidazole, and 1 mM β-mercaptoethanol), and sonicated on ice in a Branson 250 sonifier (50% duty, output 5, 2 min, three times). Insoluble material was removed by centrifugation and the supernatant was loaded onto Ni–NTA agarose (Qiagen, Basel). After washing (three column volumes with lysis buffer, 20 mM imidazole) and elution (lysis buffer, containing 250 mM imidazole), the protein was stored at −20 °C until use.

Gel filtration chromatography

The size of pure AMPK complex in solution was determined by gel filtration chromatography with a Superose 12 HR 10/30 column (Amersham-Pharmacia) connected to HPLC (BioCad, Perkin–Elmer). Minor non-protein contaminants in Ni^{2+}-purified AMPK were removed by batch affinity purification with Blue Sepharose (Amersham-Pharmacia), using elution with 50 mM sodium phosphate, 1 M NaCl, 50 mM AMP, and 1 mM β-mercaptoethanol at pH 8.5. One hundred micrograms of protein was then separated in 25 mM sodium phosphate, pH 7.0, 200 mM NaCl, and 1 mM β-mercaptoethanol at a flow rate of 0.75 ml/min and a temperature of 22 °C. The column was calibrated for Stokes radii with the following marker proteins (Amersham-Pharmacia): carbonic anhydrase (24.0 Å, 29 kDa), albumin (35.5 Å, 67 kDa), aldolase (48.1 Å, 158 kDa), catalase (52.2 Å, 232 kDa), ferritin (61 Å, 440 kDa), and thyroglobulin (70.0 Å, 669 kDa).

Western blotting

Proteins were separated by standard SDS–PAGE using 12% polyacrylamide and transferred onto polyvinylidene fluoride (PVDF) membrane. The membrane was blocked in TBS (10 mM Tris–HCl, pH 7.4, 0.5 M NaCl) with 5% low fat milk powder. The primary antibody was applied in the same buffer at various dilutions and the blot was incubated for 2–16 h. After extensive washing in TBS, the blots were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase. After further extensive washing, blots were developed using enhanced chemiluminescence (Amersham). The following antibodies raised to specific AMPK subunits were applied: α1 (sheep, TSPPDSFLDDHHLTR), β1 (rabbit, β1 GST fusion protein), and γ1 (sheep, PENEHSQETPESNS), α-subunit phospho-T172-specific antibody was obtained from Cell Signaling Technology, Beverly, MA.

Activation of AMPK and activity measurements

Ni^{2+}-purified recombinant AMPK was activated with a partially purified preparation of AMPKK (purified up to the Q-Sepharose step [14], with the exception that NaF and sodium pyrophosphate were omitted from the buffers) in the presence of 100 μM ATP, 5 mM $MgCl_2$, and 1 mM DTT in 50 mM Hepes, pH 7.4, for 30 min at 37 °C in a thermostated shaker. Samples were then diluted in Hepes assay buffer (50 mM Hepes–HCl, pH 7.4, 1 mM EDTA, 10% glycerol, 50 mM NaF, 5 mM Na-pyrophosphate, and 1 mM DTT) and assayed for AMPK activity by phosphorylation of a synthetic peptide substrate (HMRSAMSGLHLVKRR, the "SAMS" peptide) [26] in the presence or absence of various concentrations of AMP. For determination of K_m, AMPK was assayed at varying concentrations of SAMS peptide in the presence of 200 μM AMP. Partially purified AMPK from anoxic rat liver (purified up to DEAE–Sepharose [23]) was also assayed by phosphorylation of the SAMS peptide. Activities are given as mean values ± SEM of four independent experiments and expressed as nanomole phosphate incorporated per minute and microgram protein. Kinetic analysis was performed using Graphpad Prism software, as previously described [15].

Phosphorylation of AMPK

Ni^{2+}-purified recombinant AMPK was incubated in the presence or absence of AMPKK (Q-Sepharose pure

[14]) in the presence of 100 μM [γ-^{32}P]ATP (approx. 400 cpm/pmol), 100 μM AMP, 5 mM $MgCl_2$, and 1 mM DTT for 30 min at 37 °C in a thermostated shaker. After dilution, AMPK was repurified on NTA–Nickel magnetic agarose beads (Qiagen, Basel) before adding SDS sample buffer and running on 12% acrylamide SDS gels.

Results

Construction of vectors for polycistronic expression in E. coli

The polycistronic vector was constructed from individual monocistronic vectors by repetitive cycles of restriction and ligation, using only three different restriction sites. In every cycle, restriction sites are eliminated through ligation of compatible cohesive ends and a new site is introduced (summarized in Figs. 1a–c, for details see Materials and methods). For construction of the monocistronic vectors encoding only single subunits, the multiple cloning site of the pET-vectors was modified to contain *Xba*I, *Nde*I/*Nco*I, *Spe*I, and *Blp*I in this order. The ribosome-binding site resides between *Xba*I and *Nde*I/*Nco*I sites. The start codon of the open reading frame then is located inside the recognition sequence of *Nde*I or *Nco*I. Directed cloning of the open reading frames encoding AMPK subunits between *Nde*I/*Nco*I and *Spe*I yielded a set of monocistronic plasmids named pHis-α1, pβ1, and pγ1 (Fig. 1a). In pHis-α1, a hexahistidine-tag is fused to the N-terminus of the α-subunit to simplify purification of the recombinant protein complex by Ni^{2+}–NTA-affinity chromatography. Any of the monocistronic vectors, in our case pγ1, may then serve as the receiving vector for the integration of additional cistrons (Fig. 1b). In each cloning cycle, the receiving vector is digested with *Spe*I and *Blp*I, the donor vector with *Xba*I and *Blp*I. Ligation of the new cistron insert into the receiving vector destroys the recognition sequences for the compatible *Spe*I/*Xba*I sites and at the same time introduces a new *Spe*I site. Every successive cloning step adds a single cistron, carrying its individual ribosome-binding site (RBS) at an appropriate distance to the next start codon, thus forming a polycistronic vector. Following this protocol, the tricistronic vector pγ1β1His-α1 has been constructed (Fig. 1c).

Co-expression of subunits and purification of heterotrimeric AMPK complex

Induction of *E. coli* BL21(DE3) transformed with pγ1β1His-α1 led to the appearance of three new protein bands in whole cell extracts, as analyzed by SDS–PAGE (Fig. 2 lanes a and b). The same bands were purified from a clarified bacterial lysate by Ni^{2+}-affinity chromatography (Fig. 2, lane c). The three different eluted polypeptides have apparent molecular masses of 63, 39, and 36 kDa, which correspond well to the predicted values of hexahistidine-tagged α1 (63 kDa) and γ1-subunits (37 kDa). The anomalous electrophoretic mobility of the β1-subunit with an expected molecular mass of 30 kDa has also been observed with the enzyme purified from liver [27,28]. Since only His-tagged α1-subunit is expected to specifically bind to the Ni^{2+}-column, coelution of all three AMPK subunits at an apparently equimolar ratio as indicated by Coomassie blue staining shows the formation of a stable hetero-trimeric complex. The single chromatographic step led to AMPK protein with a purity of $\geqslant 95\%$, as judged by Coomassie blue staining of the gel (Fig. 2, lane c), with a total yield of about 2 mg/liter of bacterial culture. The purification is summarized in Table 2.

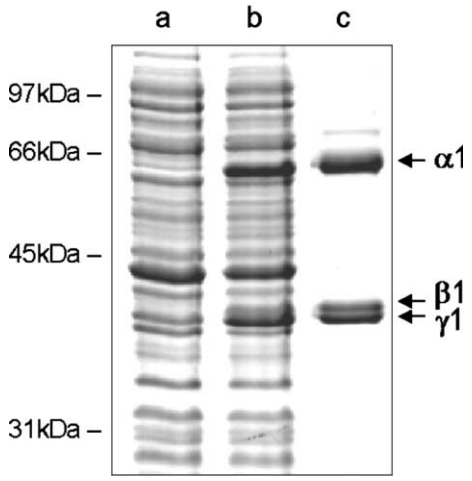

Fig. 2. SDS–PAGE of fractions from AMPK expression and purification steps, stained with Coomassie blue. Lanes: (a) whole cell extract of pγ1β1His-α1-transformed *E. coli* cells before induction with IPTG, (b) after induction with IPTG, and (c) AMPK after purification by Ni^{2+}-affinity chromatography.

Molecular properties of recombinant AMPK

The purified, recombinant complex was applied to gel filtration chromatography, using a calibrated Superose-

Table 2
Summary of purification of recombinant AMPK

No.	Purification step	Total units (U)[a]	Total protein (mg)	Specific activity (U/mg)
1	*E. coli* lysate	—[b]	243	—[b]
2	Ni^{2+}–agarose	1480	2	740

[a] Unit is defined as 1 nmol phosphate incorporated into SAMS peptide per minute.

[b] Substances in the *E. coli* lysate interfere with the activation of AMPK by its upstream kinase and/or the activity assay.

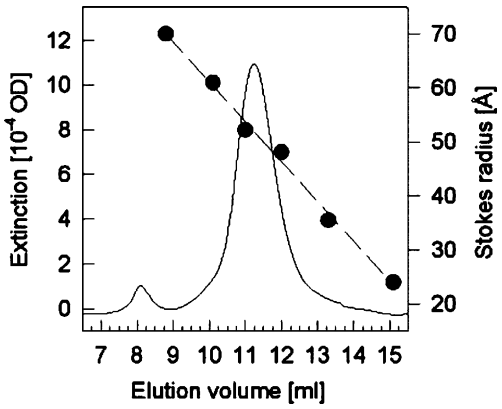

Fig. 3. Gel filtration chromatography of purified AMPK complex. About 100 μg protein (50 μl) was separated on a Superose 12 column (Pharmacia) at a flow rate of 0.75 ml/min and 22 °C. The trace (solid line) gives the absorption at 280 nm (left scale). The column was calibrated for Stokes radii (right scale) by different marker proteins (black circles and regression line, for details see Materials and methods).

12 column (Fig. 3). AMPK eluted as a single peak, indicating a largely monodisperse solution with an apparent Stokes radius of 52 Å. A similar value (54–58 Å) for the Stokes radius of the native enzyme isolated from rat liver was previously reported [24]. In that report, the authors presented evidence, suggesting that the enzyme complex forms an asymmetric structure. A small fraction of protein eluting at the size exclusion limit of the column (8 ml) corresponds to AMPK aggregates formed in the gel filtration buffer at 200 mM NaCl, as judged by Coomassie-stained SDS–PAGE of the corresponding elution fraction.

Enzymatic activity, phosphorylation, and AMP-dependence of recombinant AMPK

Kinase activity of the purified recombinant enzyme can be determined by incorporation of ^{32}P-phosphate into the SAMS peptide, a specific substrate of AMPK [26]. Initially, the purified recombinant enzyme was completely inactive, but it exhibited significant activity after incubation with an upstream kinase preparation (Fig. 4a). The kinase was activated 3.3-fold by 200 μM AMP, with a half-maximal effect at 1.5 μM. These values are similar to those previously reported for the α1 complex isolated from rat liver (1.7-fold stimulation, half-maximal effect at 12 μM [29]) and recombinant α1β1γ1 expressed in mammalian cells (3-fold stimulation, half-maximal effect at 5.7 μM [15]). The specific activity of the kinase measured in the presence of 200 μM AMP was 0.74 (±0.08) nmol/min/μg. Although this activity is lower than that previously reported for the kinase purified from rat liver [24], these results demonstrate that recombinant AMPK can be activated via phosphorylation by an upstream kinase and is sensitive to AMP. Further kinetic analysis of the recombinant AMPK complex yielded a K_m value for the SAMS peptide of 46.6 ± 9.9 μM. This is very similar to the values previously reported for the native rat liver enzyme (59 μM [30] and 26 μM [31]). Taken together, these results demonstrate the validity of the recombinant complex for studying AMPK function and regulation.

Western-blotting was used to compare the partly purified native enzyme isolated from hypoxic liver with Ni^{2+}-affinity-purified recombinant enzyme before and after incubation with the upstream kinase preparation (Fig. 4b). Subunit-specific antibodies confirmed the identity of α-, β-, and γ-subunits in the recombinant AMPK preparation. When loaded with equal amounts of AMPK activity, liver and AMPKK-activated recombinant enzyme revealed about equal amounts of β- and γ-subunits. This confirmed that both complexes could be activated to the same extent. The lower amount of α1 present in the liver sample is explained by the simultaneous presence of α2 in this preparation, which

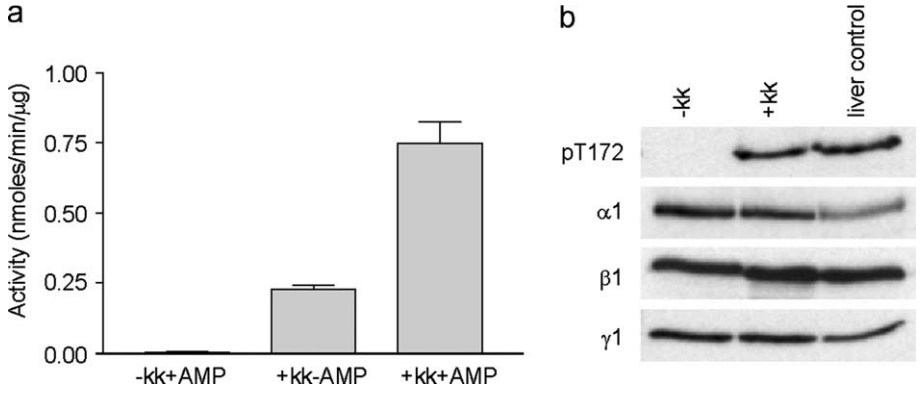

Fig. 4. Activation of recombinant AMPK (α1β1γ1). (a) Activation of AMPK, as measured by SAMS assay, following incubation in the presence or absence of the upstream kinase (kk) and the presence or absence of 200 μM AMP. Activities are given as mean values (±SEM) from three independent experiments and are plotted as nanomole phosphate incorporated per minute and microgram protein. (b) Western blot of AMPK using subunit-specific antibodies raised to either α1, β2, or γ1 or to phosphorylated T172 within the α subunit (pT172). Equal amounts of untreated (inactive) recombinant AMPK (−kk) or following phosphorylation with the upstream kinase (+kk) were used. AMPK from hypoxic rat liver (liver control) was applied at equal AMPK activity as of the recombinant AMPK activated with upstream kinase.

accounts for approximately 50% of the total activity [32]. An antibody specifically recognizing phosphorylated T172 in the α-subunit gave no signal with untreated recombinant AMPK, in agreement with the absence of any enzymatic activity in this preparation. In contrast, upon incubation with the upstream kinase preparation, both enzymatic activity (Fig. 4a) and phosphorylation of T172 (Fig. 4b) were increased. Again, liver and activated recombinant enzyme were similar with respect to the phospho-T172 signal and presumably therefore activated to the same extent.

Discussion

Although AMPK was first described in 1973, yet under a different name [33,34], and has been implicated in human health and disease [5,21,35], information on molecular structure and function of this important protein kinase is very scarce. Here, we present a strategy utilizing a polycistronic vector for expression of functional heterotrimeric AMPK. It is envisaged that this recombinant enzyme will prove to be a versatile tool to solve many of the major pertinent questions concerning AMPK, especially its biochemical and biophysical properties and the multiple mechanisms controlling its activity in vivo.

Vectors for polycistronic expression of heterologous protein complexes in *E. coli* have been described previously (e.g., [36–38]). However, our new cloning strategy for construction of polycistronic vectors combines ease of use with a minimal number of necessary restriction sites and a theoretically unlimited number of cistrons. Additionally, our polycistronic mRNA with identical 5′-sequences in every cistron, including the ribosome-binding site, may favor the stoichiometric production of all three subunits of AMPK in a single cell. Indeed, in contrast to earlier findings with tricistronic vectors [39], we found that all AMPK subunits were expressed in apparently equimolar amounts.

The three expressed polypeptides were identified as AMPK subunits by their apparent molecular weight determined by SDS–PAGE and their reaction with subunit-specific antibodies. Additionally, they co-eluted with His-tagged α1-subunit from the Ni^{2+}-affinity column. Thus, AMPK subunits assemble into heterotrimeric complex in the cytoplasm of *E. coli* without overexpression of specialized proteins that would assist in folding or assembly of the enzyme. In addition, N-terminal myristoylation of the β-subunit or N-acetylation of the γ-subunit [7,8] does not seem to be critical for either enzyme activity or for stability of the complex. A single chromatographic step was sufficient for purification of the complex to near homogeneity and yielded an almost monodisperse, homogeneous solution as seen by gel filtration chromatography. However, the molecule elutes somewhat larger than expected from the molecular mass of the complex, possibly indicating a deviation from a perfectly globular shape. Consistent with these results, an asymmetric structure was previously proposed for the enzyme isolated from rat liver [24].

The purified enzyme was then characterized in respect to activation by the upstream kinase, enzyme activity, stimulation by AMP, and determination of K_m for one of its substrates, the SAMS peptide. All of these kinase properties are in accordance with previous data of the native liver enzyme [29–31]. We conclude therefore that bacterially expressed AMPK is fully functional. In contrast to that of native AMPK purified from hypoxic liver, the recombinant enzyme is inactive and unphosphorylated at Thr172 within the α-subunit unless activated by the upstream kinase preparation. This makes the recombinant AMPK an invaluable tool for the elucidation of phosphorylation events. The unlimited availability of unphosphorylated AMPK as a substrate for AMPKK may also greatly facilitate identification of the upstream kinase(s), which so far has remained elusive.

In mammals, up to 12 different AMPK isoenzymes may occur [4]. Although AMPK is not very abundant, it is possible to purify small amounts of AMPK from eukaryotic sources [23]. However, purification of AMPK from tissue usually results in a mixture of AMPK isoenzymes, unless extensively repurified by immunoaffinity techniques (D. Carling et al., unpublished data). Expression of all three AMPK subunits via transfection of eukaryotic cells [15] gives very limited yield and a non-uniform subunit composition, since the transfection rate needs to be very high for all encoding plasmids. Otherwise, the recombinant subunits combine with the endogenous subunits present in every eukaryotic cell. An important advantage of the bacterial expression system is the absence of any close homologue of AMPK, allowing the production of AMPK with a defined subunit composition. Using our polycistronic expression system, it should also be feasible to produce other combinations of subunit-isoforms of AMPK. Such preparations of recombinant protein will allow a detailed biochemical and biophysical characterization of AMPK isoenzymes, including allosteric regulation and kinetic properties. Finally, a high yield bacterial expression has often been a prerequisite for structural characterization by X-ray crystallography. Since AMPK has become a potential target for treatment of diabetes type II, as well as the metabolic syndrome [5,18,19], a molecular structure at atomic resolution would greatly facilitate the rational design of AMPK-specific drugs.

Taken together, the recombinant enzyme generated by our new polycistronic bacterial expression system will overcome many disadvantages of other sources that have limited AMPK research for decades: (i) it can be produced in large amounts, (ii) it forms functional

heterotrimeric complexes, (iii) complexes have defined homogeneous subunit composition, and (iv) enzymatic activity is comparable to native enzyme. The expression system is therefore expected to provide a valuable and important tool for future research focusing on structure or function of AMPK [40].

Acknowledgments

The work was supported by an ETH graduate student grant (to D.N., U.S., and T.W.), the Swiss National Science Foundation (Grant No. 31-62024.00 to T.W. and U.S.), the Swiss Cancer League and the Zentralschweizer Krebsstiftung (to U.S. and T.W.), the Swiss Society for Research on Muscle Disease and the German Parent Organization Benni (to T.W.). This study was further supported by the Medical Research Council, UK (to A.W. and D.C.), and Contract QLG1-CT-2001-01488 from the European Commission (to D.C.).

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