Online Supplemental Material for

Michard et al. **http://www.jgp.org/cgi/doi/10.1085/jgp.200509413**

Generation of Mutants and Chimeras

Mutants and chimeras were created by PCR-based techniques and with the pAlter mutagenesis system (Promega) using appropriate oligonucleotides to introduce the desired mutations.

AKT2 Mutations Generated with the pAlter System

The KpnI-XbaI fragment of the AKT2 coding region was cloned into the KpnI-XbaI sites of the vector pAlter-1. The mutagenesis reactions were performed according to the user manual of the pAlter mutagenesis system. To introduce the mutations the following oligonucleotides were used: AKT2-G186R: 5′-CACTTgTAATCTCTTgCgATTACTTAgATTTTggCgACTTCgACgCgTTAAACACCTCTTCACTAggC-3′, AKT2-K197S: 5′-gggATTACTTAgATTTTggCgACTTCgACgCgTTAgTCACCTCTTCACTAggCTCgAgAAgg-3′, AKT2-K197D: 5′-gggATTACTTAgATTTTggCgACTTCgACgCgTTgATCACCTCTTCACTAggCTCgAgAAgg-3′, AKT2-H198S: 5′-gggATTACTTAgATTTTggCgACTTCgACgCgTTAAAAgCCTCTTCACTAggCTCgAgAAgg-3′, AKT2-K197S-H198S: 5′-gggATTACTTAgATTTTggCgACTTCgACgCgTTAgTAgCCTCTTCACTAggCTCgAgAAgg-3′, AKT2-S210A: 5′-CgAgAAggACATAAgATATgCCTATTTCTggATCCgCTgCTTTCgACTTCTATC-3′ AKT2-S210N: 5′-CgAgAAggACATAAgATATAACTATTTCTggATCCgCTgCTTTCgACTTCTATC-3′, AKT2-S329A: 5′-ggACTCgTCgTACCATggAATTCAggAATgCCATTgAAgCAgCgTCAAACTTTgTTAACAg-3′, AKT2-S329N: 5′-ggACTCgTCgTACCATggAATTCAggAATAACATTgAAgCAgCgTCAAACTTTgTTAACAg-3′.

Subsequently, the KpnI-XbaI cassette of the pCI-AKT2 plasmid was replaced by the respective mutagenized cassette. All mutations were verified by sequencing the mutagenized cassette. To generate the double mutant S210A-S329A the 1.7-kb EcoRV-NotI fragment of pCI-AKT2-S210A was replaced by the corresponding fragment of pCI-AKT2-S329A. The double mutant S210N-S329N was created accordingly. For the triple mutants K197S-S210A-S329A and K197S-S210N-S329N the 1.8-kb XhoI-NotI fragment of pCI-AKT2-K197S was replaced by the corresponding fragments of pCI-S210A-S329A and pCI-S210N-S329N, respectively.

KAT1 Mutations Generated with the pAlter System

The entire KAT1 coding region was cloned as a SacI-BamHI fragment into the SacI-BamHI sites of the vector pAlter-1. The mutagenesis reactions were performed according to the user manual of the pAlter mutagenesis system. To introduce the mutations the following oligonucleotides were used: KAT1-XhoI: 5′-CgCTATTTgCAAggCTCgAgAAAgATATCCgTTTCAAC-3′ and KAT1-S179K: 5′-CgTCTCCggCgAgTTAAgTCgCTATTTgCAAggCTCgAgAAAgATATCCgTTTCAAC-3′ (XhoI site underlined in primer sequence and indicated in Fig. S1 B). Subsequently, the KpnI-XcmI cassette of the pCI-KAT1 plasmid was replaced by the mutagenized cassettes. The mutations were verified by sequencing the mutagenized cassette.

Generation of AKT2-KAT1 Chimeras

The exchange of the S5-P-S6 region from AKT2 by the corresponding region of KAT1 and vice versa was achieved by polymerase chain reactions using Pfu polymerase (Promega) in two-step protocols.

Replacing the S5-P-S6 Region of AKT2 by the S5-P-S6 Region of KAT1. Two PCRs were performed using for one PCR pCI-KAT1-XhoI as template and the primer KAT1-f: 5′-CCgCAAACACATAATCTCTCCTTTTAATCC-3′ together with the chimeric primer KAT1-AKT2-r: 5′-ggTACgACgAgTCCCTTCCACCACgAggTTggTCATATTTCCAATgAgg-3′, and for the other PCR pCI-AKT2 as template and the primer AKT2-r: 5′-CAATCTCAgCTCCATCTTCATTCgTCACC-3′ together with the chimeric primer KAT1-AKT2-f: 5′-CCTCATTggAAATATgACCAACCTCgTggTggAAgggACTCgTCgTACC-3′.

The PCR products were purified and used as templates for the second elongating step again by PCR using the KAT1-f and AKT2-r flanking primers. Subsequently, the XhoI-XbaI cassette of the pCI-AKT2 plasmid was replaced by the chimeric cassette. The chimera contained additionally a restriction site for BstXI (underlined in primer sequences and indicated in Fig. S1 A). The chimera was verified by sequencing the exchanged cassette.

Replacing the S5-P-S6 Region of KAT1 by the S5-P-S6 Region of AKT2. Two PCRs were performed using for one PCR pCI-AKT2 as template and the primer AKT2-f: 5′-CCAgAATCACATCAggTCTAgTggATgg-3′ together with the chimeric

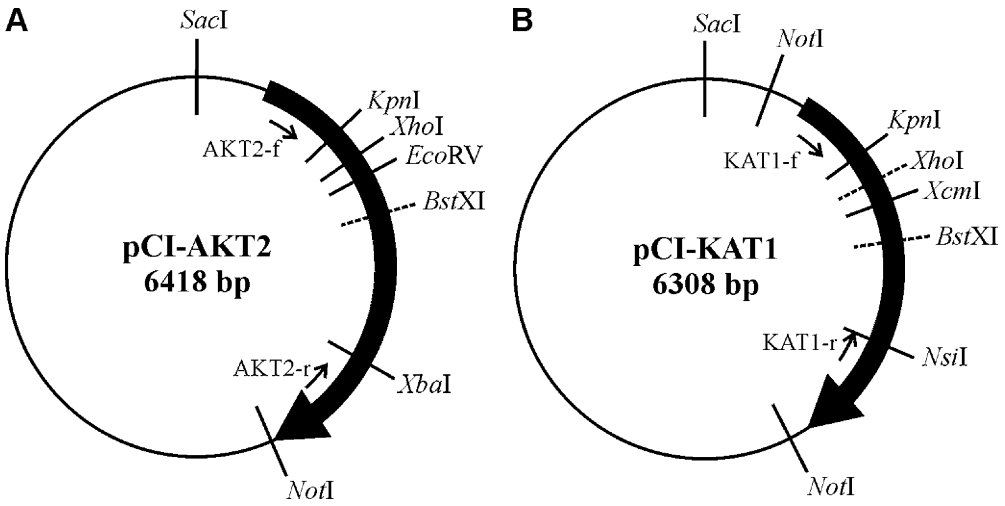

Figure S1. Vector maps. (A) Vector map of the plasmid pCI-AKT2. The coding sequence of AKT2 was cloned as SpeI-NotI fragment into the NheI-NotI sites of the pCI vector. The coding sequence of AKT2 is shown as black arrow. The restriction sites used in this study are displayed, the unique restriction sites for SacI, KpnI, XhoI, EcoRV, XbaI, and NotI. For the generation of chimeras the primers AKT2-f and AKT2-r were used. The restriction site for BstXI was introduced by silent base exchanges in chimeric plasmids (see below). (B) Vector map of the plasmid pCI-KAT1. The coding sequence of KAT1 was cloned as NotI fragment into the NotI site of a modified pCI vector. A large part of the MCS of pCI was removed by cleavage with the enzyme combination SalI/XhoI followed by religation. The coding sequence of KAT1 is shown as black arrow. The restriction sites used in this study are displayed: The unique restriction sites for SacI, KpnI, XcmI, and NsiI. The restriction site for XhoI was introduced by silent base exchanges (see below). For the generation of chimeras the primers KAT1-f and KAT1-r were used. The restriction site for BstXI was introduced by silent base exchanges in chimeric plasmids (see below).

primer AKT2-KAT1-r: 5′-ggTTCggCTAgTCCAATgAACCACgAggTTggTCATgTTACCAATAAgg-3′, and for the other PCR pCI-KAT1 as template and the primer KAT1-r: 5′-ATTgTTCATgATgACTCgTCCATCg-3′ together with the chimeric primer AKT2-KAT1-f: 5′-CCTTATTggTAACATgACCAACCTCgTggTTCATTggACTAgCCgAACC-3′.

The PCR products were purified and used as templates for the second elongating step again by PCR using the AKT2-f and KAT1-r flanking primers. Subsequently, the XhoI-NsiI cassette of the pCI-KAT1-XhoI plasmid was replaced by the chimeric cassette. The chimera contained additionally a restriction site for BstXI (underlined in primer sequences and indicated in Fig. S1 B). The chimera was verified by sequencing the exchanged cassette. All other chimeras were created by exchanging the corresponding SacI-XhoI fragments and/or the corresponding SacI-BstXI fragments.