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Plasmid construction

His6-tagged plasmid (pRE540) expressing wild-type Kes1p was constructed by in frame insertion of the 730-bp PCR fragment of KES1 NH_2-terminal coding sequence into the pQE30 vector (using primers KES-H and KES-F; Table S1) by BamHI and EcoRI. Then, the remaining COOH terminus of KES1 coding sequence was cloned into the above construct from pCTY244 by EcoRI and ClaI to form the complete coding sequence of KES1 gene.

GST-tagged KES1 plasmid (pRE682) was constructed by insertion of KES1 coding sequence from pCTY225 into pGEX-4T-3. To construct plasmids expressing GST-Kes1p(1-314), an XhoI site was engineered between K314/A315 of pRE682 by site-directed mutagenesis using primers Kes-L4 and M4 (Table S1). The mutagenized KES1 was truncated by restriction with XhoI and religated to yield pRE657. To construct the GST-Kes1p PH domain plasmid, a BamHI site was inserted between S170/F171 of pRE657 by site-directed mutagenesis using primers Kes-S2 and T2 (Table S1). KES1 codons 1–170 were deleted by restriction with BamHI and religation to yield pRE665.

To construct E. coli plasmids expressing full-length His6-tagged or GST-tagged Kes1p proteins, or appropriate truncation fragments, the desired mutations were introduced into corresponding plasmids by site-directed mutagenesis using primers as described in Table S1. All mutations were confirmed by DNA sequencing.

To construct plasmids expressing Kes1p-GFP or Kes1p-YFP in yeast, a 15-bp linker region containing BamHI and XbaI sites was inserted immediately upstream of the KES1 stop codon using primers KES1/GFP-A and -B (Table S2). A 750-bp PCR product containing appropriately clamped GFP or YFP sequences was generated using primers GFP-BamHI and -XbaI (Table S2). The PCR products were inserted into the BamHI and XbaI sites to generate the desired chimeras. Appropriate mutations were introduced into expression plasmids by site-directed mutagenesis using the primers described above.

To construct the KEX2-RFP expression plasmid, the 3×Myc fragment of YEp(KEX2-3×Myc) was replaced by a 670-bp PCR fragment of RFP (using primers RFP-5′-Kpn and RFP-3′-Bam; Table S2) clamped by KpnI and BamHI sites. Then, another 670-bp PCR fragment of RFP (using primers RFP-5′-Bam and RFP-3′-Sal; Table S2) was inserted into the BamHI and SalI sites that reside downstream of the first RFP. Thus, the KEX2-RFP gene fusion represents a fusion of KEX2 to two tandem copies of RFP.

Expression and purification of His6-tagged and GST-tagged proteins from *E. coli*

1 liter of Superbroth (12 g tryptone, 24 g yeast extract, 4 g glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, and 50 mg/ml ampicillin) was inoculated with 10 ml of an overnight culture of *E. coli* strain KK2186 harboring plasmids expressing His6- or GST-tagged proteins. The culture was grown at 37°C for 2–3 h before inducing with isopropyl b-D-thiogalactopyranoside (IPTG; 0.5 mM). After 3–5 h cells were harvested by centrifugation (5,000 rpm, 10 min). The cell pellet was resuspended with 10 ml ice-cold PBS (10 mM KPO_4, pH 7.4, 100 mM NaCl, and 2.7 mM KCl) supplemented with lysozyme, and shaken for 10 min at 25°C. PMSF (1 mM) was added and cells were disrupted by sonication (60% power, 4 bursts of 30 s with 30-s intervals on ice). Triton X-100 was added (final concentration 0.1% vol/vol), and lysate was incubated (10 min at 25°C). After addition of DNase I (10 mg/ml) and $MgCl_2$ (10 mM), lysate was centrifuged (12,000 *g*, 20 min at 4°C). The supernatant was collected and filtered through a 0.45-mm low protein binding membrane (Gelman Sciences).

To purify His6-tagged proteins, HiTrap Ni-chelating columns (Amersham Pharmacia Biotech) were equilibrated with PBS, loaded, and washed with PBS. Proteins were eluted with 500 mM imidazole in PBS. To purify GST-tagged proteins, filtrates were incubated with glutathione-Sepharose 4B beads (30 min at 4°C). Beads were loaded into a column, washed with PBS buffer, and bound proteins were eluted in 2.5 ml 10 mM glutathione, pH 8.0. Elution buffer was rapidly substituted by desalting with TE, pH 7.4 (PD-10 column; Amersham Pharmacia Biotech), and concentrated by Centricon filtration (Millipore). Protein concentration was determined by Bradford protein assay (Bio-Rad Laboratories).

PIP photolabeling assay

Lipids in chloroform were dried under a stream of N_2 gas and emulsified in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA (TE) buffer by sonication. Purified protein (0.02–0.04 nmol) was incubated in 50 μl TE, pH 7.4, buffer with [3H]BZDC-PI(4,5)P2 (final concentration 70 nM) and 0.1% Triton X-100. In displacement reactions, competitor lipids were added (final concentration of 10 mM). Reactions were equilibrated in wells of Dynatech Immulon microtiter plates for 10 min and irradiated with UV light (360 nm; 1 h). Adducts were resolved by SDS-PAGE, gels were stained with Coomassie blue, destained, treated with Entensify (NEN Life Science Products), dried, and subjected to autoradiography (80°C, 3 wk). Signal was quantified by densitometry. For IP_3 experiments, [3H]BZDC-IP_3 (400 nM) was used and Triton X-100 was omitted.

Table SI. **Primers**

Mutations	Primers	Mutations	Primers	Mutations	Primers
P30T	Kes-O3, Kes-P3	E117A	Kes-D4, Kes-E4	R236E, KK242/243EE	Kes-3E-5′, Kes-3E-3′
S43Y	Kes-Q3, Kes-R3	EQ139/140AA	Kes-E3, Kes-F3		
E107K	Kes-S3, Kes-T3	HH143/144AA	Kes-G3, Kes-H3	E312K	Kes-W3, Kes-X3
K109A	Kes-B4, Kes-C4	LH201/202FN	Kes-U3, Kes-V3	W317A	Kes-C2, Kes1-D2

Table S2. **Primer sequences**

Primers	Primer sequences (5′–3′)
Kes-H	ccggatccatgtctcaatacgcaagc
Kes-F	ccgtcgactagcctgatattgtgtataatgcctt
Kes-C2	gaaagtagaaaagccgcgtacgacgtggctggtgcc
Kes-D2	ggcaccagccacgtcgtacgcggcttttctactttc
Kes-S2	caaccaaattaaagccagtggatccttcactaaatccttaatgc
Kes-T2	gcattaaggatttagtgaaggatccactggctttaatttggttg
Kes-E3	gaaacggttttgttaagtgcggcagtttctcacc atccaccc
Kes-F3	gggtggatggtgagaaactgccgcacttaacaaaaccgtttc
Kes-G3	ttaagtgagcaagtttctgccgcaccacccgtcactgctttt
Kes-H3	aaaagcagtgacgggtggtgcggcagaaacttgctcacttaa
Kes-O3	ctttcttcgttatcggctactccattcattttatctcca
Kes-P3	tggagataaaatgaatggagtagccgataacgaagaaag
Kes-Q3	atctcattgaccgagttttctcagtactgggctgaacat
Kes-R3	atgttcagcccagtactgataaaactcggtcaatgagat
Kes-S3	aatgaatccttaggttctaagaaaaaaccttgaaccca
Kes-T3	tgggttcaaaggtttttcttagaacctaaggattcatt
Kes-U3	ttggttaccccacctccattcaatattgaaggtattcttgtc
Kes-V3	gacaagaataccttcaatattgaatggaggtggggtaaccaa
Kes-W3	gagcaacacccactggcaagtagaaaagcctggtac
Kes-X3	gtaccaggcttttctacttgccagtgggtgttgctc
Kes-B4	tccttaggttctgagaaagcacctttgaacccatttcta
Kes-C4	tagaaatgggttcaaaggtgctttctcagaacctaagga
Kes-D4	ttgaacccatttctaggtgcgttgttcgttggtaaatggg
Kes-E4	ccatttaccaacgaacaacgcacctagaaatgggttcaa
Kes-L4	ccactggaaagtagaaaactcgaggcctggtacgacgtggct
Kes-M4	agccacgtcgtaccaggcctcgagttttctactttccagtgg
Kes-3E-5′	tgtgttattgaattttcaggtgaaggctacttttctggtgaagaaaattcattcaaggcaaga
Kes-3E-3′	tcttgccttgaatgaattttcttcaccagaaaagtagccttcacctgaaaattcaataacaca
KES1/GFP-A	gaaaaggaaattgttttgggatccgcatctagataaggagaacgataaagt
KES1/GFP-B	actttatcgttctccttatctagatgcggatcccaaaacaatttccttttc
GFP-BamHI	aaaaggatccgccaccatggtgagcaagggcgag
GFP-XbaI	aaaatctagacttgtacagctcgtccatgcc
RFP-5′-Kpn	aaaaggtaccgcaggagcgggcatggtgcgctcctccaagaacgtcatc
RFP-3′-Bam	aaaaggatccactagtgcgcgggcgcaggacaggaacaggtggtggcggccctc
RFP-5′-Bam	aaaaactagtgcaggatccatggtgcgctcctccaagaacgtcatc
RFP-3′-Sal	aaaagtcgaccaggaacaggtggtggcggccctc