Comparison of Purified Recombinant AMPK $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ Activation and Inhibition using Fluorescence-based Assays

John Printen, Laurie Reichling, Steve Riddle, Rica Bruinsma, Alyssa Newport, Kristin Cleary,* Xiao-Dong Qian,* and Kurt W. Vogel

Invitrogen Corporation • 501 Charmany Dr • Madison WI *Invitrogen Corporation • 94/96 South Street • Hopkinton, MA

Introduction

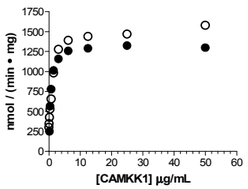
AMP activated protein kinase (AMPK) is a heterotrimeric complex consisting of a catalytic α subunit, a scaffolding β subunit, and a γ subunit capable of binding AMP or AMP analogs. AMPK is a key regulator of cellular metabolism, and its activity is modulated in part by an allosteric mechanism in which binding of AMP to the γ-subunit causes an increase in activity of the catalytic α-subunit. Because therapeutic targeting of AMPK for type-2 diabetes and other metabolic disorders aims to identify activators of the kinase, this allosteric mechanism of activation offers a unique opportunity to develop small molecules that directly stimulate AMPK activity through this mode of action. Additionally, recent work suggests that inhibitors of AMPK may be useful in targeting obesity.

To address the need for methods to identify and characterize small-molecules that show isoform-specific effects on AMPK, we have expressed and purified recombinant isoforms of AMPK, and have developed a suite of fluorescence-based assays to identify and characterize such compounds.

Development of Recombinant, Heterotrimeric AMPK

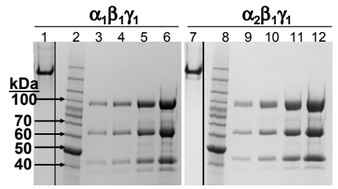
Recombinant heterotrimeric AMPK was purified from baculovirus infected insect cells. The purified AMPK was then further activated by the upstream kinase CAMKK1. After additional purification, AMPK was determined to be > 80% pure by native and denaturing PAGE analysis. ATP K_m values were then determined in the presence or absence of 100 µM AMP.

Activation by CAMKK1



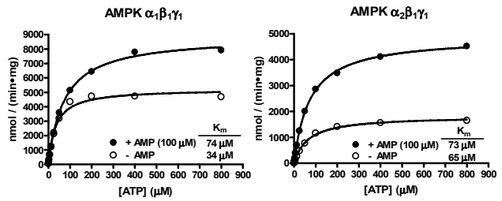
Activation of AMPK $\alpha_1\beta_1\gamma_1$ (●) and AMPK $\alpha_2\beta_1\gamma_1$ (○) by CAMKK1. Activity of AMPK was determined after a 2 hour incubation with CAMKK1 at room temperature.

Physical Purity



SDS-PAGE and native gel analysis of AMPK $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$. Lanes 1 and 7: native (non-denaturing) PAGE samples. Lanes 2 and 8: Invitrogen Benchmark protein ladder. Lanes 3 – 6: AMPK $\alpha_1\beta_1\gamma_1$ loaded at 1, 2, 5, or 10 µg / lane. Lanes 9 – 12: AMPK $\alpha_2\beta_1\gamma_1$ loaded at 1, 2, 5, or 10 µg / lane.

ATP K_m Determinations



ATP K_m values were determined for AMPK $\alpha_1\beta_1\gamma_1$ and AMPK $\alpha_2\beta_1\gamma_1$ in the presence (●) or absence (○) of 100 µM AMP. The $\alpha_1\beta_1\gamma_1$ isoform was activated less than 2 fold by AMP, whereas the $\alpha_2\beta_1\gamma_1$ isoform was activated approximately 3-fold.

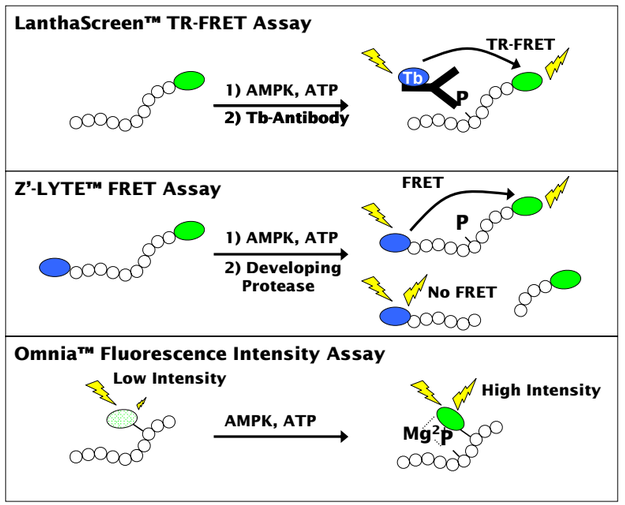
Development of Fluorescent Assays

Three fluorescence-based assays were developed to characterize small-molecule modulation of AMPK activity:

(1) A LanthaScreen™ TR-FRET assay which is ideally suited to HTS use because of its resistance to many forms of compound interference.

(2) A FRET-based Z'-LYTE™ assay that allows for compound profiling in Invitrogen's 224-kinase SelectScreen™ kinase profiling service.

(3) A real-time kinetic assay using the Omnia™ assay format. The Omnia™ assay format provides a real-time assay readout and is ideal for detailed mechanistic studies of compound activity.



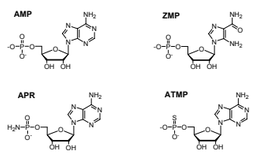
Small Molecule Activation of AMPK

Small-molecule activation of AMPK $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ was determined in a radiometric assay and in the 3 different fluorescent assay formats.

Legend:

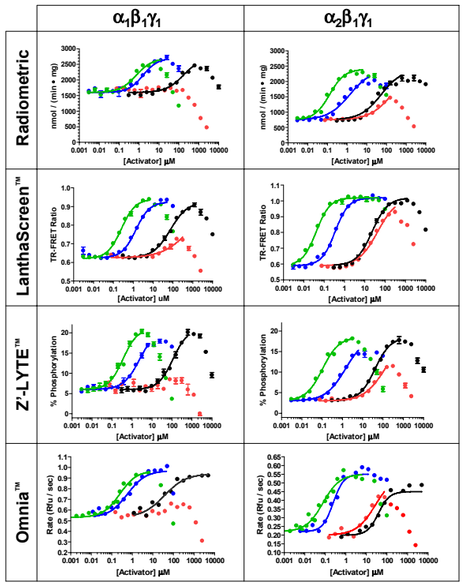
AMP (●), ZMP (●), ATMP (●), APR (●)

Structures of Activators Tested



Results

AMPK Isoform	Assay Format	Activator IC_{50} (µM) AMP	ZMP	APR	ATMP	Maximum Activation
$\alpha_1\beta_1\gamma_1$	Radiometric	1.6	165	n.d.	0.7	1.7-fold
$\alpha_1\beta_1\gamma_1$	LanthaScreen™	1.4	74	722	0.25	n.d.
$\alpha_1\beta_1\gamma_1$	Z'-Lyte™	2.5	112	n.d.	0.35	3.3-fold
$\alpha_1\beta_1\gamma_1$	Omnia™	0.58	36	n.d.	0.25	1.8-fold
$\alpha_2\beta_1\gamma_1$	Radiometric	1.4	54	194	0.13	2.9-fold
$\alpha_2\beta_1\gamma_1$	LanthaScreen™	0.35	23	43	0.04	n.d.
$\alpha_2\beta_1\gamma_1$	Z'-Lyte™	1.4	40	n.d.	0.1	6.3-fold
$\alpha_2\beta_1\gamma_1$	Omnia™	0.23	40	n.d.	0.07	2.5-fold



Small Molecule Inhibition of AMPK

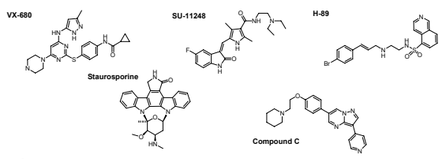
Small-molecule inhibition of AMPK $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ was determined in a radiometric assay and in the 3 different fluorescent assay formats.

Legend:

VX680 (●), SU-11248 (●), Compound C (●),

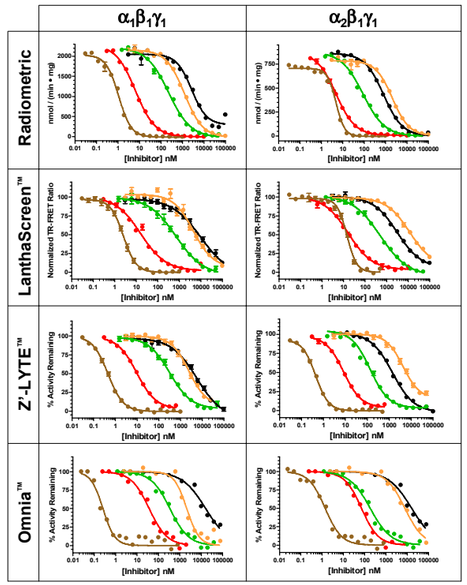
H89 (●), Staurosporine (●)

Structures of Activators Tested



Results

AMPK Isoform	Assay Format	Inhibitor IC_{50} VX-680	SU-11248	Cpd C	H89	Stauro-sporine
$\alpha_1\beta_1\gamma_1$	Radiometric	2.7 µM	6.5 nM	230 nM	1.2 µM	1.0 nM
$\alpha_1\beta_1\gamma_1$	LanthaScreen™	9.8 µM	16 nM	700 nM	4.5 µM	2.5 nM
$\alpha_1\beta_1\gamma_1$	Z'-Lyte™	5.7 µM	9.6 nM	320 nM	2.5 µM	0.5 nM
$\alpha_1\beta_1\gamma_1$	Omnia™	16 µM	37 nM	340 nM	2.1 µM	0.3 nM
$\alpha_2\beta_1\gamma_1$	Radiometric	0.8 µM	4.8 nM	88 nM	2.1 µM	4.8 nM
$\alpha_2\beta_1\gamma_1$	LanthaScreen™	3.1 µM	16 nM	500 nM	12 µM	14 nM
$\alpha_2\beta_1\gamma_1$	Z'-Lyte™	1.4 µM	8.3 nM	140 nM	4.2 µM	0.44 nM
$\alpha_2\beta_1\gamma_1$	Omnia™	16 µM	72 nM	184 nM	3.1 µM	1.4 nM



Results and Conclusions

Recombinant human AMPK has been expressed and purified, and convenient fluorescent assays to monitor its activation or inhibition have been developed, with good correlation between the different assay formats. Comparison of the $\alpha_1\beta_1\gamma_1$ and $\alpha_2\beta_1\gamma_1$ isoforms of AMPK reveals isoform-specific responses to small molecule activators and inhibitors. The inhibitors VX-680 and Compound C show a slight potency preference towards the $\alpha_2\beta_1\gamma_1$ isoform, whereas H89 shows a slight preference towards the $\alpha_1\beta_1\gamma_1$ isoform. All of the activators tested showed a potency preference towards the $\alpha_2\beta_1\gamma_1$ isoform, and activity of the $\alpha_2\beta_1\gamma_1$ was able to be stimulated to a greater extent than seen for the $\alpha_1\beta_1\gamma_1$ isoform.

Additional Information

(1) www.invitrogen.com/kinase
(2) www.invitrogen.com/lanthascreen
(3) www.invitrogen.com/omnia
(4) Riddle, S.M. *et al.* "TR-FRET kinase assays using physiological protein substrates: application of terbium-fluorescein and terbium-GFP FRET pairs" *Anal. Biochem*, 2006 (356) 108-116.
(5) Shults, M. D. *et al.* "A Multiplexed Homogenous Fluorescence-Based Assay for Protein Kinase Activity in Cell Lysates" *Nature Methods*, 2005 (2) 277-284.

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