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Ligand Binding to the AMP-activated Protein Kinase Active Site Mediates Protection of the Activation Loop from Dephosphorylation*[S]

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Background: Snf1, the AMP-activated protein kinase of yeast, is regulated at the level of dephosphorylation.
Results: Ligand binding to the Snf1 active site mediates protection from dephosphorylation.
Conclusion: Energy charge of the adenosine nucleotide bound at the kinase active site determines rate of dephosphorylation.
Significance: Adenylate binding to Snf1 connects kinase activity to energy availability.

The AMP-activated protein kinase (AMPK) is a conserved signaling molecule in a pathway that maintains adenosine triphosphate homeostasis. Recent studies have suggested that low energy adenylate ligands bound to one or more sites in the γ subunit of AMPK promote the formation of an active, phosphatase-resistant conformation. We propose an alternative model in which the kinase domain association with the heterotrimer core results in activation of the kinase catalytic activity, whereas low energy adenylate ligands bound in the kinase active site promote phosphatase resistance. Purified Snf1 α subunit with a conservative, single amino acid substitution in the kinase domain is protected from dephosphorylation by adenosine diphosphate in the complete absence of the β and γ subunits. Staurosporine, a compound known to bind to the active site of many protein kinases, mediates strong protection from dephosphorylation to yeast and mammalian AMPK enzymes. The analog-sensitive Snf1-I132G protein but not wild type Snf1 exhibits protection from dephosphorylation when bound by the adenosine analog 2NM-PP1 *in vitro* and *in vivo*. These data demonstrate that ligand binding to the Snf1 active site can mediate phosphatase resistance. Finally, Snf1 kinase with an amino acid substitution at the interface of the kinase domain and the heterotrimer core exhibits normal regulation of phosphorylation *in vivo* but greatly reduced Snf1 kinase activity, supporting a model in which kinase domain association with the heterotrimer core is needed for kinase activation.

Members of the AMPK[2] family of protein kinases respond to energy stress by activating pathways that restore levels of ATP and inhibit those that consume ATP (1). The hallmark of the AMPK family is the oligomeric structure of the kinase as an αβγ heterotrimer (2). The α subunit contains a typical serine/threonine kinase domain at its N terminus, a conserved C-terminal domain that interacts with the γ subunit, and the C terminus of the β subunit to form a folded structure known as the heterotrimer core (3, 4). The kinase domain and the heterotrimer core are joined by the central region of the α subunit, a domain that is predicted to be disordered (5).

AMPK is regulated primarily by phosphorylation of its activation loop. Phosphorylation of AMPK results in a 500-fold increase in activity, whereas the AMP allosteric stimulation is only a 2-fold increase in activity (6, 7). More recent studies have shown that the phosphorylation status of mammalian and yeast AMPK is regulated at the level of dephosphorylation (6, 8). Purified AMPK enzymes adopt a phosphatase-resistant conformation upon binding to low energy adenylate ligands (6, 7). Biochemical studies (9, 10) and protein crystallography (4, 11) have shown that high and low energy adenylate ligands can bind to one or more of the four cystathionine-β-synthase domains present in the γ subunit. However, understanding the mechanism by which adenylate energy charge is sensed is not simple because the γ subunit binds to both high and low energy adenylate ligands and does not appreciably change shape in response to the energy charge of the bound adenylate ligand(s) (4, 11). Townley and Shapiro (4) have proposed that the high and low energy adenylate ligands are sensed by changes in the electrostatic charge on the surface of the γ subunit. An alternate model has been proposed by Carling and Gamblin and co-workers (12) in which a portion of the α subunit, known as the α-hook, contacts the surface of the γ subunit and interrogates the adenylate ligand bound in site 3. The α-hook model is particularly appealing because it provides an explanation for the mechanisms by which adenylate charge is sensed and by which the kinase attains its phosphatase-resistant conformation.

In this study we examined in further detail the role played by the γ subunit in the activation of Snf1 and the protection from dephosphorylation. We found that the γ subunit is not required for adenylate-mediated protection from dephosphorylation *in vitro*. Ligands binding to the kinase active site mediate protection of the kinase from dephosphorylation. We propose a new model for the regulation of AMPK in which association of the

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[2] The abbreviations used are: AMPK, AMP-activated protein kinase; TAP, tandem affinity purification.

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kinase domain with the heterotrimer core mediates activation of the kinase catalytic activity.

EXPERIMENTAL PROCEDURES

Yeast Strains and Genetic Methods—The yeast strains used in this study were all derivatives of S228C. Wild type Snf1 complexes were purified from FY1193 (*MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 snf1Δ10*) transformed with pSNF1-TAP (13). Transformation of yeast strains used the lithium acetate method (14). Snf1 complexes with specific β subunits were purified from MSY560 (*MATa ura3-52 leu2Δ0 his3Δ200 sip1Δ::HIS3 sip2Δ::HIS3 gal83Δ::HIS3 snf1Δ10*) transformed with plasmids that express Snf1-TAP and a specific β subunit. Cells were grown at 30 °C using standard media (15). Oligonucleotide-directed mutagenesis was performed with *Pfu* polymerase followed by DpnI digestion of the plasmid template (16). All of the mutations were confirmed by DNA sequencing.

Protein Purifications—Snf1 kinase complexes were isolated by tandem affinity purification (TAP) (17) from yeast cells lacking endogenous Snf1 and expressing Snf1-TAP from a low copy number plasmid (13). The cDNA for human phosphatase PP2Cα was purchased from Open Biosystems (IHS1382-8646531) and inserted into the bacterial expression plasmid pET14b (Novagen). Bacterial cells were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 2.5 h at 26 °C. Extracts were prepared by sonication, and the His-tagged PP2Cα was purified using nickel-nitrilotriacetic acid-agarose (Qiagen). Rat AMPK composed of the α1, β1, and γ1 subunits was purified as described (18). His-tagged rat calcium/calmodulin-dependent protein kinase kinase β was purified from bacteria and used to phosphorylate the activation loop of recombinant rat AMPK as described (19). His-tagged Mig1 protein (residues 207–413) was expressed in bacteria and purified using nickel-nitrilotriacetic acid-agarose (Qiagen).

Western Blotting—Snf1-HA was detected with a 1:3000 dilution of HA probe (Santa Cruz). Goat anti-mouse IgG DyLight 680 (Thermo) diluted 1:10,000 was used as the secondary antibody. For detection of phosphorylated Snf1, Phospho-AMPKα (Thr-172) antibody (Cell Signaling) diluted 1:1000 was used. Goat anti-rabbit IRDye 800CW (Li-Cor) (1:10,000 dilution) was used as the secondary antibody. The blots were processed by using the Snap identification system (Millipore) and scanned by using an Odyssey scanner (Li-Cor). Integrated intensity values of bands were quantified by using Odyssey scanning software. Snf1 activation loop (Thr-210) phosphorylation *in vivo* was determined by Western blotting of extracts prepared using the boiling method described by Kuchin and co-workers (20). In experiments using the analog-sensitive *SNF1-L183I* allele, 2NM-PP1 dissolved in dimethyl sulfoxide was added to the culture medium to a final concentration of 20 μM for 5 min prior to protein extraction.

Dephosphorylation Assays—Dephosphorylation reactions (10 μl) contained purified Snf1 proteins or mammalian AMPK (~50 ng) in reaction buffer (20 mM HEPES, pH 7.0, 0.1 M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 5 mM magnesium acetate). Adenosine diphosphate (Sigma) was dissolved in TE (10 mM Tris pH 8, 1 mM EDTA), adjusted to neutral pH, and added to reactions at a final concentration of 0.8 mM unless otherwise stated. 2NM-PP1 and staurosporine were dissolved in dimethyl sulfoxide and added to reactions at concentrations of 1 μM. Titrations of purified PP2C were performed to determine the appropriate dilution needed to remove 80–90% of the Snf1 phosphorylation. Purified PP2C phosphatase was diluted in reaction buffer and added to the reactions, which were then incubated at 37 °C for 10 min. The reactions were stopped by the addition of SDS sample buffer. Total and phosphorylated Snf1 and AMPK were analyzed by quantitative Western blotting. Phosphatase assays using the chromogenic substrate *para*-nitrophenyl-phosphate were conducted in 50 μl reactions containing 20 mM HEPES pH 8.0, 0.5 mM EDTA, 0.5 mM dithiothreitol, 2 mM $MnCl_2$, 0.1 M NaCl, 5% (v/v) glycerol and 20 mM *para*-nitrophenyl-phosphate. The reactions were incubated at 37 °C for 10 min and stopped with the addition of 1 ml of 1 M KPO_4 pH 8. Absorbance at 405 nm was read, and the number of moles of *para*-nitrophenyl-phosphate hydrolyzed was calculated using the molar extinction coefficient of $1.78 \times 10^4\ M^{-1}\ cm^{-1}$.

Kinase Assays—*In vitro* kinase reactions (10 μl) contained 0.2 mM [γ-^{32}P]ATP (1000 cpm/pmol), 20 mM HEPES, pH 7.0, 0.1 M NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 5 mM magnesium acetate, and His-tagged Mig1 protein (residues 207–413) at ~50 μg/ml. The reactions were incubated at 30 °C for 30 min and stopped by the addition of SDS sample buffer. Proteins were resolved on an SDS-polyacrylamide gel. Incorporation of ^{32}P was quantified using a Typhoon 9400 PhosphorImager (Amersham Biosciences). In kinetic analyses, kinase assays using the SAMS peptide as the substrate were performed as described (21, 22). Snf1 enzymes were TAP-purified from yeast cells (13) and rat AMPK was purified from bacteria (5). The K_m values for ATP and peptide were calculated by measuring initial reaction velocities at varying concentrations of substrate. K_i for ADP was determined by measuring the apparent K_m (K_m') for ATP in the presence of 0.4 mM ADP using the equation for a competitive inhibitor: $K_m' = K_m(1 + [I]/K_i)$. The slopes and intercepts of all double reciprocal plots were determined with linear regression analysis and had R values of 0.995 or greater.

Statistical Analysis—For all bar plots, the mean values using a minimum of three independent measurements are plotted with *error bars* representing one standard error. Statistical significance was determined using the Student's t test for unpaired variables with equal variance unless otherwise indicated. Statistical significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, $p > 0.05$.

RESULTS

The Snf1 Linker Region Is Not Required for Snf1 Regulation or ADP-mediated Protection from Dephosphorylation—The linker region that connects the AMPK kinase domain to its C terminus has been proposed to play a key role in regulating the activity of AMPK. In one study it was suggested to bind the kinase domain and inhibit kinase activation (23), whereas another study has proposed that the linker lies along the surface of the γ subunit in the active conformation (12). We showed that deletion of Snf1 residues 381–488 that comprise a large portion of the linker region had no effect on Snf1 regulation *in vivo* and ADP-mediated protection *in vitro* (5). Because the 381–488 deletion left portions of the Snf1 linker intact includ-

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ing the ubiquitin-association domain (23), the possibility remained that the residual linker sequences could be playing a role in Snf1 regulation and adenylate sensing. Therefore, we extended the size of the linker deletion in both the N-terminal direction to residue 320 and in the C-terminal direction to residue 505. Residues 320 and 505 are both visible in structural models and are present in folded domains (3, 24). Cells expressing Snf1 enzymes with linker residues 320–488 and 381–505 were functional as judged by growth on alternative carbons sources, normal regulation of activation loop phosphorylation *in vivo*, and ADP-mediated protection *in vitro* (supplemental Fig. S1). Therefore, the linker region of the α subunit (residues 320–505) is not required for function *in vivo* or adenylate-mediated protection of the Snf1 enzyme *in vitro*.

Point Mutations Targeting the Adenylate-binding Sites in the γ Subunit Do Not Affect Its Function—Following reports of adenylate binding to the γ subunits of mammalian and *Schizosaccharomyces pombe* AMPK (4, 10, 11), we were eager to determine whether adenylate binding to the yeast γ subunit was important for its function. Point mutations were engineered into low copy number plasmids expressing the γ subunit of yeast AMPK and introduced into cells lacking the γ subunit gene (*snf4Δ1*). To our surprise and consternation, all of the point mutations were functional and supported growth on alternative carbon sources (supplemental Fig. S2). The mutations tested included residues that contact the phosphates and ribose moieties of bound adenylates (4, 11) and tryptophan substitutions in all four cystathionine-β-synthase domains designed to sterically block adenylate binding. None of these changes had a significant effect on Snf4 function *in vivo*. In contrast, analogous mutations had large effects on the γ subunit function of the mammalian enzyme (6, 10). In studies of the *S. pombe* AMPK, adenylate binding to sites 2 and 4 were detected. For site 2, the critical aspartate that contacts the ribose moiety is provided not by the γ subunit but by a region of the β subunit named the β flap (4). We engineered a precise deletion of the β flap in the yeast Gal83 protein and found that this deletion had no effect on yeast growth assays and showed regulated activation loop phosphorylation in response to changes in glucose *in vivo* (supplemental Fig. S3). Furthermore, purified heterotrimers containing Gal83 with the β flap deleted were competent for adenylate-mediated protection from dephosphorylation *in vitro*. Taken together, these data forced us to conclude either that the yeast γ subunit did not require adenylate binding for its function or that the adenylate-binding sites in the yeast γ subunit were significantly different from their mammalian and *S. pombe* homologs.

Snf1-L183I Exhibits ADP-mediated Protection from Dephosphorylation in the Absence of the β and γ Subunits—In an earlier study, we found that substitution of Snf1 leucine 183 with isoleucine rendered the Snf1 kinase functional in the absence of the γ subunit (25). Snf1-L183I is highly functional in absence of γ and partially functional in the absence of both β and γ (Fig. 1*A*). We purified the Snf1-L183I kinase complex from cells that expressed different combinations of β and γ subunits yielding enzymes composed of the αβγ, αβ, and α subunits (Fig. 1*B*). *In vitro* phosphatase protection assays conducted with these enzymes showed that ADP-mediated protection was observed

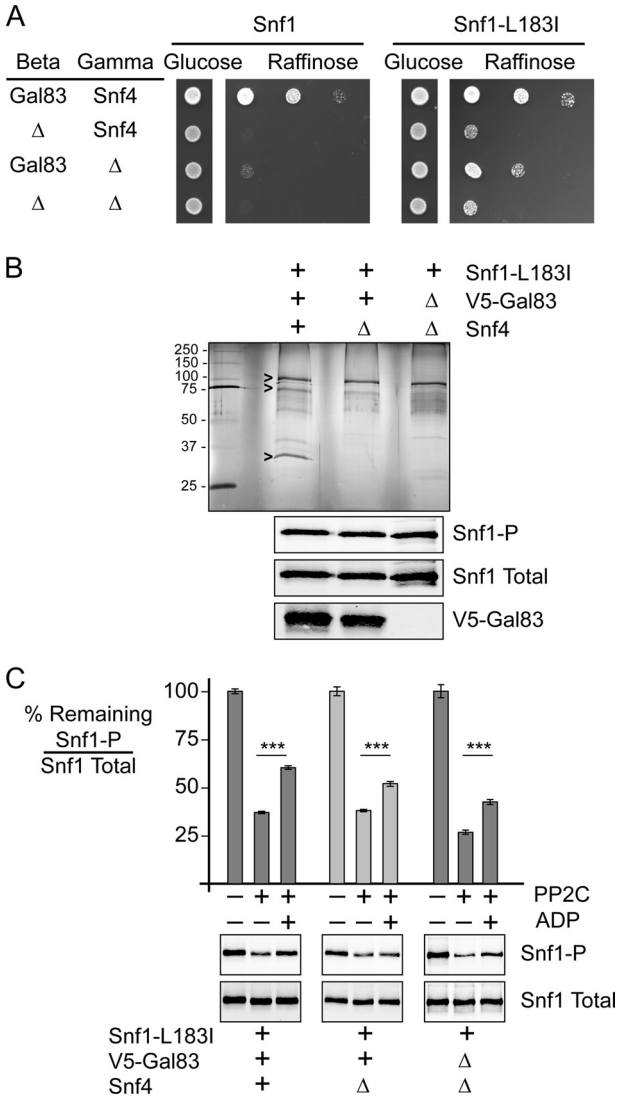

FIGURE 1. **Snf1-L183I is functional in the absence of the β and γ subunits.** *A*, growth assay of cells expressing either wild type Snf1 or Snf1-L183I in the presence or absence of the β and γ subunits as shown. *B*, purification of Snf1 complexes from cells expressing Snf1-L183I with V5-tagged Gal83 and Snf4 subunits as indicated. Western blots of the purified enzymes are shown in the *lower panels* using antibodies that detect phosphorylated Snf1 (*Snf1-P*), total Snf1, or V5-Gal83 as indicated. *C*, adenylate-mediated protection assays using the purified complexes shown in *B*. The reactions were conducted in triplicate with recombinant PP2C phosphatase and 0.8 mM ADP as shown. The mean ratio of phosphorylated Snf1 (*Snf1-P*) to total Snf1 is plotted as the percentage remaining after phosphatase treatment. Representative blots are shown below. Statistical significance between phosphatase-treated samples with and without ADP is indicated.

with the Snf1-L183I αβγ heterotrimer, with an αβ dimer and with the Snf1-L183I α subunit by itself (Fig. 1*C*). Therefore, the β and γ subunits are not required for ADP-mediated protection of the Snf1-L183I kinase. Previously we showed that the wild type α subunit was not competent for adenylate-mediated protection (5). These data indicate that the Snf1-L183I mutant protein bypasses a γ-dependent step and is able to bind ADP and exhibit protection from dephosphorylation.

Kinetic Analysis of Wild Type Snf1 and Snf1-L183I Enzymes—Our results with Snf1-L183I suggested that the adenylate-binding site that mediates protection from dephosphorylation is present in the α subunit. The obvious candidate for this site is

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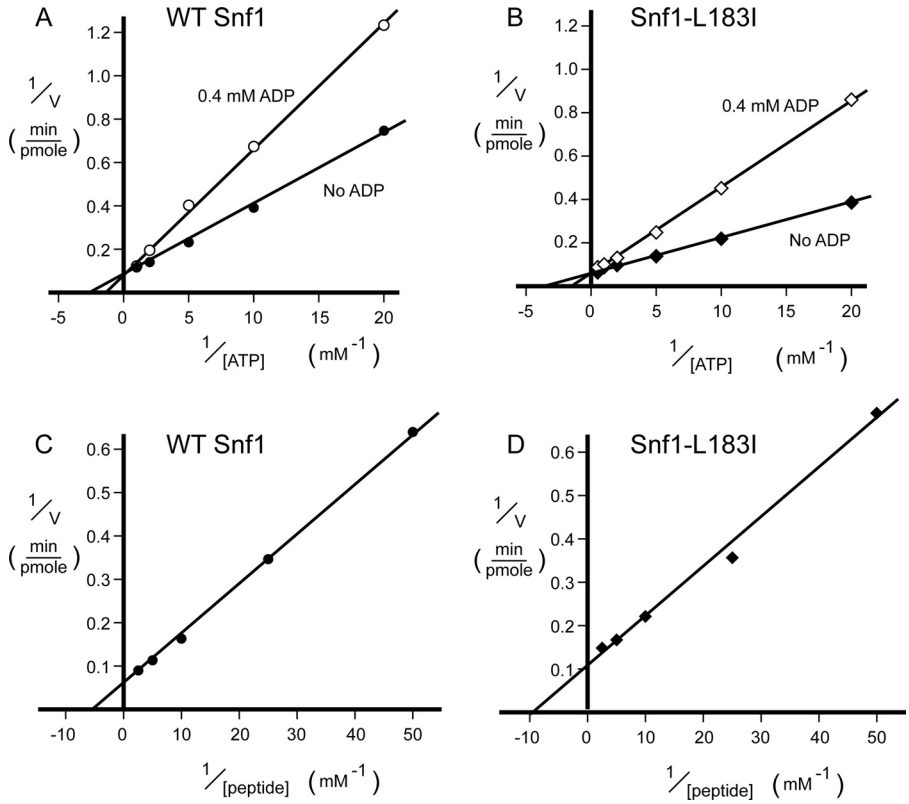

FIGURE 2. **Kinetic analysis of the wild type Snf1 and Snf1-L183I enzymes.** Wild type Snf1 (*A* and *C*) and Snf1-L183I heterotrimers (*B* and *D*) containing all three subunits (Snf1, Gal83, and Snf4) were assayed for the ability to phosphorylate the SAMS peptide. Initial reaction velocities were measured and plotted as a function of substrate concentration using the double reciprocal format. Reactions with varying concentrations of ATP were conducted in the presence or absence of 0.4 mM ADP (*A* and *B*). Reactions with varying concentrations of peptide substrate were conducted in the presence of 1 mM ATP (*C* and *D*).

the kinase active site. To characterize the interaction of nucleotides with the active sites of the wild type and L183I enzymes, we conducted kinetic analyses of purified enzymes using phosphorylation of the SAMS peptide as our assay (21, 22). The initial reaction velocities of the enzymes were measured as a function of the concentration of ATP in the presence or absence of ADP. The double reciprocal plots of the data show that ADP is a competitive inhibitor of Snf1, confirming the idea that ADP binds at the Snf1 active site (Fig. 2). The substrate and inhibitor constants determined from these data are shown in Table 1. The observed K_i values for ADP (Table 1) are consistent with the concentrations of ADP needed to mediate protection *in vitro* (5). Also, the L183I mutation significantly reduces the K_m for ATP and peptide, as well as the K_i for ADP. Thus the L183I mutation promotes or stabilizes a conformation of the Snf1 active site with higher affinity for the peptide substrate and for adenosine nucleotides. The wild type Snf1 K_m value for ATP is significantly higher than that measured for recombinant rat AMPK (Table 1 and supplemental Fig. S4), as well as native AMPK purified from rat liver (26). The weaker binding of adenylate nucleotides to the yeast active site is consistent with our earlier study showing that yeast Snf1 required a higher concentration of ADP than mammalian AMPK to mediate protection from dephosphorylation (19).

Staurosporine Mediates Protection from Dephosphorylation to Both Yeast and Mammalian AMPK Enzymes—Our results with Snf1-L183I suggested that the adenylate-binding site that mediates protection from dephosphorylation is present in the α subunit. The obvious candidate for this site is the kinase active site. We predicted that ligands known to bind to the kinase active site might also confer protection from dephosphorylation. Staurosporine, a natural alkaloid isolated from *Streptomyces* bacteria, is a known inhibitor of eukaryotic kinases that is competitive with ATP (27) and binds in the kinase active site (28). In fact, the recent structure of the active conformation of mammalian AMPK was crystallized with staurosporine bound in its active site (12). We conducted phosphatase protection assays with staurosporine and found that this compound conferred strong protection from dephosphorylation to the yeast and mammalian enzymes (Fig. 3, *A* and *B*). Staurosporine conferred protection to the wild type yeast heterotrimer, as well as the Snf1-L183I α subunit. Staurosporine had no effect on the PP2C phosphatase catalytic activity when assayed for the ability to hydrolyze para-nitrophenylphosphate (Fig. 3*C*). We conclude that staurosporine binding to yeast and mammalian AMPK enzymes confers protection from dephosphorylation.

TABLE 1
Substrate and inhibitor constants for Snf1 and AMPK enzymes

Enzyme	Subunits			K_m		K_i
	α	β	γ	ATP	Peptide	ADP
				mM	mM	mM
Yeast Snf1	Snf1	Gal83	Snf4	0.44	0.23	0.81
Yeast Snf1	Snf1-L183I	Gal83	Snf4	0.27	0.12	0.25
Yeast Snf1	Snf1	Gal83-H384A	Snf4	1.2	0.41	
Rat AMPK	α1	β1	γ1	0.076	0.044	

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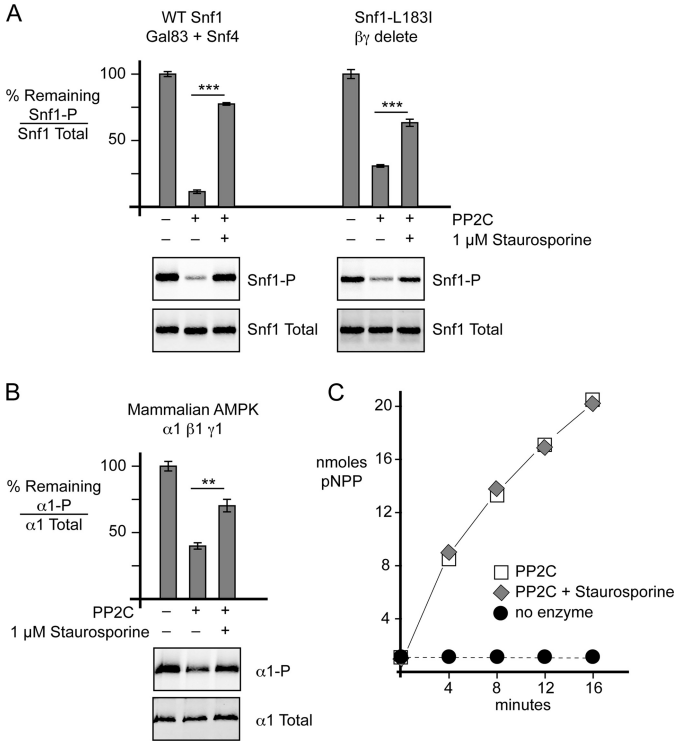

FIGURE 3. **Staurosporine mediates protection of yeast and mammalian AMPK activation loops from dephosphorylation.** *A* and *B*, wild type Snf1 heterotrimer containing all three subunits (Snf1, Gal83, and Snf4) and Snf1-L183I enzyme without any β or γ subunits (*A*) or recombinant rat AMPK (*B*) were subjected to dephosphorylation in the presence or absence of 1 μM staurosporine. The reactions were conducted in triplicate, and the mean values ± S.E. are plotted. Representative Western blots are shown in the *lower panels*. *C*, phosphatase assay measuring hydrolysis of *p*-nitrophenylphosphate by PP2C in the presence or absence of 1 μM staurosporine. The mean values from duplicate reactions are plotted.

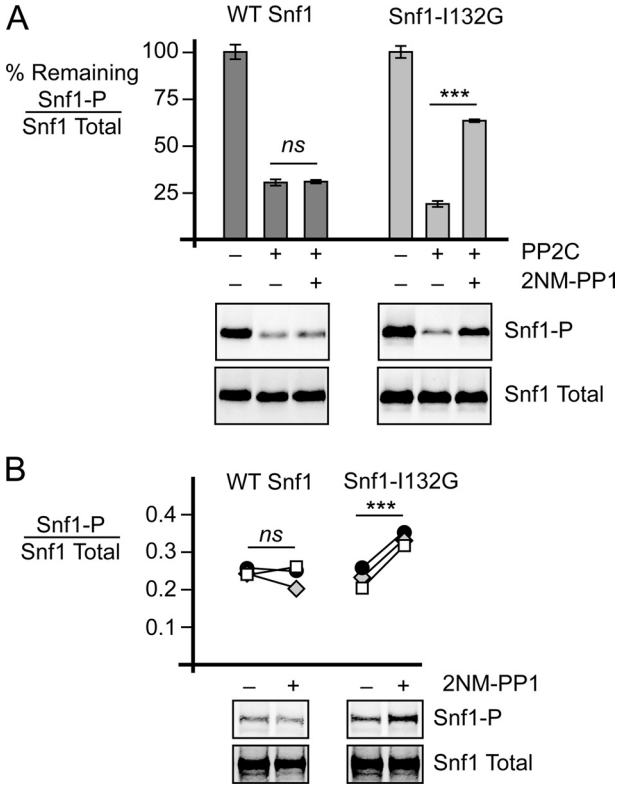

FIGURE 4. **2NM-PP1 binding protects the analog-sensitive but not the wild type Snf1 *in vitro* and *in vivo*.** *A*, *in vitro* phosphatase protection assays were conducted with PP2C, 2NM-PP1, and AMPK heterotrimers containing either the wild type Snf1 or the analog-sensitive Snf1 (I132G). The reactions were conducted in triplicate. The mean values ± S.E. are plotted. Representative Western blots are shown below. *B*, 2NM-PP1 mediates protection of Snf1 from dephosphorylation *in vivo*. Cells expressing either wild type or Snf1-I132G were grown in high glucose medium, split into two cultures, and treated with Me_2SO (−) or 2NM-PP1 dissolved in Me_2SO (+) for 5 min prior to harvest. The cell extracts were prepared and analyzed by Western blotting. The ratio of phosphorylated Snf1 over total Snf1 is plotted for experiments conducted in triplicate. Statistical significance using the Student's *t* test for paired values is indicated. Representative blots are shown in the *lower panels*.

Analog-sensitive Allele of Yeast Snf1 Is Protected by Adenosine Analog 2NM-PP1 in Vitro and in Vivo—The idea that ligand binding to the kinase active site mediates protection from dephosphorylation is counter to the prevailing models that envision ligand interactions with the γ subunit. To definitively prove that the ligand interaction that mediates protection from dephosphorylation is at the kinase active site, we employed the analog-sensitive allele of the yeast AMPK enzyme, *SNF1-I132G*. This system utilizes a modified kinase with a mutation in the adenylate-binding pocket of the kinase active site such that the altered kinase can accommodate adenylate analogs that the wild type kinase cannot (29). We and others have used this system to identify genes whose expression patterns are altered by acute inhibition of the Snf1 kinase (30–32). In this experiment, we purified the wild type and Snf1-I132G heterotrimers and measured their dephosphorylation *in vitro* in the presence of the adenylate analog 2NM-PP1 (Fig. 4*A*). When the Snf1 enzyme was dephosphorylated *in vitro*, the wild type enzyme was unaffected by the presence of 2NM-PP1, whereas the analog-specific form (I132G) showed strong protection by 2NM-PP1. We next 7asked whether 2NM-PP1 could afford protection from dephosphorylation *in vivo*. Cells expressing either wild type or the analog-sensitive allele of Snf1 were grown in high glucose and exposed to 20 μM 2NM-PP1 for 5 min prior to harvest. 2NM-PP1 had no effect on the phosphorylation status of the wild type enzyme but caused a significant increase in the phosphorylation status of the analog-sensitive allele (Fig. 4*B*). Therefore, ligand engagement with the Snf1 active site *in vitro* and *in vivo* can mediate protection from dephosphorylation.

A Mutation at the Interface of the Kinase Domain with the Heterotrimer Core Prevents Kinase Activation—Our studies demonstrate that ligand binding to the kinase active site mediated protection from dephosphorylation. We next investigated the purpose of the AMPK kinase domain association with the heterotrimer core. The structure of the active form of AMPK shows that the interface between the kinase domain and the heterotrimer core is composed of residues that are highly conserved between AMPK enzymes from all species and include the activation loop of the kinase and the C terminus of the β subunit (12). A histidine residue present in the C terminus of the β subunit is conserved throughout all species and makes contact with the activation loop of the kinase domain when associated with the heterotrimer core. Substitution of this residue with alanine reduces adenylate-mediated protection from dephosphorylation in both yeast and mammalian AMPK (12, 19). Here we analyzed the effect of this mutation on Snf1 func-

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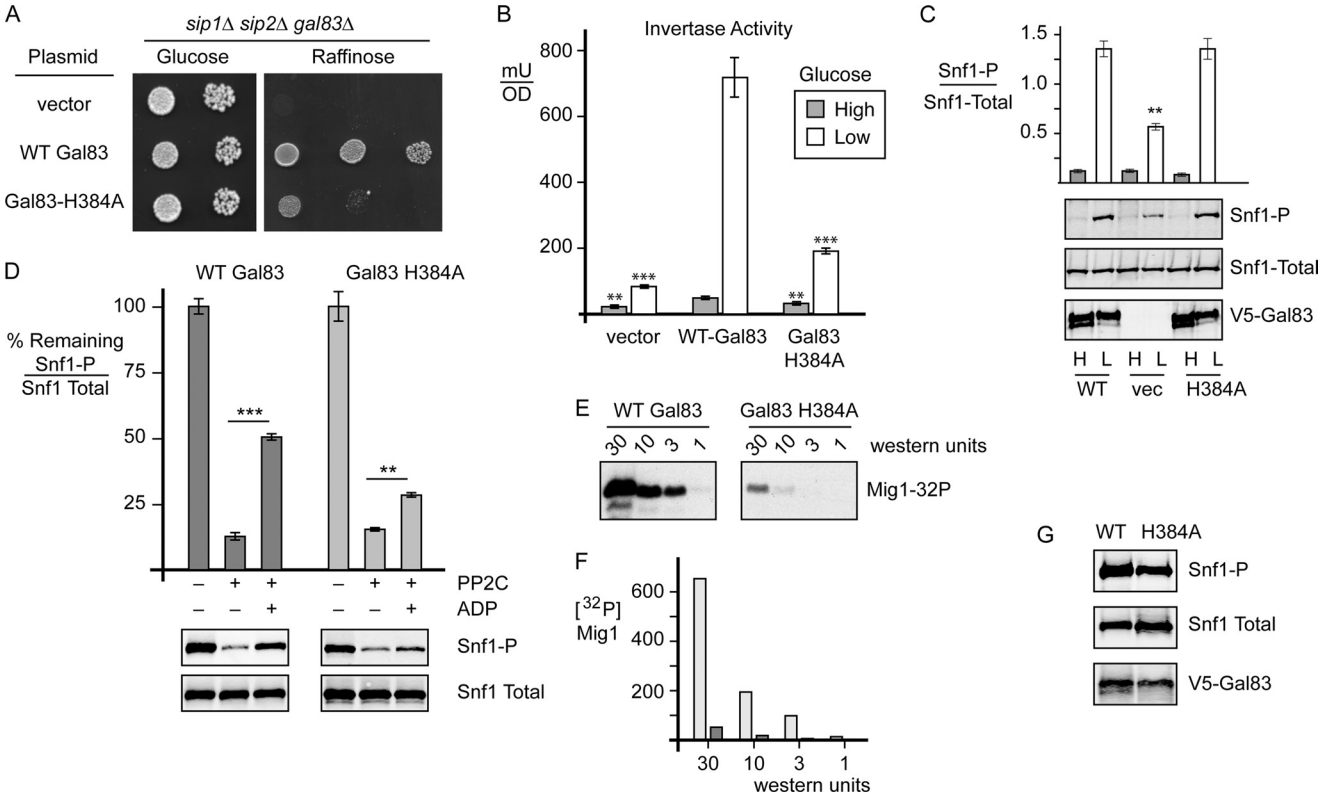

FIGURE 5. **A mutation in the kinase domain-heterotrimer interface affects Snf1 kinase activation but not phosphorylation status.** *A*, cell growth assays were conducted with cells lacking all three genes for the β subunits and transformed with a plasmid expressing wild type Gal83, Gal83-H384A, or empty vector as shown. 10-fold dilutions of cells were spotted onto solid medium with glucose or raffinose as the carbon source. *B*, invertase assay of cells grown on high glucose medium or 2 h after shifting to low glucose. The assays were conducted in triplicate, and the mean values are plotted ± S.E. The values that are statistically different from wild type are indicated. *C*, phosphorylation of the Snf1 activation loop was measured in triplicate in cells grown on media containing high glucose (*H*) or 30 min after shifting to low glucose (*L*). Western blots used antibodies that detect phosphorylated Snf1 (*Snf1-P*), total Snf1, or V5-tagged Gal83 proteins. The mean ratio of phosphorylated over total Snf1 is plotted ± S.E. The values statistically different from wild type are indicated. Representative Western blots are shown below. *vec*, vector. *D*, *in vitro* phosphatase protection assays were conducted in triplicate using Snf1 heterotrimers purified from yeast. The mean values are plotted ± S.E. ADP-mediated protection was statistically significant for both enzymes. *E*, *in vitro* kinase assays using a recombinant Mig1 protein as substrate. Reactions with a titration of enzyme levels are shown. Enzymes were normalized such that equivalent levels of phosphorylated Snf1 were present. *F*, quantitation of the [^{32}P]Mig1 from reactions shown in *E*. *G*, Western blots of the purified Snf1 heterotrimers used in this experiment with antibodies that detect phosphorylated Snf1 (*Snf1-P*), total Snf1, or V5-tagged Gal83 proteins.

tion *in vivo*, its phosphorylation status, and its biochemical properties *in vitro*.

To assess β subunit function, we started with a yeast strain lacking the genes for all three β subunits (*gal83*Δ *sip1*Δ *sip2*Δ) and introduced a low copy number plasmid expressing wild type Gal83, Gal83-H384A, or empty plasmid vector. Cells expressing the Gal83-H384A protein exhibited greatly reduced Snf1 kinase function as judged by growth on raffinose medium and invertase expression (Fig. 5, *A* and *B*). In contrast, the Gal83-H384A mutation had no effect on the phosphorylation status of the Snf1 activation loop *in vivo* in response to glucose concentration (Fig. 5*C*). When cells are shifted to low glucose, the phosphorylation of the activation loop increases by about 10-fold in cells expressing wild type Gal83 and Gal83-H384A, whereas cells lacking any β subunit exhibit much smaller increase activation loop phosphorylation. These results demonstrate that the phosphorylation of the activation loop is by itself not sufficient for the activation of Snf1 kinase *in vivo*. Cells expressing Gal83-H384A show normal levels of phosphorylated Snf1 but are unable to grow on raffinose or induce invertase.

Snf1 heterotrimers containing wild type Gal83 or Gal83-H384A were TAP-purified from yeast cells and characterized *in vitro*. Both enzymes exhibited adenylate-mediated protection from dephosphorylation (Fig. 5*D*), although the Gal83-H384A mutant displayed a reduced ability to be protected from dephosphorylation. A similar deficit in adenylate-mediated protection has been observed with the analogous mutation in the β subunit of mammalian AMPK (12) and with recombinant Snf1 heterotrimers containing Sip2-H379A as the β subunit (19). We estimate that the reduced protection by ADP is on the order of 2-fold calculated as the difference between wild type and mutant enzyme ratio of phosphorylated kinase domain with and without ADP. The catalytic activity of these enzymes was measured by incubating them with [γ-^{32}P]ATP and recombinant Mig1 protein, a known target of Snf1 kinase *in vivo* (33, 34). Incorporation of ^{32}P into Mig1 was measured by autoradiography (Fig. 5*E*), quantified by phosphorimaging analysis (Fig. 5*F*), and plotted as a function of the amount of phosphorylated Snf1 subunit present in each reaction (Fig. 5*G*). The specific activity of the Snf1 enzyme was reduced by 10-fold when the Snf1 heterotrimer contained the Gal83-H384A protein. Kinetic analysis of the Snf1 enzyme containing Gal83-H384A was conducted using the SAMS peptide assay (supplemental Fig. S5). Mutation of the Gal83 His-384 residue resulted in a significant increase in the K_m for both peptide and ATP.

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Thus alteration of the kinase domain interface with the heterotrimer core had a more severe reduction in catalytic activity and a decreased affinity for substrates. We and others (35–37) have previously shown that deletion of the β and γ subunits reduces Snf1 kinase activity without substantially affecting the regulation of Snf1 phosphorylation *in vivo*. Taken together, our data with the Gal83-H384A mutant and its location at the kinase domain-heterotrimer interface indicate that association of the kinase domain with the heterotrimer core promotes activation of the Snf1 kinase catalytic activity.

DISCUSSION

Binding of ADP to Snf1 confers protection from dephosphorylation (5, 19). One unanswered question from these studies was the site of ADP binding that conferred protection from dephosphorylation. The γ subunit contains four potential adenylate-binding sites (38). Mammalian γ binds adenylates in sites 1, 3, and 4 (11), whereas *S. pombe* γ uses sites 2 and 4 (39). Snf4, the *Saccharomyces cerevisiae* γ, binds adenylates with much lower affinity in two sites, site 4 and a second site yet to be determined (19). In an effort to identify the site(s) that bind ADP and confers protection from dephosphorylation to the Snf1 kinase, we conducted an extensive mutagenesis of the γ subunit targeting residues that were predicted to contact bound adenylate nucleotides in each of the four potential sites. None of the mutations affected the function of the γ subunit *in vivo* (supplemental Fig. S2), and many had no effect *in vitro* (19). These results forced us to consider the possibility that adenylate binding to the yeast γ subunit is not required for its ability to protect Snf1 kinase from dephosphorylation. Here we provide strong evidence that adenylate-mediated protection of Snf1 from dephosphorylation is caused by ligand engagement with the kinase active site. This conclusion is based on the following results: 1) ADP-mediated protection is observed in the Snf1-L183I kinase in the complete absence of a γ subunit; 2) staurosporine, a compound known to bind to kinase active sites, affords strong protection of Snf1 from dephosphorylation; and 3) the adenosine analog 2NM-PP1 mediates protection from dephosphorylation only to the analog-sensitive form of Snf1 containing an amino acid substitution in its active site. The ability of adenylate nucleotides bound in a kinase active site to mediate protection of the activation loop from dephosphorylation has recently been described for the mammalian AKT kinase (40, 41).

If adenylate-mediated protection from dephosphorylation is the result of adenylate binding to the kinase active site, then what is the purpose of adenylate binding to the γ subunit? The mammalian AMPK enzyme is exquisitely responsive to cellular energy charge using at least three distinct mechanisms to respond to changes in adenylate concentrations. Low energy adenylate ligands stimulate AMPK phosphorylation by the activating kinase LKB1 (42), cause allosteric activation of the catalytic activity, and confer resistance to dephosphorylation (6, 7). In yeast, two of these three mechanisms have been reported. The protection of Snf1 from dephosphorylation has been observed *in vivo* (8) and *in vitro* (19), and the phosphorylation of Snf1 by its upstream kinase, Sak1, appears be regulated by the Ras-PKA pathway (43). However, allosteric activation of Snf1 by adenylate ligands has not been observed, and the yeast γ subunit binds adenylates with much lower affinity (19). Perhaps these two observations are connected. Allosteric activation of AMPK may be mediated by adenylate binding to the γ subunit. Yeast AMPK may have already lost the ability to respond to adenylate energy charge through allosteric activation and is thus unaffected by mutations affecting the vestigial adenylate-binding sites in its γ subunit (supplemental Fig. S2).

Structural studies of the AMPK enzymes from several species have shown that the enzyme forms two folded domains, comprised of the kinase domain and the heterotrimer core, and that each domain can be crystallized independently (44). A new structure presented by Gamblin and Carling and co-workers (12) as the active form of AMPK shows the kinase domain bound to the heterotrimer core. Two lines of evidence support the idea that the compact conformation with the kinase domain bound to the heterotrimer core is in fact the active conformation. First, the crystallization of the compact conformation required phosphorylation of the activation loop threonine (12), a modification known to be required for full kinase activity. The second line of evidence comes from the studies of Taylor and Kornev (45), who identified four hydrophobic residues in kinase domains that form a regulatory spine and come into alignment when kinases are in the active conformation. These residues are not aligned in the crystal structures of the isolated AMPK kinase domains (24, 46) but come into alignment when the kinase domain binds to the heterotrimer core (12). In this study we show that an amino acid substitution at the interface between the kinase domain and the heterotrimer core greatly reduces catalytic activity without affecting the phosphorylation of the activation loop (Fig. 5). Thus phosphorylation of the Snf1 activation loop is not by itself sufficient for kinase activation. We propose that kinase domain association with the heterotrimer core is required for adoption of the catalytic conformation with a stabilized activation loop and the alignment of the regulatory spine (Fig. 6). This idea is supported by our earlier study in which we selected for amino acid substitutions in the Snf1 kinase that rendered it active in the absence of the γ subunit (25). We identified residues in the kinase domain hydrophobic core that were either a part of or were adjacent to the catalytic spine as defined by Taylor and Kornev (45). Similarly, Momcilovic *et al.* (21) showed that substitutions in the hydrophobic core of the Snf1 kinase domain adjacent to the regulatory spine resulted in activation of Snf1 catalytic activity and increased phosphorylation. Previously we showed that activation of the Snf1 kinase required two steps: the phosphorylation of the activation loop and a second step mediated by the γ subunit (35). We propose that the activation step provided by the γ subunit is the conformational rearrangement of the kinase domain that occurs upon association with the heterotrimer core.

Our findings led us to propose a new model for the regulation of Snf1 kinase. In this model, the Snf1 kinase domain is tethered to the heterotrimer core via the disordered linker domain (Fig. 6*A*). In the extended conformation, the activation loop threonine is exposed and can be phosphorylated and dephosphorylated by the Snf1-activating kinases and by the protein phosphatases PP1 and Sit4, a yeast PP2A-like phosphatase (37, 47).

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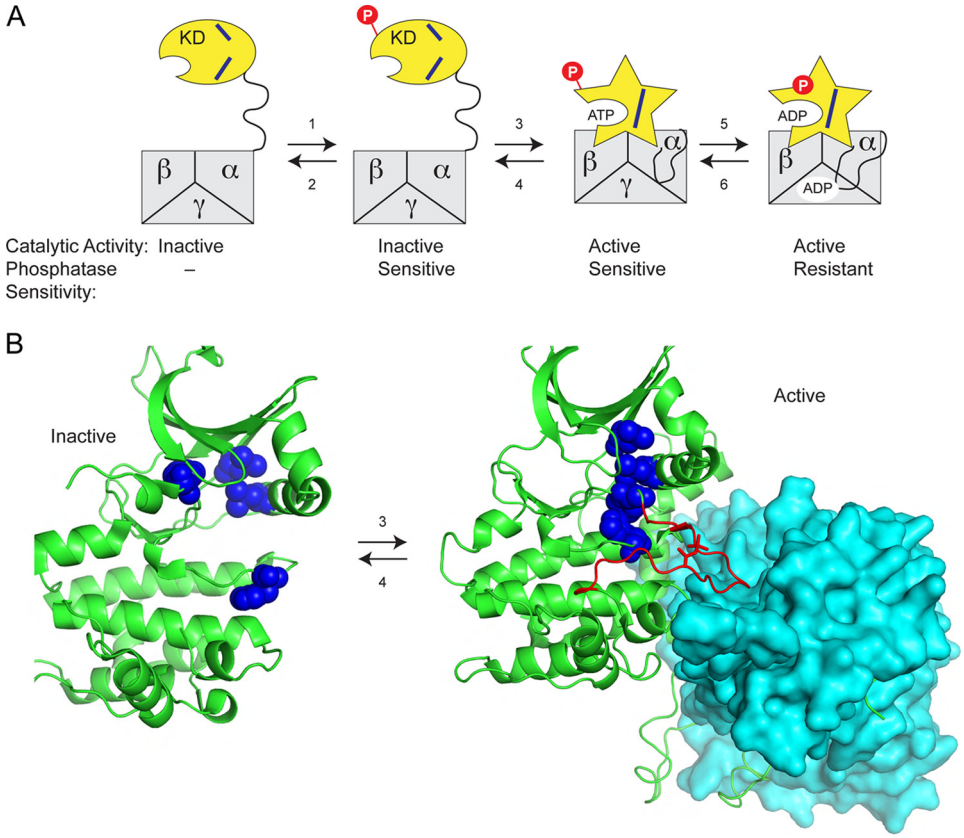

FIGURE 6. **Model for activation of Snf1 kinase and acquisition of phosphatase resistance.** *A*, the Snf1 kinase domain (K_D) is loosely tethered to the heterotrimer core ($\alpha\beta\gamma$) via a disordered linker domain with the activation loop accessible to phosphorylation (*reaction 1*) and dephosphorylation (*reaction 2*). Once it is phosphorylated, the kinase domain can associate with the heterotrimer core (*reaction 3*) and adopt the active conformation (*star shape*) with the alignment of the regulatory spine (*blue lines*). Binding of low energy adenylate ligands to the active site and/or the γ subunit promotes formation of a phosphatase resistant conformation (*reaction 5*), indicated here as the phosphate (*red P*) burrowing into the K_D. *B*, structure of the mammalian AMPK kinase domain (*green cartoon representation*) in the inactive and active states showing the position of the residues (*blue spheres*) that comprise the regulatory spine (residues Leu-68, Leu-79, Phe-158, and His-137 in mammalian AMPK). Upon association with the heterotrimer core (*cyan surface representation*), the kinase domain adopts an active conformation with the regulatory spine in alignment. The activation loop (shown in *red*) becomes structured when it is bound in a cleft in the heterotrimer core. Protein Data Bank files used to generate this figure were 2Y94 and 2H6D.

Phosphorylation of the activation loop is not sufficient for kinase activation because the regulatory spine is not properly aligned. However, phosphorylation of the activation loop is a prerequisite for the association of the kinase domain with the heterotrimer core. Once bound to the heterotrimer core, the kinase domain shifts to the active conformation with the alignment of the regulatory spine (Fig. 6*B*). In the active conformation, the Snf1 kinase binds ATP in the active site and transfers the terminal phosphate to its substrates leaving ADP in the active site. In this ADP-bound state, the kinase is resistant to inactivation by dephosphorylation. Under low glucose and low ATP conditions, it is possible that the Snf1 kinase spends most of its time in the ADP-bound state. For several protein kinases, including PKA, S6K1, Tie2, and Csk kinases, the dissociation of ADP from the active site is the rate-limiting step in catalysis (48–51). When the kinase domain is bound to the heterotrimer core, the phosphorylated threonine in the activation loop interacts with the arginine residue of the conserved HRD motif (12). The interaction of the phosphorylated threonine with the HRD arginine is critical for the acquisition of phosphatase resistance by AKT (41). In this model the reduced rate of dephosphorylation of Snf1 in the low glucose state is due to the additive effects of adenylate-mediated protection (8) and decreased phosphatase activity (37, 47).

Does this model pertain to mammalian AMPK, or is it restricted to yeast AMPK? The heterotrimeric structure of all AMPK enzymes is highly conserved, suggesting a conservation of regulatory mechanisms. We showed that staurosporine mediates protection to the mammalian enzyme (Fig. 3*B*), demonstrating that ligand engagement with the mammalian AMPK active site can confer protection from dephosphorylation. On the other hand, mutations in the mammalian γ subunit that reduce adenylate binding (10) also reduce adenylate-mediated protection (6). These data seem to suggest that mammalian AMPK could utilize two different mechanisms for ligands to confer protection: one through ligand engagement with the active site and one through engagement with sites in the γ subunit. However, these two mechanisms could be unified if one proposed that adenylate binding to the γ subunit favors association with the kinase domain to form the compact and active conformation. If the compact and active conformation is a prerequisite for the formation of the protected conformation (Fig. 6*A*), then mutations in the γ subunit could have deficits in both activation of the kinase catalytic activity and in adenylate-mediated protection. Further studies with both mammalian and yeast AMPK will be needed to determine the importance of ligand binding to the kinase active site.

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Ligand binding to the AMPK active site mediates protection of the activation loop from dephosphorylation

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Supplementary Material

FIGURE S1. **Snf1 linker is not required for regulation of activation loop phosphorylation.** (*A*) Schematic representation of the Snf1 subunit showing the location of the kinase domain (green), the βγ interaction domain (βγ ID; blue) and the linker region (red). Surface representation of the Snf1 heterotrimer based on the structure of mammalian AMPK in the active conformation (PDB file 2Y94) with β and γ subunits shown in cyan. The α subunit is comprised of the kinase domain (green), the βγ interaction domain (βγ ID; blue) connected by the linker region (red). The position of the ubiquitin association domain homology (UBA) is indicated. The position of residues 320 and 505 that define the deletion endpoints are indicated on a structural model of active AMPK with lines and distinct coloring. (*B*) Growth phenotypes of *snf1Δ* cells transformed with low-copy plasmids expressing wild type Snf1 (WT Snf1), one of the linker deletion mutants of Snf1 or no Snf1 (vector). Cells were spotted onto solid media with either glucose or sucrose as the carbon source. (*C*) Phosphorylation of Snf1 in vivo. Extracts were prepared from cells grown in media with high glucose (H) or 30 minutes after shifting to low glucose (L). Western blots were probed with antibodies to phosphorylated Snf1 (Snf1-P) or total Snf1 as shown. Quantitation of the western blots signals were expressed as the ration of phosphorylated Snf1 over total Snf1. (*D*) purification of Snf1 heterotrimers with linker deletion mutations. An SDS polyacrylamide gel stained with silver nitrate is shown the mobility of the Snf1, Gal83 and Snf4 proteins indicated on the right. Molecular weight markers (M) are shown on the left. (*E*) Snf1 dephosphorylation assays were conducted in triplicate with recombinant PP2C phosphatase and 0.8 mM ADP as shown. The mean ratio ± SE of phosphorylated Snf1 (Snf1-P) to total Snf1 is plotted as the percentage remaining after phosphatase treatment. Representative blots are shown below. Statistical significance between phosphatase treated samples with and without ADP are indicated.

FIGURE S2. **Mutations in the adenylate binding pockets of the yeast γ subunit do not impair its function.** Cells lacking the γ subunit gene (*snf4Δ1*) were transformed with low-copy number plasmids expressing wild type Snf4 (WT-Snf4), Snf4 with the point mutation indicated or with empty vector. Cells were spotted onto agar media with either glucose or raffinose as the carbon source. Snf4-3D contains three mutations (D83A, D240A and D312A). Snf4-4W contains four mutations (L78W, V164W, V235W and V307W).

FIGURE S3. **Gal83 β flap is not required for function.** (*A*) Structure of the *S. pombe* AMPK heterotrimer showing adenosine diphosphate (ADP) bound in CBS2. The α and γ subunits are shown in yellow and cyan surface representation, respectively. The β subunit is represented in blue ribbons with the β flap in red. The aspartate residue (D250) that contacts the ribose moiety is indicated. The β subunit histidine residues that are predicted to contact the kinase activation loop (H264 and H259) are indicated. (*B*) Alignment of the β flap region of the *S. pombe* β subunit and *S. cerevisiae* Gal83 indicating the flap

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region that was deleted from Gal83, the aspartate residue (*) that contacts the ribose and the conserved histidine residues (arrows). (*C*) Growth assay of cells lacking all three β subunit genes and transformed with low-copy number plasmids that express wild type Gal83, Gal83 lacking the β-flap or empty plasmid vector. Cells were spotted onto media with glucose or raffinose as the carbon source. (*D*) Snf1 phosphorylation in vivo was measured by western blotting using extracts from cells grown on high glucose (H) or 30 minutes after shifting to low (L) glucose. Extracts were prepared in triplicate with the mean ratio of phosphorylated Snf1 (Snf1-P) over total Snf1 plotted ±SE. Representative blots are shown below. Values significantly different from wild type are indicated. (*E*) In vitro phosphatase protection assay using purified Snf1 heterotrimer containing Gal83-Δflap. Reactions were conducted in triplicate with mean values plotted ±SE. Statistical significance of adenylate mediated protection is indicated.

FIGURE S4. **Kinetic analysis of recombinant rat AMPK α1 β1 γ1.** Rat AMPK was assayed for catalytic activity using the SAMS peptide assay. Initial reaction velocities were measured as a function of ATP (A) and SAMS peptide (B) concentrations. Results were plotted using the double reciprocal format and used to calculate Km for ATP and peptide.

FIGURE S5. **Kinetic analysis of the Snf1 kinase complex containing the Gal83-H384A subunit.** The Snf1 complex containing the Gal83-H384A subunit was assayed for catalytic activity using the SAMS peptide assay. Initial reaction velocities were measured as a function of ATP (A) and SAMS peptide (B) concentrations. Results were plotted using the double reciprocal format and used to calculate Km for ATP and peptide.

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FIGURE S1.

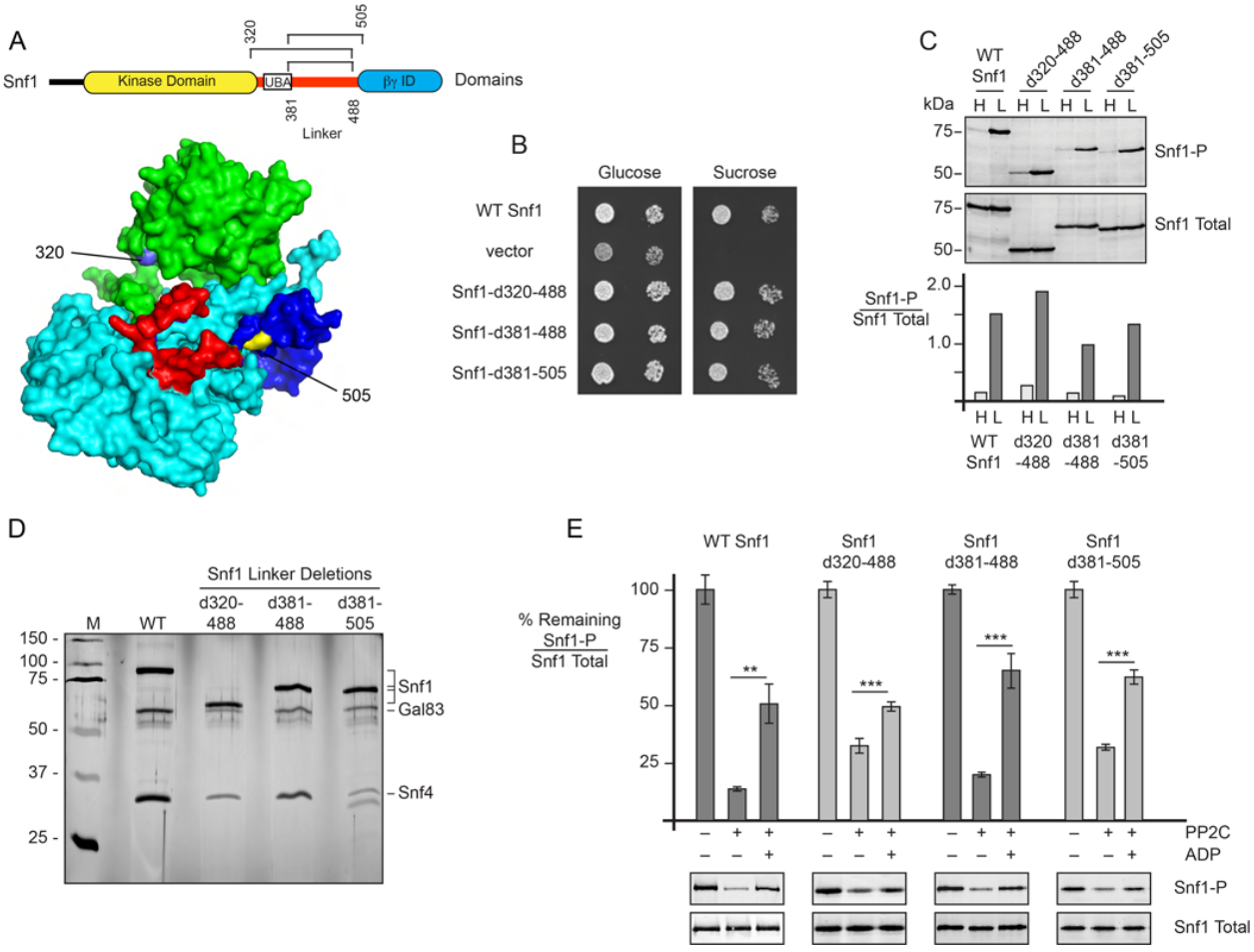

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FIGURE S2.

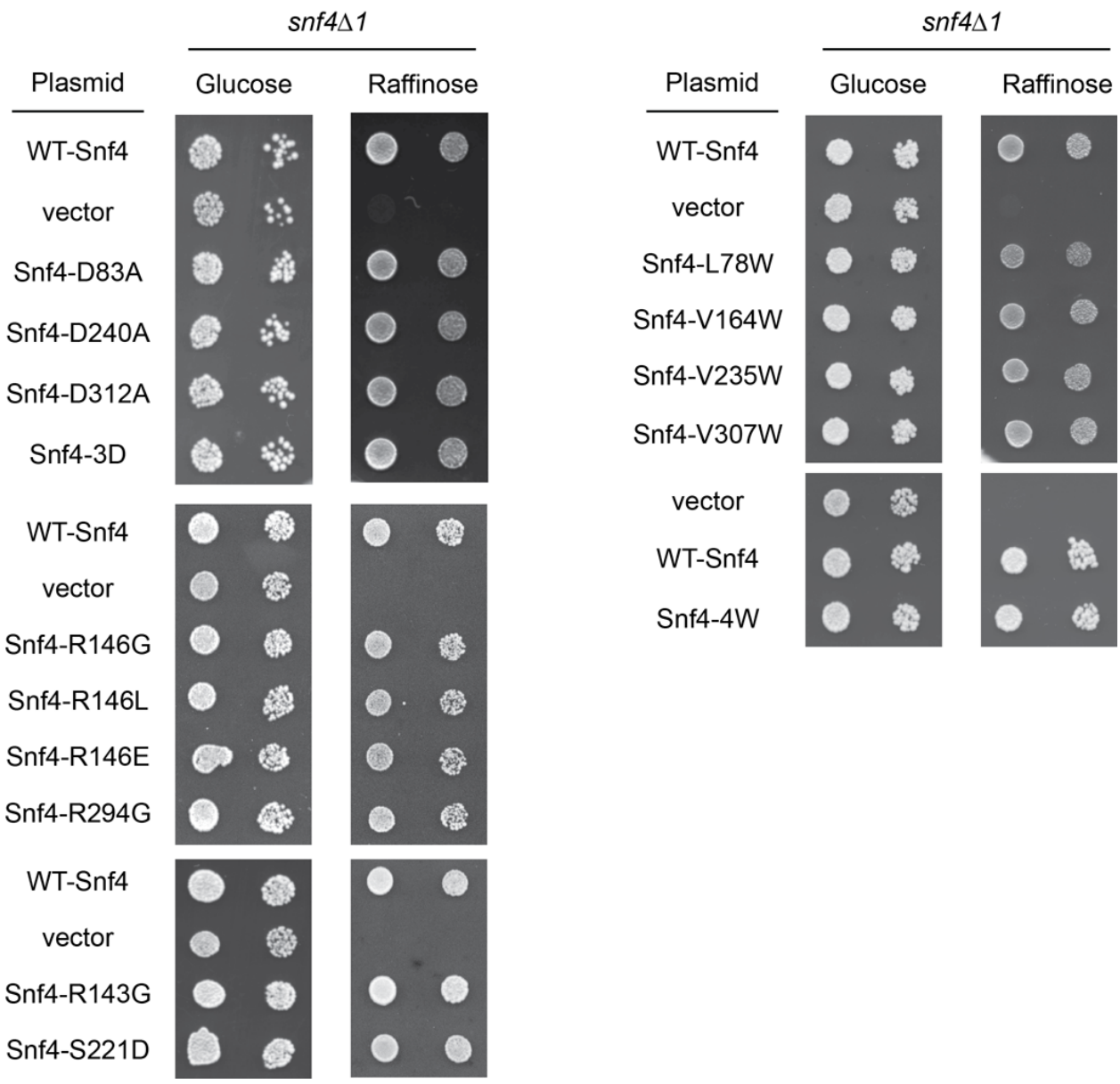

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FIGURE S3.

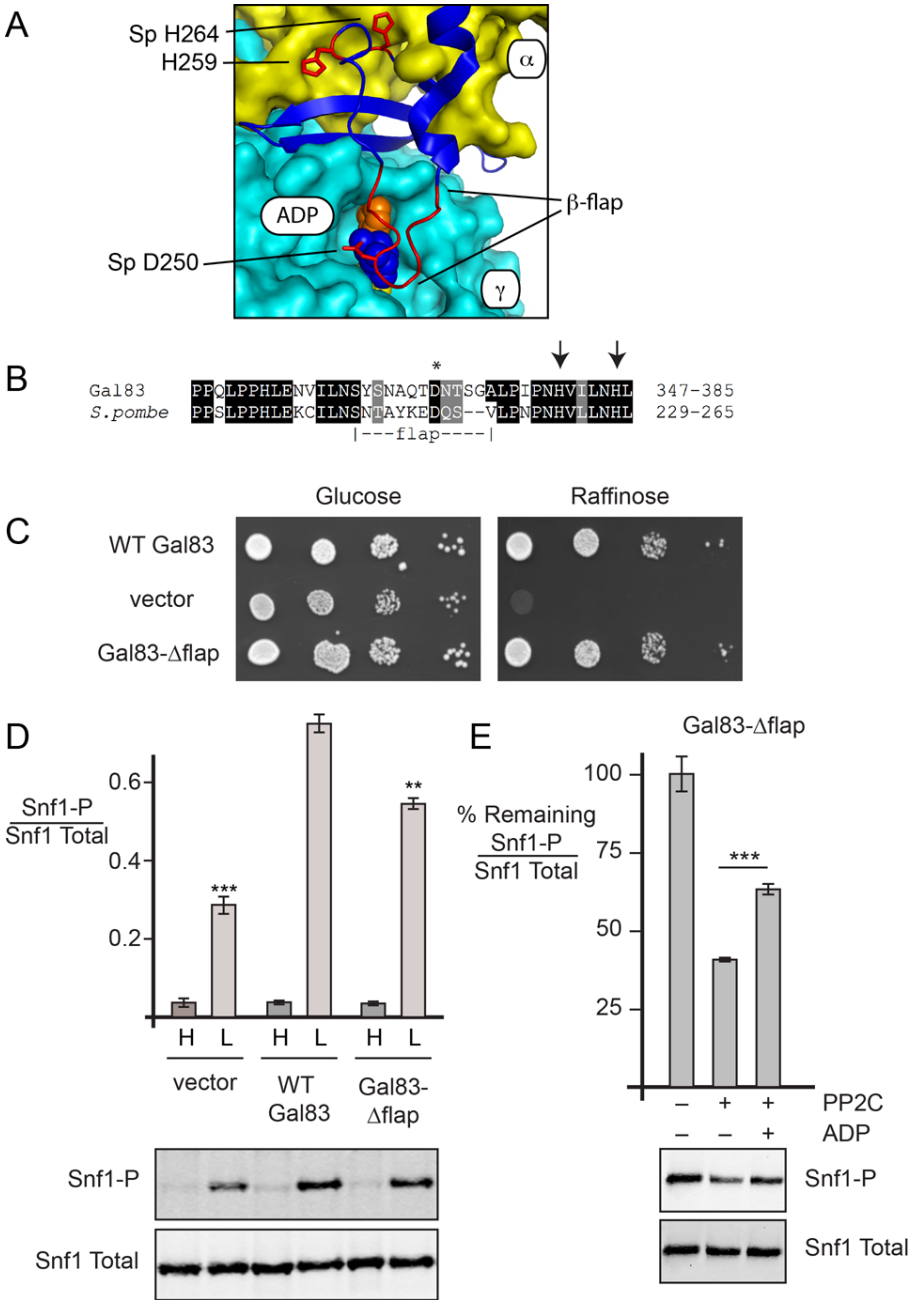

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FIGURE S4.

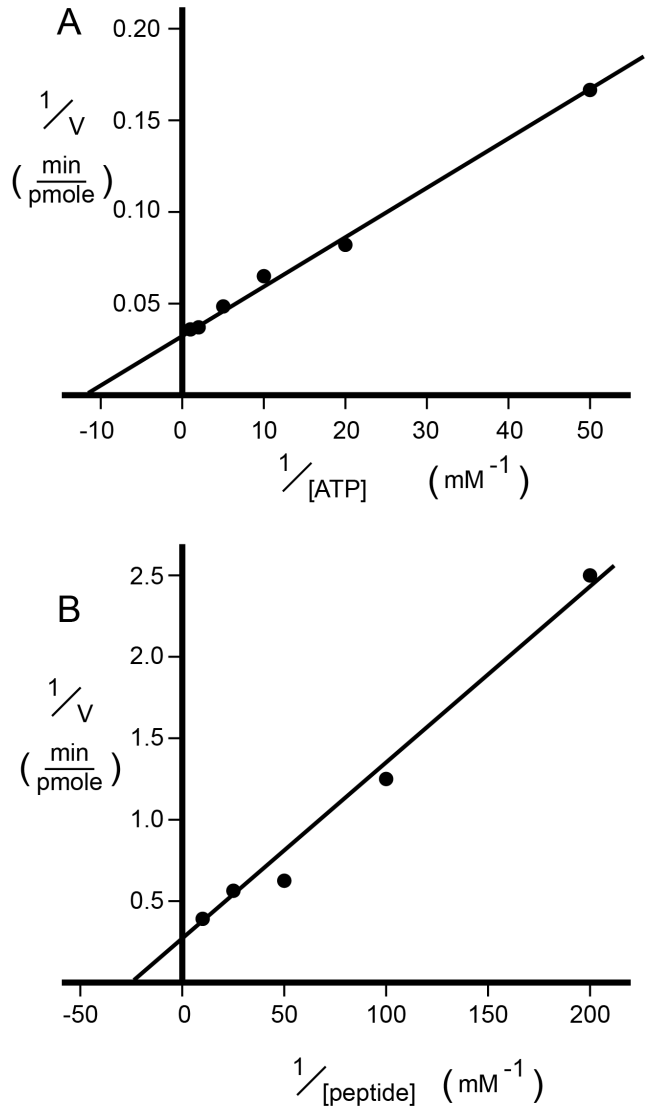

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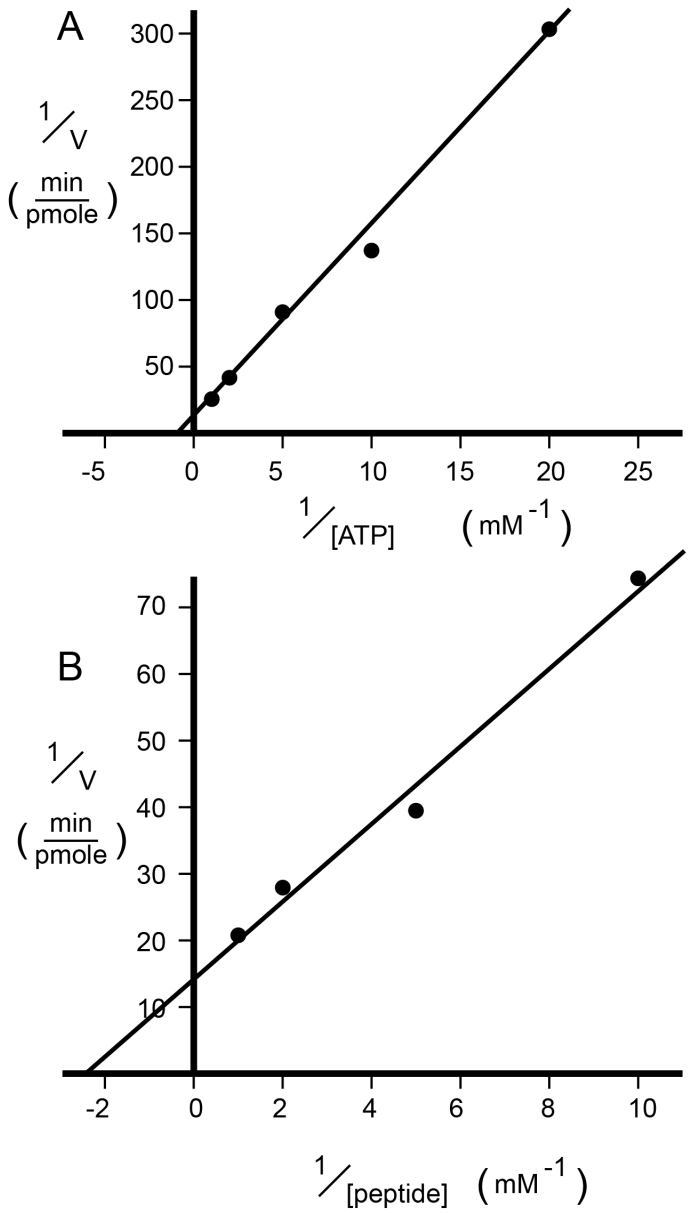
FIGURE S5.



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