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ATP sensitive bi-quinoline activator of the AMP-activated protein kinase

John W. Scott [a,*], Jonathan S. Oakhill [a], Naomi X.Y. Ling [a], Christopher G. Langendorf [a], Richard C. Foitzik [b], Bruce E. Kemp [a], Olaf-Georg Issinger [c]

[a] St. Vincent's Institute and Department of Medicine, University of Melbourne, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia
[b] Cancer Therapeutics CRC Pty. Ltd., Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville 3052, Victoria, Australia
[c] Biomedical Research Group, BMB, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark

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ABSTRACT

The AMP-activated protein kinase (AMPK) regulates cellular and whole-body energy balance in response to changes in adenylate charge and hormonal signals. Activation of AMPK in tissues such as skeletal muscle and liver reverses many of the metabolic defects associated with obesity and Type 2 diabetes. Here we report a bi-quinoline (JJO-1) that allosterically activates all AMPK αβγ isoforms *in vitro* except complexes containing the γ3 subunit. JJO-1 does not directly activate the autoinhibited α subunit kinase domain and differs among other known direct activators of AMPK in that allosteric activation occurs only at low ATP concentrations, and is not influenced by either mutation of the γ subunit adenylate-nucleotide binding sites or deletion of the β subunit carbohydrate-binding module. Our findings indicate that AMPK has multiple modes of allosteric activation that may be exploited to design isoform-specific activators as potential therapeutics for metabolic diseases.

1. Introduction

The AMP-activated protein kinase (AMPK) is an important regulator of cellular and whole-body energy homeostasis that coordinates metabolic pathways to balance energy supply with demand. At the cellular level, AMPK functions primarily as a fuel gauge monitoring the relative levels of adenylate nucleotides (ATP/ADP/AMP), which can be regarded as a molecular read-out of cellular energy charge [1,2]. AMPK protects cells from stresses (fuel deprivation, hypoxia, exercise) that lower cellular energy charge by orchestrating a switch in metabolism in favour of ATP-producing catabolic pathways while inhibiting ATP-consuming anabolic processes. It achieves this largely by direct phosphorylation of enzymes that catalyse the rate-limiting steps of major metabolic pathways such as fatty acid, sterol, and carbohydrate metabolism. AMPK also plays an adaptive role to chronic energy challenges by reprogramming the expression of metabolic genes via phosphorylation of transcription factors and co-regulators [3,4]. In addition to regulating energy balance at the cellular level, AMPK is also a central regulator of whole-body energy metabolism, integrating a variety of hormonal (leptin, ghrelin and adiponectin) and nutritional signals in the central nervous system and periphery to control feeding behaviour and body weight [5]. Consequently, there is now considerable interest in developing direct activators of AMPK for the treatment of metabolic diseases including Type 2 diabetes and obesity.

AMPK exists as a heterotrimeric complex composed of an α subunit that contains the kinase and autoinhibitory domains (AID), as well as regulatory β and γ subunits that contain a carbohydrate-binding module (CBM) and the allosteric adenylate nucleotide-binding sites, respectively. Multiple isoforms exist for each subunit in mammals (α1, α2, β1, β2, γ1, γ2 and γ3), therefore at least twelve heterotrimeric combinations are possible giving rise to differences in tissue distribution, regulation and function. There are four potential adenylate-binding sites on the γ subunit, however site 2 does not appear to be functional [6–8]. Sites 1 and 3 are exchangeable sites that can bind either ATP, ADP or AMP, whereas binding of ATP to site 4 occludes nucleotide binding at site 3 [9]. The key step in the activation of AMPK is phosphorylation of Thr172 in the activation loop of the α subunit by either LKB1 or Ca^{2+}/calmodulin-dependent protein kinase kinase-2 (CaMKK2) [1]. Binding of ADP or AMP to the γ subunit promotes phosphorylation of Thr172 by the upstream kinases provided that the β subunit is myristoylated [6,10]. In addition, both ADP and AMP maintain the active state of AMPK by inhibiting dephosphorylation of Thr172 by protein phosphatases [8]. AMP, but not ADP, causes a further allosteric activation of phosphorylated AMPK, however this effect is relatively modest compared with Thr172 phosphorylation but sufficient to stimulate downstream signalling in cells [11].

The first small-molecule direct activator of AMPK reported was the thienopyridone drug A769662 [12]. Whereas AMP can activate all twelve possible αβγ isoform combinations of AMPK, A769662

* Corresponding author. Fax: +61 39416 2676.
E-mail address: jscott@svi.edu.au (J.W. Scott).

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selectively activates complexes containing the β1 subunit [13]. Activation of AMPK-β1 containing complexes by A769662 is dependent on the CBM and, in particular, the Ser108 autophosphorylation site that resides within this domain [13,14]. Several direct small-molecule AMPK activators have since been reported including the furanothiazolidine derivative PT1 and the naturally-occurring molecule sanguinarine [15–17]. PT1 is thought to activate AMPK by relieving inhibition of kinase activity by the AID in the α subunit. Sanguinarine selectively activates AMPK complexes containing the α1 and γ1 subunits, but is not selective for the β1 isoform like A769662. These findings provide a potential pathway for the development of isoform-selective activators of AMPK and may ultimately be exploited to target the activation of AMPK in specific tissues.

In the present study, we report the discovery of a small-molecule (JJO-1) that directly activates AMPK in cell free assays but only at low ATP concentrations. We show that JJO-1 activates all AMPK αβγ isoforms except complexes containing the γ3 subunit, and that activation occurs independently of the β subunit CBM. JJO-1 now joins a growing list of isoform-selective activators of AMPK.

2. Materials and methods

2.1. Molecular biology

Plasmid constructs for AMPK α1, α2, β1, β2 and γ1, γ2 and γ3 subunits for expression in COS7 cells were generated as described previously [13]. Point mutations were generated using Quikchange site-directed mutagenesis (Stratagene) and deletion mutants as described previously [6,13]. All plasmid constructs were verified by sequencing the entire open reading frame. DNA for transfection was prepared using HiSpeed MaxiPrep kit (QIAGEN) and quantified by absorbance at 260 nm.

2.2. Expression of recombinant AMPK and mutant variants

COS7 cells were grown in Dulbecco's Modified Eagle Medium (Sigma) with 10% foetal calf serum at 37 °C with 5% CO_2. Cells were transfected at 60% confluency with 1 μg of the various plasmids expressing human AMPK subunits (pcDNA3 GSTα1, GSTα2, myc-tagged β1, myc-tagged β2 and pMT2 HA-tagged γ1, γ2 and γ3) and deletion/point mutants using FuGene 6 (Roche) according to the manufacturers instructions. Transfected cells were harvested after 36 h by washing with ice-cold phosphate-buffered saline (PBS) followed by rapid lysis *in situ* using 1 ml of lysis buffer (50 mM Tris.HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 1 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% [v/v] Triton X-100) containing protease inhibitors (Roche). For drug treatments, transfected COS7 cells (36 h post-transfection) were incubated with fresh media for 1 h after which either JJO-1, phenformin (Sigma) or a DMSO control was added for a further 1 h, then harvested as described above. Cellular debris was removed by centrifugation and total protein was determined using the Bradford protein assay (Bio-Rad).

2.3. AMPK purification and activity assays

AMPK complexes were purified by glutathione-Sepharose pull down from 50 μg of lysate using 10 μl of a 50% slurry of glutathione Sepharose (GE Healthcare) pre-equilibrated with lysis buffer (see above), followed by successive washes in lysis buffer containing 1 M NaCl and finally into assay buffer (50 mM HEPES [pH 7.4], 1 mM DTT, 0.02% [v/v] Brij-35). AMPK activity was determined in the pulldowns by phosphorylation of the SAMS peptide using 100 μM SAMS, 200 μM [γ-^{32}P]-ATP (Perkin Elmer), 5 mM $MgCl_2$ in the presence and absence of either JJO-1, A769662 (Creagen), PT1 (Tocris Bioscience) or AMP (Roche) in a standard 25 μl volume assay at 30 °C. Reactions were terminated after 10 min by spotting 15 μl onto P81 phosphocellulose paper (Whatman) and washing in 1% phosphoric acid (Merck). Radioactivity was quantified by scintillation counting. Kinase activity was corrected for variations in protein expression by immunoblotting for the α1 subunit and densitometry. AMPK phosphorylation and dephosphorylation assays were performed as described previously [6]. For the chemical library screen, AMPK activity was determined using a luminescence ATP detection assay system (Perkin Elmer) following the manufacturers instructions.

2.4. Immunoblotting

Glutathione-Sepharose purified or immunoprecipitated AMPK was denatured in SDS–PAGE sample buffer, separated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h in PBS-T supplemented with 2% non-fat milk, and then incubated with conjugated primary anti-α1 (IR800 dye) and PT172 (IR680 dye) antibodies. Binding was detected using the LiCor Odyssey Infra-red dual detection system.

2.5. Statistical analysis

Results are expressed as the mean ± standard error of mean (SEM). Statistical analysis, where indicated, was performed using one-way analysis of variance (ANOVA), with the alpha level set at 0.05 for each test. *P*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Identification of a novel direct activator of AMPK

Using recombinant AMPK-α1β1γ1 and an ATP depletion luminescence kinase assay, we screened the Diverse Compound Library Set 1 from the National Cancer Institute and identified a bi-quinoline compound (JJO-1) as a direct activator of AMPK (Fig. 1A). To confirm the screening hit, we resynthesised the compound and measured activation of AMPK using the standard [γ-^{32}P]-ATP based assay. Although JJO-1 activated AMPK using the luminescence assay, we were unable to demonstrate activation using the standard assay. A key difference between the assays is the final concentration of ATP that is used (1 μM for the luminescent assay compared with 200 μM for the standard assay), therefore we investigated whether this could explain the discrepancy. Indeed, ATP concentrations of 50 μM or lower in the standard assay restored the activation of AMPK by JJO-1 (Fig. 1B). At 20 μM ATP, JJO-1 activated AMPK approximately 2.7-fold with a half-maximal concentration of 1.8 ± 0.2 μM (Fig. 1C).

The subunit isoform composition of an AMPK complex is an important factor in determining sensitivity to AMP and A769662 [13,18]. To test whether activation of AMPK by JJO-1 is influenced by the presence of a particular subunit isoform, we expressed all twelve possible AMPK αβγ and measured kinase activity in the presence and absence of either JJO-1, A769662 or AMP at 20 μM ATP. Unlike A769662, which selectively activates AMPK-β1 containing complexes, JJO-1 activated all AMPK complexes except those containing the γ3 subunit (Fig. 1D). In contrast, A769662 did not activate β2 containing complexes but did activate β1γ3 containing complexes, whereas AMP activated all the subunit isoform combinations.