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Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway

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The protection against apoptosis provided by growth factors in several cell lines is due to stimulation of the phosphatidylinositol-3-OH kinase (PI(3)K) pathway, which results in activation of protein kinase B[1,2] (PKB; also known as c-Akt and Rac) and phosphorylation and sequestration to protein 14-3-3 of the pro-apoptotic Bcl-2-family member BAD[3–7]. A modest increase in intracellular Ca^{2+} concentration also promotes survival of some cultured neurons[8,9] through a pathway that requires calmodulin but is independent of PI(3)K and the MAP kinases[10,11]. Here we report that Ca^{2+}/calmodulin-dependent protein kinase kinase (CaM-KK) activates PKB directly, resulting in phosphorylation of BAD on serine residue 136 and the interaction of BAD with protein 14-3-3. Serum withdrawal induced a three- to fourfold increase in cell death of NG108 neuroblastoma cells, and this apoptosis was largely blocked by increasing the intracellular Ca^{2+} concentration with NMDA (*N*-methyl-D-aspartate) or KCl or by transfection with constitutively active CaM-KK. The effect of NMDA on cell survival was blocked by transfection with dominant-negative forms of CaM-KK or PKB. These results identify a Ca^{2+}-triggered signalling cascade in which CaM-KK activates PKB, which in turn phosphorylates BAD and protects cells from apoptosis.

To identify a Ca^{2+}-mediated, PI(3)K-independent cell-survival mechanism, we tested whether CaM-KK might phosphorylate the 'activation-loop' residue T308 in PKB[12–15]—CaM-KK phosphorylates similar activation-loop sites in calmodulin-dependent protein kinases I and IV (refs 16–18). CaM-KK alone exhibited strong *in vitro* autophosphorylation[19] on multiple sites whereas a fusion protein containing glutathione-*S*-transferase and PKB exhibited little autophosphorylation; incubation of CaM-KK and GST–PKB together produced phosphorylation by CaM-KK of the PKB (Fig. 1a). Analysis of ^{32}P-labelled amino acids showed that the phosphorylated residue of PKB was a threonine (data not shown), presumably T308 because the T308A mutant was not phosphorylated by CaM-KK (Fig. 1a).

Phosphorylation of T308 should result in activation of PKB[12–15]. α-CaM-KK or PKB alone each exhibited low kinase activity towards histone H2B, but *in vitro* incubation of these proteins together resulted in a synergistic increase in kinase activity towards histone H2B (Fig. 1b). The T308A PKB mutant, which has normal basal PKB activity, showed little enhancement of activity in response to α-CaMKK, indicating that CaM-KK was probably activating PKB. We confirmed this using a two-step assay: haemagglutinin (HA)-tagged PKB was expressed in COS-7 cells, immunoprecipitated and incubated for 20 min without or with recombinant α-CaM-KK, Ca^{2+}/calmodulin and Mg^{2+}/ATP. After washing the immune complex to remove CaM-KK, we studied PKB activity by assaying histone phosphorylation in the presence of EGTA, which inhibits any residual CaM-KK. CaM-KK catalysed strong activation of wild-type PKB in a Ca^{2+}/calmodulin-dependent manner (Fig. 1c). Phosphorylation and activation of the T308A PKB mutant did not occur (Fig. 1a–c) and activation of wild-type PKB was reversed by treatment with protein phosphatase 2A (Fig. 1d), showing that activation of PKB by CaM-KK was due to phosphorylation, consistent with Fig. 1a. Expressed HA–PKB showed significant basal phosphorylation of its other regulatory site, S473 (ref. 15), determined using a phospho-specific antibody. Phosphorylation of S473 in HA–PKB was not increased by co-transfected CaM-KK activated by ionomycin but was markedly increased by treatment with platelet-derived growth factor (PDGF) (data not shown). The results of Fig. 1 show directly that recombinant CaM-KK can activate recombinant PKB *in vitro* through phosphorylation of T308.

Can CaM-KK activate PKB in cells? We co-transfected COS-7 cells with HA–PKB and either wild-type or constitutively active CaM-KK. In the absence of CaM-KK there was low PKB activity in the immune complex assay described above, but upon co-transfection with constitutively active CaM-KK (Fig. 2a) or wild-type CaM-KK plus ionomycin treatment (Fig. 2b) there was marked activation of wild-type PKB but not of the T308A mutant (Fig. 2a). Activation of PKB by CaM-KK was not sensitive to the PI(3)K inhibitor wortmannin (Fig. 2b, c). The rate of activation (Fig. 2c) was dependent on the amount of CaM-KK plasmid used (data not shown), and activation was reversed by subsequent *in vitro* treatment with protein phosphatase 2A (Fig. 2d).

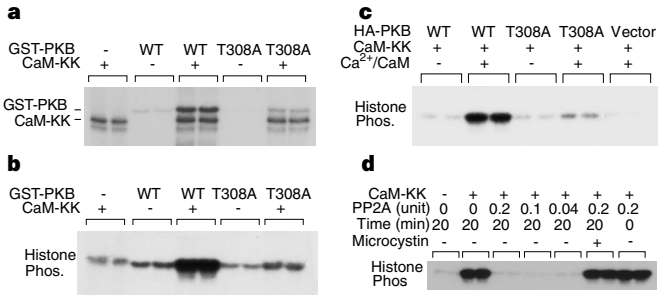

Figure 1 Phosphorylation and activation of recombinant PKB by CaM-KK *in vitro*. Each experiment in Figs 1 and 2 was replicated at least three times. **a**, GST–PKB (wild-type, WT, or T308A; 0.6 μg plasmid) was ^{32}P-labelled by CaM-KK (72 nM). Reaction products were analysed by SDS–PAGE/autoradiography. Duplicate samples are shown. **b**, GST–PKB (WT or T308A; 0.6 μg) was phosphorylated by CaM-KK (72 nM) in the presence of the PKB substrate histone H2B (0.2 mg ml^{-1}) and ^{32}P-ATP before analysis by SDS–PAGE/autoradiography. **c**, Immunoprecipitated HA–PKB (WT or T308A) was phosphorylated by CaM-KK (0.36 μM) and washed to remove CaM-KK, and the immune complex was assayed for PKB activity using histone H2B as a substrate in the presence of 2 mM EGTA, a Ca^{2+} chelator. **d**, HA–PKB (WT) was activated by CaM-KK and washed as in **c**. The indicated units of protein phosphatase 2A (PP2A) catalytic subunit were incubated for 0 or 20 min in the absence or presence of 1 μM of the PP2A inhibitor microcystin. The immune complex was washed again, and PKB activity was assayed, using histone H2B as a substrate, in the presence of 1 μM microcystin. CaM, calmodulin.

COS-7 cells do not contain detectable endogenous CaM-KK, so ionomycin treatment in the absence of transfected CaM-KK did not result in PKB activation (Fig. 2c), as shown previously for 3T3 cells[20]. Therefore, in further experiments we used the neuroblastoma NG108 cell line, which contains endogenous CaM-KK[21] and Ca^{2+}-permeable NMDA-type ionotropic glutamate receptors. Stimulation of NG108 cells with NMDA or by depolarization with KCl resulted in a three- to fourfold activation of transfected or endogenous PKB that peaked at 1 hour after stimulation and remained elevated for up to 6 hours (Fig. 3a). The activation of HA–PKB by NMDA was blocked by dominant-negative PKB (Fig. 3c) or addition of EGTA (20 mM) or the NMDA antagonist MK-801 (10 μM) to the medium (data not shown), and was apparently achieved by endogenous CaM-KK, as it was blocked by co-transfection with a dominant-negative CaM-KK (Fig. 3b). Activation of HA–PKB (sevenfold) by insulin-like growth factor (IGF), which was inhibited by wortmannin, was not blocked by dominant-negative CaM-KK (data not shown). HA–PKB activation by NMDA at 30 min was roughly twofold in either the presence (Fig. 3a) or the absence (Fig. 3b) of wortmannin, indicating that this is a PI(3)K-independent pathway.

PKB activation by CaM-KK should result in phosphorylation of BAD[3,4] on residue S136 and its sequestration through interaction with protein 14-3-3 (refs 22, 23). We tested the ^{32}P-phosphorylation of GST–BAD *in vitro*; CaM-KK was not a catalyst, but PKB activated by CaM-KK was effective (data not shown). When NG108 cells were transfected with GST–BAD and then either transfected with constitutively active CaM-KK (Fig. 3d) or treated with NMDA (Fig. 3e), the phosphorylation of S136 in BAD was enhanced; the effect of NMDA was blocked by dominant-negative CaM-KK (Fig. 3e). Lastly, when endogenous BAD from NMDA-treated cells was immunoprecipitated, there was a time-dependent increase in co-precipitation of protein 14-3-3 (Fig. 3f).

We next studied apoptosis in NG108 cells upon serum withdrawal, using co-expressed green fluorescent protein (GFP) to identify cells transfected with the proteins mentioned below. Serum withdrawal for 24 hours induced a three- to fourfold increase in apoptosis; this enhanced apoptosis was blocked by 60–70% by treatment with Ca^{2+}-mobilizing agonists that activate PKB, such as NMDA (Fig. 4a) or 25 mM KCl (data not shown). The protective effect of NMDA was blocked by transfection with dominant-negative PKB (Fig. 4a), supporting the idea that PKB is involved in this pathway. These results indicate that apoptosis of these cells in

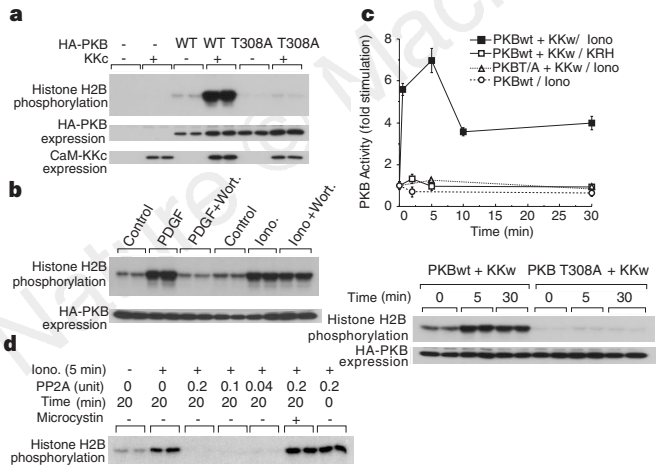

Figure 2 Activation of PKB by CaM-KK in transfected COS-7 cells. **a**, Cells were transfected with HA–PKB (WT or T308A; 0.6 μg plasmid) without or with constitutively active CaM-KK (KKc) (0.6 μg). HA–PKB was immunoprecipitated and assayed for activity with the substrate histone H2B (top). Western blots confirmed the expression levels of HA–PKB (centre) and CaM-KK (bottom). **b**, Cells transfected with HA–PKB (WT; 0.6 μg) and CaM-KK (WT; 0.6 μg) were treated for 10 min with PDGF or ionomycin with or without wortmannin. Immunoprecipitated PKB was assayed for activity (top) and level of expression (bottom). **c**, Cells were transfected with the indicated constructs (PKBT/A is the T308A PKB mutant; KKw is wild-type CaM-KK), and treated with KRH buffer containing 200 nM wortmannin without or with 1 μM ionomycin. Top, immunoprecipitated HA–PKB was assayed and normalized to time zero; centre, autoradiograph of a typical experiment including ionomycin; bottom, levels of HA–PKB expression. **d**, Cells transfected with HA–PKB and CaM-KK were treated for 5 min with 1 μM ionomycin. HA–PKB was immunoprecipitated and incubated with the indicated units of PP2A as in Fig. 1d; PKB activity towards histone H2B was measured.

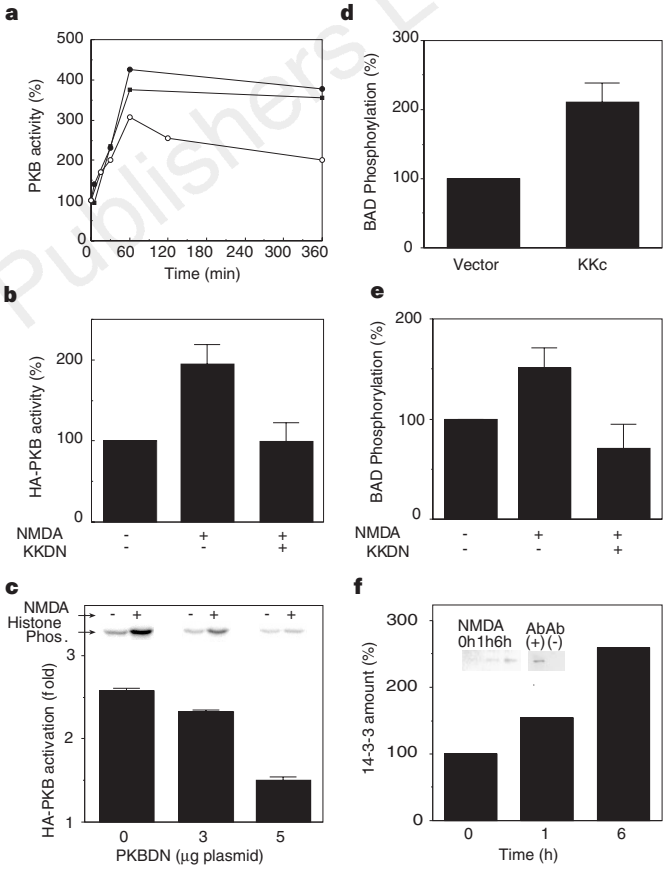

Figure 3 Phosphorylation of PKB and BAD as a result of CaM-KK activity in NG108 cells. **a**, Endogenous PKB (filled symbols) or transfected HA–PKB (1 μg plasmid; open circles) was immunoprecipitated and assayed from NG108 cells treated with 100 μM NMDA (circles) or 25 mM KCl (squares) in Mg^{2+}-free KRH buffer containing 200 nM wortmannin. **b**, **c**, NG108 cells, transfected with HA–PKB (1 μg plasmid) and either vector alone or vector expressing dominant-negative CaM-KK (KKDN; **b**; 5 μg plasmid) or dominant-negative PKB (PKBDN, **c**), were treated with or without NMDA for 30 min; immunoprecipitated HA–PKB activity towards histone H2B was assayed. The inset in **c** shows one experiment ± NMDA. **d**, **e**, Cells transfected with GST–BAD (1 μg plasmid) and either empty vector, constitutively active CaM-KK (KKc, **d**; 1 μg plasmid) or KKDN (**e**; 5 μg plasmid) were either non-stimulated (**d**) or treated for 1 h with or without NMDA (**e**). GST–BAD was precipitated and subjected to western analysis using an antibody specific for phosphorylated residue S136. **f**, Endogenous BAD was immunoprecipitated after treatment of cells with NMDA for 0, 1 or 6 h. The immune complex was subjected to western analysis of co-precipitated protein 14-3-3 (measured relative to time zero). Bars show averages from two experiments. Inset, the left three lanes show the results obtained from one experiment; the right two lanes show specific co-precipitation of 14-3-3 only, when anti-BAD antibody (Ab) was used for immunoprecipitation.

response to serum deprivation can be largely reversed by a Ca^{2+}-dependent, PKB-mediated pathway that may involve CaM-KK. If true, co-transfection with constitutively active CaM-KK should mimic the effect of NMDA treatment whereas dominant-negative CaM-KK should block the protective effect of NMDA. These were exactly the results observed (Fig. 4b). However, protection against apoptosis by treatment with IGF, which activates a PI(3)K-dependent pathway, was not reversed by dominant-negative CaM-KK (data not shown). In Fig. 4c, apoptosis of GFP-positive cells expressing dominant-negative CaM-KK (white dots with arrows) was about the same with (bottom panel) or without (middle panel) NMDA treatment, whereas in non-transfected cells (those not expressing GFP or dominant-negative CaM-KK) NMDA markedly protected against apoptosis (compare white dots without arrows in middle and bottom panels).

Programmed neuronal cell death is important in vertebrate neural development, during which roughly half of all neurons die. Survival usually results from the presence of neurotrophic factors provided by the target tissue or from persistent neuronal activity.

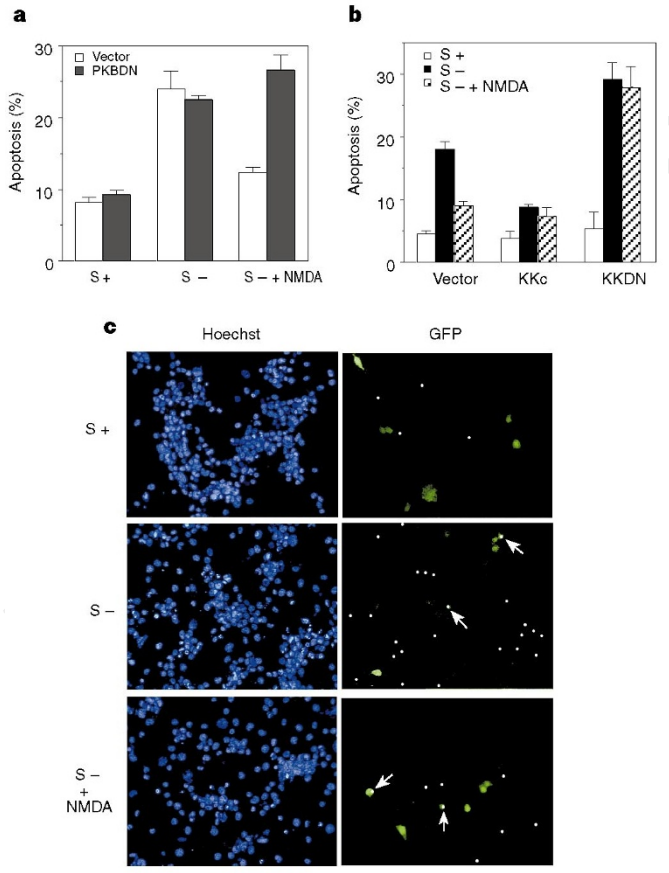

Figure 4 Regulation of apoptosis in NG108 cells. **a**, **b**, Cells transfected with GFP and either vector alone or vector expressing constitutively active CaM-KK (KKc; 1 μg), dominant-negative PKB (PKBDN) (5 μg) or dominant-negative CaM-KK (KKDN) (5 μg) were maintained in serum for 24 h (S+) or were serum-starved for 24 h in the absence (S–) or presence (S– NMDA+) of NMDA. GFP-positive cells were analysed for apoptosis by Hoechst staining: small, bright, condensed nuclei indicate apoptotic cells (see **c**). Apoptosis was quantified for each transfection/treatment condition in three plates using 150–200 GFP-positive cells. **c**, Selected fields of cells transfected with GFP (0.6 μg) and KKDN (5 μg) and treated as indicated (from the experiment giving the data shown in the right-hand three bars of **b**). Apoptotic cells were identified by Hoechst staining (left) and marked on computer images with a white dot, which was then overlayed with GFP immunofluorescence (right). Apoptotic GFP-positive cells are denoted by arrows.

For example, blockage of electrical activity in some types of developing neuron reduces their chance of survival during the period of programmed death[24]. We propose a molecular model in which neuronal activity, by increasing the intracellular Ca^{2+} concentration to a modest degree, can protect cells against apoptosis. The increased intracellular Ca^{2+} concentration would stimulate CaM-KK, which in turn activates PKB. Activated PKB phosphorylates BAD[3,4], resulting in its sequestration by protein 14-3-3 (refs 22, 23). These results also indicate a broader role for CaM-KK, as it can phosphorylate and activate a cellular target protein (PKB) that is not also activated by Ca^{2+}/calmodulin, as is the case for calmodulin-dependent protein kinases I and IV (refs 16–18).

Methods

General. Protein kinase B (α-isoform) complementary DNA[25,26] was cloned from rat brain messenger RNA using reverse transcription with polymerase chain reaction, and the T308A mutant was made by standard *in vitro* mutagenesis (5Prime → 3Prime, Inc.) and sequenced to confirm the mutation. The HA-tagged dominant-negative PKB double mutant (K179A (refs 1, 7) plus T308A) was catalytically inactive when expressed in COS-7 cells and assayed in the immune complex. The expression plasmids for wild-type CaM-KK and constitutively active CaM-KK have been described[21]. For *in vitro* experiments, CaM-KK was expressed in Sf9 cells and purified on CaM–Sepharose. Dominant-negative CaM-KK contained a K157A mutation. For transfections of COS-7 and NG108 cells, we used LipofectAMINE[27]. HA–PKB was immunoprecipitated with monoclonal antibody 12CA5 (Boehringer) and endogenous PKB in NG108 cells was immunoprecipitated with an antibody against the pleckstrin-homology domain (Upstate Biotechnology). For experiments in which HA–PKB was activated by CaM-KK in COS-7 cells, transfected cells were pre-incubated for 1 h in Krebs–Ringer HEPES (KRH) containing 128 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 2.7 mM $CaCl_2$, 10 mM glucose and 20 mM HEPES, pH 7.4, with or without 200 nM wortmannin for the last 10 min, and then treated with 20 ng ml^{-1} PDGF or 1 μM ionomycin in KRH for the indicated time, frozen and lysed. For western blotting of HA–PKB and CaM-KK, 10 μg cell lysate was subjected to SDS–PAGE and probed with 1,000-fold-diluted 12CA5 antibody (Boehringer) or anti-CaM-KK antibody (Transduction Laboratories), respectively. All treatments with NMDA also contained 1 μM glycine.

PKB phosphorylation and activation. *In vitro* activation and phosphorylation of glutathione–Sepharose-purified GST–PKB or immunoprecipitated HA–PKB by recombinant CaM-KK was done for 20–30 min with 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 3 μM calmodulin, 400 μM ^{32}P-ATP (1,000 c.p.m. $pmol^{-1}$) and baculovirus-expressed CaM-KK. Reactions were terminated by 20-fold dilution[27]. PKB activity was then assayed for 30 min in 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM DTT, 1 μM protein kinase A inhibitor peptide, 400 μM ^{32}P-ATP (1,000 c.p.m. $pmol^{-1}$) and 0.2 mg ml^{-1} histone H2B; reaction products were separated by SDS–PAGE. Treatment of activated PKB with protein phosphatase 2A (ref. 28) was for 20 min. CaM-KK did not phosphorylate GST.

BAD phosphorylation. NG108 cells were transfected with GST–BAD (pEBG-MBad; New England Biolabs), serum-starved for 6 h and then stimulated with NMDA for the indicated times. Precipitated GST–BAD was subjected to western blotting with phospho-BAD (S136) antibody (Upstate Biotechnology), and values were normalized to the total amount of expressed BAD using general BAD antibody (Transduction Laboratories). Data expressed are from four to five independent experiments. For co-precipitation of 14-3-3 protein with BAD, non-transfected NG108 cells stimulated with 100 μM NMDA for the indicated times were lysed; endogenous BAD was then immunoprecipitated with 2 μg general BAD antibody (goat IgG; Santa Cruz). The immune complex was subjected to western blotting with anti-14-3-3 antibody (rabbit IgG; Upstate Biotechnology).

NG108 cell experiments and apoptosis. NG108-15 cells were cultured on 35-mm dishes[21]. For experiments with dominant-negative CaM-KK, cells were transfected with 1 μg HA–PKB plasmid and 5 μg plasmid containing dominant-negative CaM-KK or vector plasmid. To study apoptosis, we transfected NG108 cells on 35-mm dishes with 0.6 μg of GFP plasmid (pEGFP-C1;

Clontech) and either 1 μg pcDNA3 (Invitrogen) vector expressing constitutively active CaM-KK or 5 μg vector expressing dominant-negative CaM-KK or dominant-negative PKB (in all cases, equal amounts of vector alone were used for controls). The next day, media were changed to DMEM plus 10% serum, DMEM alone or DMEM containing 100 μM NMDA plus 1 μM glycine. After 24 h, cells were fixed with 4% paraformaldehyde for 15 min, stained for 15 min with 1 μg ml^{-1} Hoechst 33258 (CalBiochem) to visualize the nucleus, and stored in 50% glycerol in PBS at 4 °C. Apoptotic cells were visualized at 350 nm as small, bright, condensed nuclei, and GFP-positive cells were observed at 475 nm with a fluorescence microscope. Five fields from each dish were chosen randomly and captured in video image. The percentage of GFP-positive cells that were apoptotic was determined from three independent dishes per treatment condition. Typically 10–15% of cells were transfected, and 150–200 GFP-positive cells were scored for each treatment.

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Decoupling of nucleotide- and microtubule-binding sites in a kinesin mutant

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Molecular motors require ATP to move along microtubules or actin filaments. To understand how molecular motors function, it is crucial to know how binding of the motor to its filamentous track stimulates the hydrolysis of ATP by the motor, enabling it to move along the filament. A mechanism for the enhanced ATP hydrolysis has not been elucidated, but it is generally accepted that conformational changes in the motor proteins[1–3] occur when they bind to microtubules or actin filaments, facilitating the release of ADP. Here we report that a mutation in the motor domain of the microtubule motor proteins Kar3 and Ncd uncouples nucleotide- and microtubule-binding by the proteins, preventing activation of the motor ATPase by microtubules. Unlike the wild-type motors, the mutants bind tightly to both ADP and microtubules, indicating that interactions between the nucleotide- and microtubule-binding sites are blocked. The region of the motor that includes the mutated amino acid could transmit or undergo a conformational change required to convert the motor ATPase into a microtubule-stimulated state.

Kar3, a minus-end-directed kinesin motor of *Saccharomyces cerevisiae*, is essential in karyogamy (nuclear fusion) following mating and in meiosis, and has a non-essential role in mitosis[4–6]. Kar3 is thought to counteract two plus-end-directed kinesin motors, Cin8 and Kip1, in the mitotic spindle[6,7]. The mutants recovered in a screen for suppressors of a *cin8 kip1* double mutant

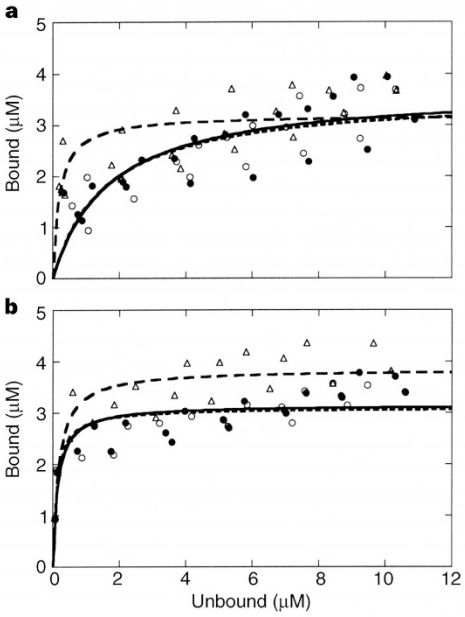

Figure 1 Microtubule binding by wild-type Kar3 and Kar3-898. Microtubule-binding assays were done without added nucleotide or in the presence of 1 mM Mg-ADP or 1 mM Mg-ATP. Data from three separate experiments for each nucleotide condition were combined and a hyperbolic curve was fitted to the data points. **a**, Kar3 and **b**, Kar3-898 protein. Open triangles and curves comprising long dashes, no added nucleotide; open circles and solid curves, Mg-ADP; filled circles and curves with short dashes, Mg-ATP.