SUPPLEMENTAL INFORMATION

In solution, PAK2 may exist as a dimer (1-3). Thus, it would be of interest to know whether the enzyme activity is affected by different oligomeric states of PAK2. To address this issue, we first characterized the oligomeric state of unphosphorylated PAK2 in solution by size-exclusion chromatography. Experiments were performed with two different protein concentrations. The concentration of PAK2 at the time of elution was approximately 200 nM (blue profile, supplemental Fig. S1) and 20 nM (red profile, supplemental Fig. S1). As shown in these figures, the unphosphorylated PAK2 (His-tagged full-length protein) eluted as a single peak at both concentrations, with a retention volume corresponding to an apparent molecular weight of 120 kDa, consistent with its calculated dimeric molecular mass of 125 kDa. These results also indicate that the PAK2 dimer does not dissociates into monomers even at low protein concentration.

To examine the effect of Cdc42 on dimerization of PAK2, the stability of PAK2 dimer in solution was assessed by gel filtration in the absence or presence of Cdc42. Cdc42 alone eluted with the expected elution volume (supplemental Fig. S2). In the absence and presence of Cdc42, PAK2 proteins eluted with a similar retention time (as a dimer) and no PAK2-Cdc42 complex was observed (supplemental Fig. S2). This is most likely due to the dynamic nature of complex formation and only micromolar binding affinity between Cdc42 and full-length PAK2. Gel filtration profiles showed that FL-PAK2 and Cdc42 co-migrated to earlier fractions than PAK2 dimer after incubation of PAK2 with both Cdc42 and ATP (supplemental Fig. S2), in line with a higher affinity expected for an activated kinase. The apparent molecular mass of the PAK2-Cdc42 complex appeared to be approximately 200 kDa, as assessed from the elution volume of the protein complex peak compared with the protein standards, indicating that complex formation with Cdc42 does apparently not dissociate the autophosphorylated PAK2 dimer under these conditions. The apparent molecular weight of the PAK2-Cdc42 complex is significantly larger than that of the tetramer complex, probably because PAK2 are in an extended conformation after the PAK2 autophosphorylation.

The previous studies have shown that the crystallized RD(70-149)-CD(249-545) complex forms tetramer in solution, and the dimerized PAK1 adopts a *trans*-inhibited conformation: the N-terminal inhibitory portion of one PAK1 molecule in the dimer binds and inhibits the catalytic domain of the other (1,3). In addition, the binding of the active form of Cdc42 did not dissociate

the RD(57-200)-CD(201-491) complex of PAK1, and the two fragments separate from each other only after treatment with ATP, which leads to autophosphorylation of the kinase (2). Considering the fact that the dimerized PAK1 adopts a *trans*-inhibited conformation (3), this result indicated that the interaction with Cdc42 does not cause dissociation of the inactive PAK1 dimer. Thus, as in the case of FL-PAK1, we have to assume a 2:2 stoichiometry with two independent binding sites for the affinity measurements between FL-PAK2 and Cdc42, since there is no indication for any other behavior from the Cdc42-binding curve (2)

With regard to phosphorylated and activated PAK2, although our gel filtration experiment showed that the activated PAK2-Cdc42 complex is a tetramer, autophosphorylation of FL-PAK2 may dissociates the PAK2 dimer at low enzyme concentration. To examine the possible effects of PAK2 concentration on the activated enzyme activity, we measured the initial velocity of activated PAK2-catalyzed reaction as a function of PAK concentration, using MLCtide as substrate (supplemental Fig. S3). The linear relationship between velocity and enzyme concentration suggests that under the conditions used in the present study, either no monomer-dimer conversion occurs in the range of enzyme concentrations used (3-30 nM), or dimer-dissociation could occur without changing the activity of activated FL-PAK2.

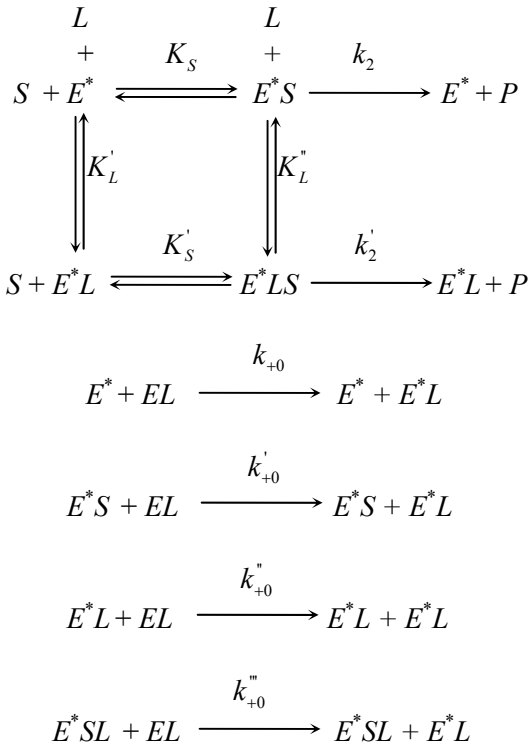
In summary, our data suggest that unphosphorylated PAK2 (substrate) forms tight dimer even in the presence of Cdc42, and phosphorylated PAK2 (enzyme) can be regarded as a kinetically normal enzyme whether the dimerized PAK2 proteins dissociates or not. Thus, each molecule in the PAK2 dimer can be identified as a catalytically independent enzyme. Therefore, in the presence of exogenous substrate and activator Cdc42, the general mechanism for PAK2 autoactivation can be written as

$$E + L \overset{K_L}{\rightleftharpoons} EL$$

$$EL \xrightarrow{k_e} E^*L \qquad \text{(for intra-initiation model)}$$

or

$$EL + EL \xrightarrow{k_e} E^*L + E^*L \qquad \text{(for inter-initiation model)}$$

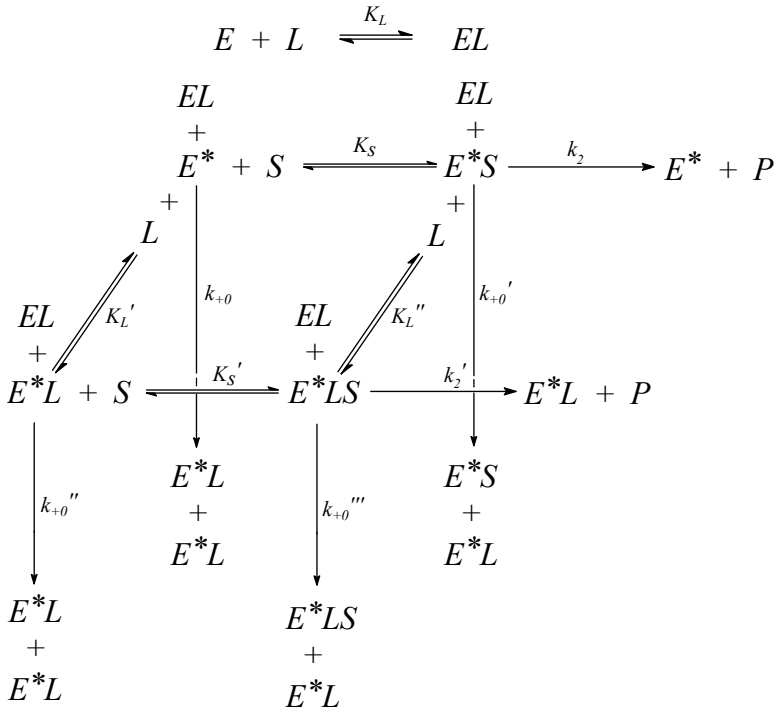


(Scheme 1)

where E and E^* represent unphosphorylated and phosphorylated enzyme, L represent activator, and S and P represent exogenous substrate and its corresponding product, respectively. In general, there are three possible mechanisms through which the unphosphoryalted kinase can be activated by *trans*-phosphorylation.

A. Kinetics of ligand-induced intermolecular autophosphorylation (catalyzed by a small amount of active enzyme)

In some cases, the unphosphorylated enzyme is completely inactive and its activation is catalyzed by a small quantity of contamination, the phosphorylated active enzyme. In the presence of exogenous substrate and effector, the general mechanism of the intermolecular autophosphorylation at low enzyme concentrations is given by:



(Scheme A)

where k_{+0} , $k_{+0}^{'}$, $k_{+0}^{''}$ and $k_{+0}^{'''}$ are the apparent second-order rate constants for autoactivation reactions, E and E^* represent unphosphorylated and phosphorylated enzyme, L represent activator, and S and P represent exogenous substrate and its corresponding product, respectively. The other substrates and products for the phosphorylation and dephosphorylation reactions such as ATP, H_2O, etc. are present at constant levels and can therefore be included in the kinetic constants without loss of generality. On the basis of the Scheme A, we have

$$[E_T^*]=[E^*]+[E^*S]+[E^*L]+[E^*LS] \quad \text{(A1)}$$

$$[E_T]=[E]+[EL] \quad \text{(A2)}$$

The total concentration of enzyme is

$$[T]_0=[E_T^*]+[E_T] \quad \text{(A3)}$$

If we assume that (1) the enzymatic reaction is irreversible and not inhibited by the product formed, (2) the substrate concentration remains constant throughout the activity measurement, and (3) there is equilibrium as far as EL, E^*S, E^*L and E^*LS are concerned; i.e., k_2 is sufficiently small as not disturb the equilibrium, and the formation of these complexes are fast reactions relative to the chemical reaction step, the following relations hold at any time:

$$[E] = \frac{K_L[E_T]}{K_L + [L]} \tag{A4}$$

$$[EL] = \frac{[L][E_T]}{K_L + [L]} \tag{A5}$$

and

$$[E^*] = \frac{K_s' K_L' [E_T^*]}{K_s' K_L' + K_s'[L] + K_L''[S] + [S][L]} \tag{A6}$$

$$[E^* L] = \frac{K_s'[L][E_T^*]}{K_s' K_L' + K_s'[L] + K_L''[S] + [S][L]} \tag{A7}$$

$$[E^* S] = \frac{K_L''[S][E_T^*]}{K_s' K_L' + K_s'[L] + K_L''[S] + [S][L]} \tag{A8}$$

$$[E^* LS] = \frac{[S][L][E_T^*]}{K_s' K_L' + K_s'[L] + K_L''[S] + [S][L]} \tag{A9}$$

A special case of interest arises when $K_s = K_s'$, $k_2 = k_2'$, $k_{+0} = k_{+0}''$, and $k_{+0}' = k_{+0}'''$, which gives rise to that $K_L' = K_L''$; this means that the binding of L to E^* does not affect either the ease of binding of substrates or the first order rates for the chemical conversion of the enzyme-substrate complexes to products. Equations (A6) to (A9) then factorize:

$$[E^*] = \frac{K_s K_L' [E_T^*]}{\left(K_s + [S]\right)\left(K_L' + [L]\right)} \tag{A10}$$

$$[E^* L] = \frac{K_s [L][E_T^*]}{\left(K_s + [S]\right)\left(K_L' + [L]\right)} \tag{A11}$$

$$[E^* S] = \frac{K_L'[S][E_T^*]}{\left(K_s + [S]\right)\left(K_L' + [L]\right)} \tag{A12}$$

$$[E^* LS] = \frac{[L][S][E_T^*]}{\left(K_s + [S]\right)\left(K_L' + [L]\right)} \tag{A13}$$

The dissociation constant K_s for [S] is then independent of [L], and K_L' is independent of [S].

The rate of the phosphorylated enzyme formation is given by

$$\frac{d[E_T^*]}{dt} = k_{+0}[EL]\left([E^*] + [E^* L]\right) + k_{+0}'[EL]\left([E^* S] + [E^* LS]\right)$$

$$= \frac{[L]\left([T]_0 - [E_T^*]\right)}{K_L + [L]} \left(\frac{k_{+0}K_s + k'_{+0}[S]}{K_s + [S]} \right) [E_T^*]$$

$$= \theta A [E_T^*] \left([T]_0 - [E_T^*] \right) \qquad \text{(A14)}$$

where

$$A = \frac{k_{+0}K_s + k'_{+0}[S]}{K_s + [S]}$$

$$\theta = \frac{[L]}{K_L + [L]}$$

With the boundary conditions, $t = 0$, $[E_T^*] = [E^*]_0$, equation (A14) can be integrated to give

$$[E_T^*] = \frac{[E^*]_0 [T]_0}{[E^*]_0 + \left([T]_0 - [E^*]_0 \right) e^{-\theta A [T]_0 t}} \qquad \text{(A15)}$$

The rate of product formation while the enzyme is being modified is

$$\frac{d[P]}{dt} = k_2 \left([E^* S] + [E^* LS] \right)$$

$$= \frac{k_2 [S][E_T^*]}{K_s + [S]}$$

$$= \frac{k_2 [S]}{K_s + [S]} \left\{ \frac{[E^*]_0 [T]_0}{[E^*]_0 + \left([T]_0 - [E^*]_0 \right) e^{-\theta A [T]_0 t}} \right\} \qquad \text{(A16)}$$

The concentration of product formed at time t is given by

$$[P] = v_s t + \frac{v_s}{\theta A [T]_0} \ln \left\{ \frac{[E^*]_0 + \left([T]_0 - [E^*]_0 \right) e^{-\theta A [T]_0 t}}{[T]_0} \right\} \qquad \text{(A17)}$$

or given by

$$[P] = v_s t + \frac{v_s}{k_{obs}} \ln \left\{ \alpha + (1 - \alpha) e^{-k_{obs} t} \right\} \qquad \text{(A18)}$$

where

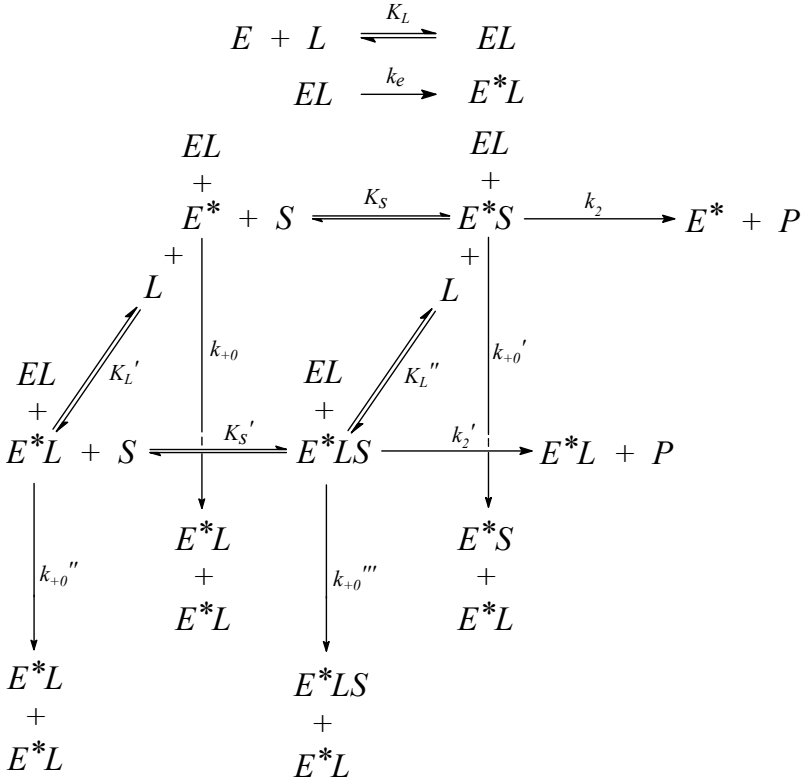
$$k_{obs} = \theta A [T]_0$$

$$\alpha = \frac{[E^*]_0}{[T]_0}$$

$$v_s = \frac{k_2[T]_0[S]}{K_s + [S]}$$

B. Kinetics of ligand-induced intermolecular autophosphorylation (with an intramolecular initiation)

In some cases, the unphosphorylated enzyme has intrinsic activity to catalyze the autophosphorylation by itself. The initial amount of phosphorylated enzyme can be generated through an intramolecular reaction, and then the phosphorylated enzyme can catalyze the phosphorylation of other inactive enzyme through an intermolecular way. In the presence of exogenous substrate and effector, the general mechanism of the intermolecular autophosphorylation at low enzyme concentrations is given by:



(Scheme B)

With the same assumptions as described in Scheme A, the rate of the phosphorylated enzyme formation is given by:

$$\frac{d[E_T^*]}{dt} = k_{+0}[EL]\left([E^*] + [E^*L]\right) + k_{+0}^{'}[EL]\left([E^*S] + [E^*LS]\right) + k_e[EL]$$

$$= \frac{[L]\left([T]_0 - [E_T^*]\right)}{K_L + [L]} \left\{ \frac{k_{+0}K_s + k_{+0}^{'}[S]}{K_s + [S]} [E_T^*] + k_e \right\}$$

$$= \theta\left([T]_0 - [E_T^*]\right)\left(A[E_T^*] + k_e\right) \qquad \text{(B1)}$$

where

$$A = \frac{k_{+0}K_s + k_{+0}^{'}[S]}{K_s + [S]}$$

$$\theta = \frac{[L]}{K_L + [L]}$$

With the boundary conditions, $t = 0$, $[E_T^*] = 0$, equation (B1) can be integrated to give

$$[E_T^*] = [T]_0 - \frac{\left(A[T]_0 + k_e\right)[T]_0}{A[T]_0 + k_e e^{\theta(A[T]_0 + k_e)t}} \qquad \text{(B2)}$$

The rate of product formation while the enzyme is being modified is

$$\frac{d[P]}{dt} = k_2\left([E^*S] + [E^*LS]\right)$$

$$= \frac{k_2[S][E_T^*]}{K_s + [S]}$$

$$= \frac{k_2[S]}{K_s + [S]} \left\{ [T]_0 - \frac{\left(A[T]_0 + k_e\right)[T]_0}{A[T]_0 + k_e e^{\theta(A[T]_0 + k_e)t}} \right\} \qquad \text{(B3)}$$

The concentration of product formed at time t is given by

$$[P] = v_s t + \frac{v_s}{\theta A[T]_0} \ln\left\{ \frac{A[T]_0 e^{-\theta(k_e + A[T]_0)t} + k_e}{A[T]_0 + k_e} \right\} \qquad \text{(B4)}$$

or given by

$$[P] = v_s t + \frac{v_s}{k_{obs} - \beta} \ln\left\{ \frac{\left(k_{obs} - \beta\right)e^{-k_{obs}t} + \beta}{k_{obs}} \right\} \qquad \text{(B5)}$$

where

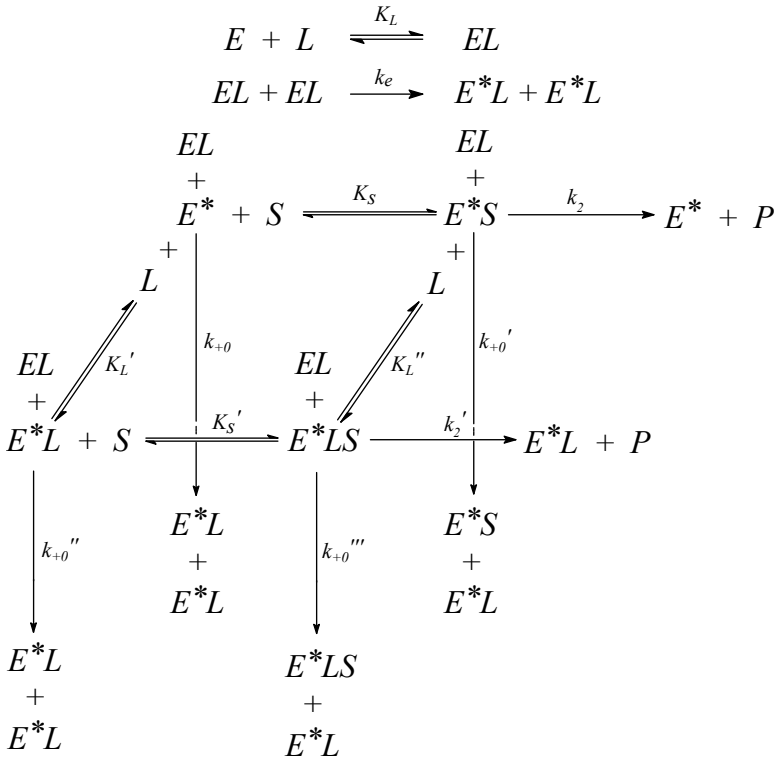
$$k_{obs} = \theta\left(A[T]_0 + k_e\right)$$

$$\beta = \theta k_e$$

$$v_s = \frac{k_2[T]_0[S]}{K_s + [S]}$$

C. Kinetics of ligand-induced intermolecular autophosphorylation (with intermolecular initiation)

In the presence of exogenous substrate and effector, the general mechanism of the intermolecular autophosphorylation at low enzyme concentrations is given by:



(Scheme C)

With the same assumptions described above, the rate of the phosphorylated enzyme formation is given by:

$$\frac{d[E_T^*]}{dt} = k_{+0}[EL]\left([E^*]+[E^*L]\right) + k'_{+0}[EL]\left([E^*S]+[E^*LS]\right) + k_e[EL][EL]$$

$$= \frac{[L]\left([T]_0 - [E_T^*]\right)}{K_L + [L]}\left\{\frac{k_{+0}K_s + k'_{+0}[S]}{K_s + [S]}[E_T^*] + k_e\frac{[L]([T]_0 - [E_T^*])}{K_L + [L]}\right\}$$

$$= \theta([T]_0 - [E_T^*])\{A[E_T^*] + k_e\theta([T]_0 - [E_T^*])\} \qquad \text{(C1)}$$

where

$$A = \frac{k_{+0}K_s + k'_{+0}[S]}{K_s + [S]}$$

$$\theta = \frac{[L]}{K_L + [L]}$$

With the boundary conditions, $t = 0$, $[E_T^*] = 0$, equation (C1) can be integrated to give

$$[E_T^*] = [T]_0 - \frac{A[T]_0}{(A - \theta k_e) + \theta k_e e^{A\theta[T]_0 t}} \qquad \text{(C2)}$$

The rate of product formation while the enzyme is being modified is

$$\frac{d[P]}{dt} = k_2([E^*S] + [E^*LS])$$

$$= \frac{k_2[S][E_T^*]}{K_s + [S]}$$

$$= \frac{k_2[S]}{K_s + [S]}\left\{[T]_0 - \frac{A[T]_0}{(A - \theta k_e) + \theta k_e e^{A\theta[T]_0 t}}\right\} \qquad \text{(C3)}$$

The concentration of product formed at time t is given by

$$[P] = v_s t + \frac{v_s}{\theta A[T]_0 - \theta^2 k_e[T]_0} \ln\left\{\frac{(A - \theta k_e)e^{-\theta A[T]_0 t} + \theta k_e}{A}\right\}$$

or given by

$$[P] = v_s t + \frac{v_s}{k_{obs} - \beta} \ln\left\{\frac{(k_{obs} - \beta)e^{-k_{obs}t} + \beta}{k_{obs}}\right\} \qquad \text{(C4)}$$

where

$$k_{obs} = \theta A[T]_0$$

$$\beta = \theta^2 k_e[T]_0$$

$$v_s = \frac{k_2[T]_0[S]}{K_s + [S]}$$

SUPPLEMENTAL FIGURES

Supplemental Figure S1

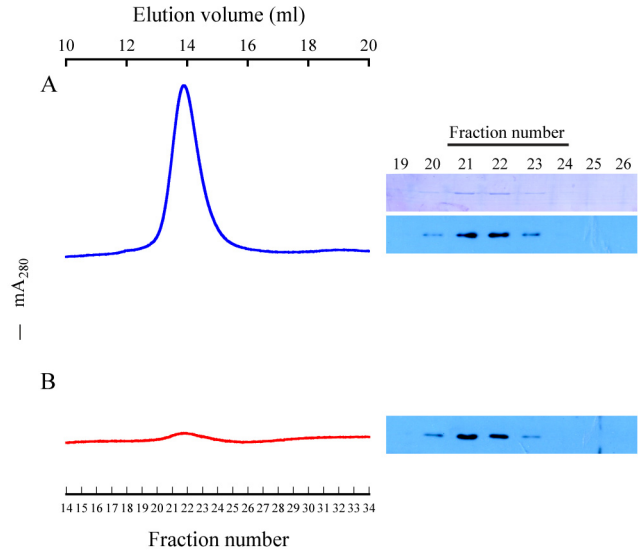


Fig. S1. Dimerization of PAK2 in solution

The His_6-tagged full-length PAK2 used here was expressed and purified as described before, and the purified PAK2 is catalytically active (4). To generate the unphosphorylated protein, the purified PAK2 was treated with PP1α at 25°C for 60 min, at which time the dephosphorylation was nearly 100% complete. The unphosphorylated PAK2 was separated from the untagged phosphatase and then subjected to the gel filtration analysis. The size exclusion chromatography was performed, using a Superdex 200 10/300 column on an ÄKTA FPLC (GE Healthcare), to determine the apparent molecular weight of this unphosphorylated PAK2 at two concentrations. Bar = 0.4 mAU (280 nm). The column was equilibrated with the buffer containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2 mM dithiothreitol (DTT) at a flow rate of 0.5 ml/min and calibrated with molecular mass standards. Each sample, at a volume of 500 µl, was loaded to the column and fractions of 0.5 ml each were collected. The concentrations of His_6-tagged PAK2 at the time of elution were approximately 200 nM (A) and 20 nM (B), respectively. In panel A, aliquots of relevant fractions were subjected to SDS-PAGE and the proteins were visualized by Coomassie Blue staining (upper) and by western blotting (anti His_6-tag, lower). The protein amounts visualized by western blotting were 1/10 that by Coomassie Blue staining. In panel B fractions were only detected by western blotting owing to the low protein concentration (20 nM).

Supplemental Figure S2

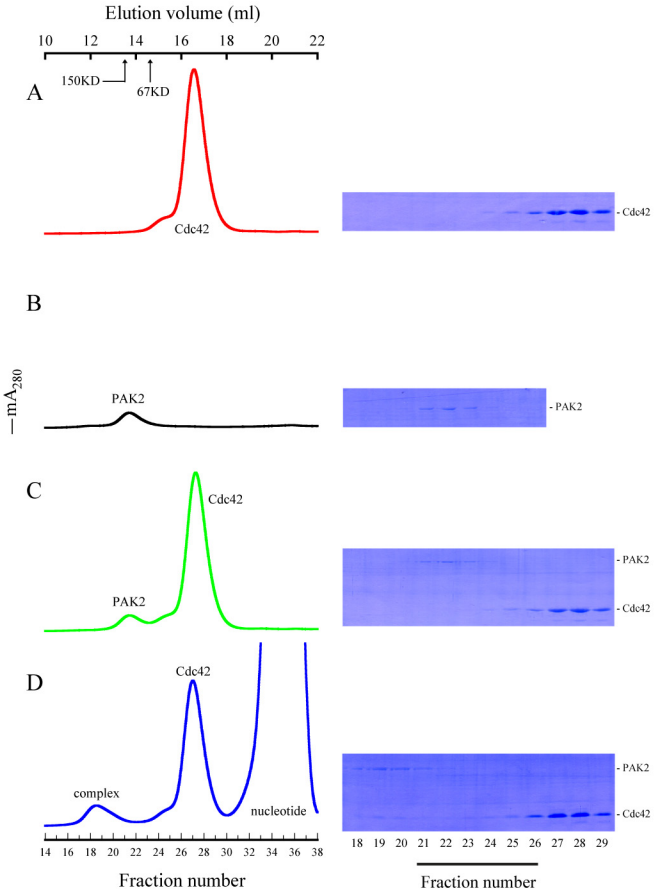


Fig. S2. Interaction between unphosphorylated/phosphorylated PAK2 and Cdc42

Size exclusion chromatography was carried out to assess the interaction between His_6-tagged PAK2 and His_6-tagged Cdc42(Q61L). Bar = 5 mAU (280nm). The proteins used were 20 µM His_6-tagged Cdc42(Q61L) (A), 600 nM His6-tagged full-length PAK2 (B), and the mixture of 20 µM Cdc42(Q61L) and 600 nM PAK2 in the absence (C) or presence (D) of 5 mM ATP. All protein samples were incubated for at least 30 min to allow equilibrium (and autophosphorylation) to be reached. Fractions were collected at 0.5 ml each, and aliquots of relevant fractions were subjected to SDS-PAGE followed by Coomassie Blue staining.

Supplemental Figure S3

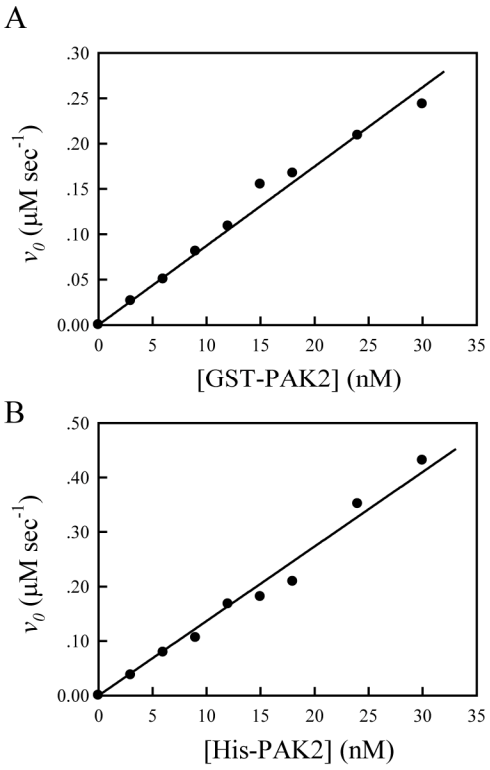


Fig. S3. Reaction of MLCtide phosphorylation by PAK2

Both GST-tagged PAK2 (A) and His-tagged PAK2 (B) were activated by Cdc42 *in vitro* as described in the main text. Phosphorylation of the substrate MLCtide was monitored through the enzyme-coupled assay described in the experimental procedures. There was a linear correlation between the initial velocity of the reaction and the enzyme concentration for both GST- and His-tagged PAK2.

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