In vivo activation of recombinant cAPK catalytic subunit active site mutants by coexpression of the wild-type enzyme, evidence for intermolecular cotranslational phosphorylation

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Abstract The catalytic subunit of cAMP dependent protein kinase (cAPK) carries two stable autophosphorylated residues. One of them, Thr[197], resides in the so-called protein kinase activation segment, and needs to be phosphorylated for full activity and protein kinase inhibitor binding of the enzyme. While wild-type recombinant mammalian C-subunit, expressed in *E. coli*, can fully autoactivate itself by phosphorylation at Thr[197], many active site mutants lack this autophosphorylation activity, so that the primary effects of the mutations become obscured. Two active site mutants of bovine C-subunit, defective in protein kinase inhibitor peptide binding, were activated by wild-type enzyme in vivo, but could not be activated in vitro, demonstrating intermolecular and presumably cotranslational autophosphorylation. The results may delineate strategies for the expression and mutagenesis of other protein kinases with requirements for activation segment phosphorylation.

Key words: Protein kinase A; Site directed mutagenesis; Active site; Autophosphorylation; Enzyme activation; Protein kinase inhibitor binding

1. Introduction

Many members of the protein kinase family need to be phosphorylated for full activity at a tyrosine or threonine residue in the so-called activation segment between the conserved sequence motifs DFG and APE in Hanks subdomain VIII [1,2]. Expression of these kinases in *E. coli* consequently and inevitably leads to enzymes with impaired activity, because most kinases are not able to autoactivate themselves, and *E. coli* does not possess the required protein kinases. This is an enormous obstacle to studies on enzyme kinetics, site-directed mutagenesis, and in investigations of protein crystallization, where large amounts of pure protein are required, normally a major advantage of the *E. coli* system. Indeed, with one exception all protein kinases for which the crystal structure has been solved so far have been crystallized from recombinant sources and most of them in an inactive state because these enzymes lack the phosphorylation of their activation segment (for review see [3]). Examples are CDK2, which requires a phosphorylation at Thr[160] [4], IRK, which requires autophosphorylation in *trans* at Tyr[162] [5], ERK2, which needs to be phosphorylated at Thr[183] and Thr[185] [6] or CaMKI at Thr[177] [7]. The active state is known only for those kinases that do not require phosphorylation for activity, like CK1 [8] or PhK γ-subunit [9]. However, there is one exception to date.

The catalytic subunit of cAPK, which requires phosphorylation of Thr[197] for full activity [10–12], has been crystallized in its fully active phospho-form [13,14]. This was possible for two reasons: First, sufficient amounts of pure enzyme in the phosphorylated active state can be obtained from mammalian sources to allow growth of highly ordered single crystals [14] and second, in *E. coli* the enzyme is able to autophosphorylate itself at residue Thr[197] [15,16], though it is still unclear whether this is an intra- or intermolecular event. The situation, however, worsens seriously if the activity of the C-subunit is impaired by mutations of active site residues. Apparently, active site mutants lose their ability to autoactivate, making it impossible to judge the specific effect of the mutation alone. So far, no mutants of active site residues of mammalian cAPK catalytic subunit have been described.

We report here two active site mutants of bovine catalytic subunit which initially were unable to bind to protein kinase inhibitor peptide (PKI(5–24)), a property of correctly folded C-subunit [11].

Coexpression together with the wild-type enzyme in *E. coli*, but not incubation with MgATP or wild-type enzyme in vitro, caused mutant activity, indicating intermolecular and presumably cotranslational phosphorylation. The results delineate possible strategies for the activation of other members of the kinase family, which have not yet been expressed in *E. coli* in fully active form due to lack of phosphorylation at their activation segment.

2. Materials and methods

2.1. Mutagenesis and enzyme expression

Mutagenesis was performed according to Kunkel [24] in *E. coli* strains CJ236 *dut*$^-$, *ung*$^-$ F′ (BioRad), XL1Blue *rec*A1 *lac*$^-$; F′[lacI^q *lac*ZΔM15] (Stratagene) using the phagemid pBluescript KS(+) (Stratagene). A bovine Cα full length 1.1 kbp 5′ *Nde*I/3′ *Bam*HI fragment [17] was mutated by using the oligonucleotide primers 5′-AAC CAC TAC **GTC** ATG AAG AT (GCC→GTC) (Ala[70]Val) and 5′-CG TTC CTT **GAC** AAA CTC G (GTC→GAC) (Val[104]Asp). For enzyme expression strain BL21(DE3)LysS F, *omp*T *hsd*S$_B$($r_B^- m_B^-$) (DE3) pLysS(Cm^R) (Novagen) and plasmids pT7-7 (Ap^R) (Stanley Tabor, Harvard Medical School, Boston, or pET-28b(+) (Kan^R) (Novagen, Madison) were used. In the pET-28b(+) vector the Cα gene was fused in frame to a sequence coding for Met-Gly-Ser-Ser-His(6)-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His--Met (His-tag). The following C-subunit forms were used in this study: wild-type: rCαWT, rCαWT-His; mutants: rCα(Ala[70]Val), rCα(Ala[70]Val)-His, rCα(Val[104]Asp), rCα(Val[104]Asp)-His. Bacteria were grown in L-broth containing chloramphenicol (30 μg/ml) and carbenicillin (50 μg/ml), and/or kanamycin (5O μg/ml) to an OD_{660} of 0.6 and induced for protein expression at 24°C for 16 h in the presence of 0.4 mM IPTG to an OD_{660} of 2–3.

2.2. PKI(5–24) affinity chromatography

Kinase expressing cells were collected and resuspended to an OD_{660}

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of 40 in 50 mM KCl, 1 mM EDTA, 1 mM DTT, 30 mM MES, pH 6.45. Cells were broken, and centrifuged at 80 000 × *g* for 45 min. To the supernatant 2 mM ATP, 3 mM $MgCl_2$ and 0.1% CHAPS were added for affinity chromatography on protein kinase inhibitor peptide PKI(5–24), coupled to Affigel 10 (BioRad) (modified from [18]). About 10 ml supernatant were incubated with 1 ml affinity matrix for 1 h. After washes in TMN (50 mM and 250 mM NaCl, 400 μM ATP, 2 mM $MgCl_2$, 0.1% Chaps, 20 mM Tris-Cl, pH 7.4), catalytic subunit was eluted with 200 mM arginine, 50 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 20 mM Tris-Cl, pH 7.4.

2.3. Nickel chelate affinity chromatography

His-tagged protein was separated from unmodified protein in the PKI(5–24) eluates using a Ni^{2+}-loaded His·Bind resin column (Novagen) after exchanging the buffer against binding buffer according to the protocol of the supplier and eluted with buffer containing 1 M imidazole. For the NI^{2+} affinity purification of His-tagged Cα-mutant protein directly from cell extracts, cells were resuspended to OD_{660} 40 in binding buffer, broken, and centrifuged as before. 10 ml of the supernatant were added to 1 ml Ni^{2+} chelate matrix. After washing with binding buffer and wash buffer with 30 mM imidazole the fusion proteins were eluted with 100 mM imidazole, 500 mM NaCl, 20 mM Tris-Cl, pH 7.9.

2.4. In vitro approach for activation of mutant enzyme

The buffer of eluates from the Ni^{2+} affinity column was exchanged for 150 mM LiCl, 1 mM DTT, 0.1 mM EDTA, 100 mM MOPS-NaOH, pH 6.8. After adding 2 mM ATP and 3 mM $MgCl_2$ the protein solution, containing approx. 50 μg/ml C-subunit, was incubated at 30°C for 20 min to 3 h. Alternatively, catalytic amounts (0.5 μg/ml) of native bovine catalytic subunit were added to this mixture. After incubation the solution was diluted 2-fold in H_20 (2 mM ATP, 3 mM $MgCl_2$) and subjected to PKI(5–24) affinity chromatography as described.

2.5. Protein kinase assays

Phosphorylation reactions were carried out for 3 min at 30°C in a mixture (100 μl) containing 50 mM MOPS (morpholinopropanesulfonic acid), pH 6.8, 10 mM MgAc, 20 ng enzyme, 0.5–2 μCi [γ-^{32}P]ATP, Kemptide substrate (Sigma) and ATP as indicated. The reactions were initiated by the addition of ATP and terminated by pipetting of 80 μl reaction mixture on Whatman P81 phosphocellulose paper (2 × 2 cm) (modified from [19]) Afterwards the paper squares were washed four times with 30% acetic acid. The amount of radioactive phospho-Kemptide was determined by liquid scintillation counting of the papers in 4 ml scintillation fluid (Zinsser Aquasafe 500).

3. Results

3.1. Site-directed mutagenesis and expression of cAPK C-subunit

Two mutants of cAPK bovine Cα catalytic subunit with exchanges of active site residues were generated. Ala^{70} was replaced with valine, and Val^{104} with aspartate. Both residues belong to the ATP binding pocket and interact with the purine of ATP [14,13,20]. The alanine, and the hydrophobicity of Val^{104} are almost invariant, being conserved in the protein kinase family [1]. The coding regions of the mutant and wild-type DNAs were sequenced and the proteins expressed in *E. coli*, predominantly in a soluble form. Fig. 1 shows an immunoblot using polyclonal antibody against Cα, and a corresponding SDS gel of purified enzymes and cellular extracts, (lanes 3,4 for the control without expression vector). The wild-type recombinant Cα proteins (lane 1,2) were purified to homogeneity in one step on a column with immobilized protein kinase inhibitor peptide PKI(5–24), and shown to be equally active with Kemptide as a substrate (Table 1). The His-tagged mutant proteins migrated somewhat faster than rCαWT-His (lanes 6,8,10,11, see also Fig. 2, lanes 14b,15). Comparable behavior has been observed previously and seen

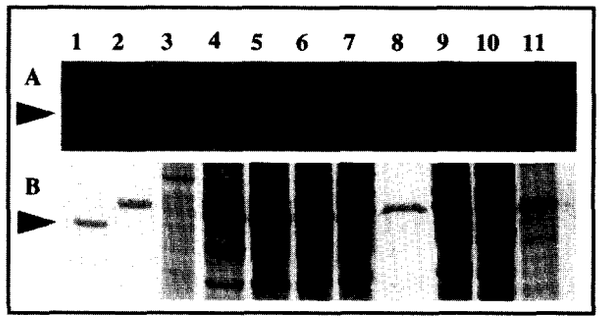

Fig. 1. Expression of Cα mutant proteins in *E. coli*. (A) Western blot with rabbit antibodies raised against bovine Cα. (B) Parallel Coomassie stained SDS-polyacrylamide gel. Lanes: 1, rCαWT; indicated by arrow; 2, rCαWT-His; 3, BL21(DE3)pLysS without vector, high speed pellet of cell extract; 4, BL21(DE3)pLysS without vector, supernatant of cell extract; 5, rCα(Ala^{70}Val)-His and rCaWT coexpression, supernatant of cell extract; 6, rCα(Ala^{70}Val)-His single expression, supernatant of cell extract; 7, rCα(Ala^{70}Val)-His and rCα(Ala^{70}Val) coexpression, supernatant of cell extract; 8, rCα(Ala^{70}Val)-His single expression, Ni^{2+} chelate affinity chromatography; 9, rCα(Val^{104}Asp)-His and rCαWT coexpression, supernatant of cell extract; 10, rCα(Val^{104}Asp)-His single expression, supernatant of cell extract; 11, rCα(Val^{104}Asp)-His single expression, Ni^{2+} chelate affinity chromatography.

as an indication of Thr^{197} being unphosphorylated [11,21]. Steinberg et al. [11] observed, after short-term expression of the mouse Cα protein in *E. coli*, a fast form of the enzyme which did not contain threonine phosphate and did not bind to a PKI(5–22) affinity column. The active site mutants rCα(Ala^{70}Val)-His and rCα(Val^{104}Asp)-His, when singly expressed, also did not bind to the PKI(5–24) affinity column (Fig. 3, lanes 7,12). This could be a primary effect of the mutations introduced or a secondary effect due to inability of the mutant enzymes to autophosphorylate at Thr^{197}, thereby obscuring the primary effect of the mutation. Singly expressed mutants were enriched on a Ni^{2+} chelate affinity column (Fig. 1, lanes 8,9, see also Fig. 3, lanes 6,11).

3.2. Attempts to restore kinase activity

We suspected that the absence of Thr^{197} phosphorylation was responsible for the deficiency in peptide binding of the mutant enzymes, therefore we tried to activate the mutant enzymes in vitro. The mutant rCα(Ala^{70}Val)-His, enriched on a Ni^{2+} affinity column (to minimize secondary effects of *E. coli* proteins, Fig. 1, lane 8, Fig. 3, lane 6), was incubated in the presence of 2 mM MgATP (Fig. 2, lanes 1,4). As this did not induce PKI(5–24) binding (lanes 2,3,5,6) an attempt was made to phosphorylate the mutant by catalytic amounts (0.5 μg/ml) of native bovine heart catalytic subunit in the presence of 2 mM MgATP. Even after 3 h incubation the mutant enzyme did not gain significant PKI(5–24) binding (lanes 8–13). The viability of the wild-type enzyme in the activation assay was verified in a subsequent kinase assay with Kemptide as a substrate (not shown). In contrast to the results with mouse wild-type enzyme lacking a Thr^{197} phosphate [11], the rCα-(Ala^{70}Val)-His mutant protein did not convert to a slow migrating form under these conditions. This could indicate that the mutant has not been phosphorylated intermolecularly at Thr^{197} in vitro, or that it did not change its migration behavior in the SDS gel or acquire the ability to bind to the PKI(5–24)-column. Lanes 15–18 of Fig. 2 show the negative

Table 1
K_m values of wild-type and mutant proteins for MgATP and Kemptide

	K_m MgATP (μM)	K_m Kemptide (μM)
rCαWT	9.1 ± 0.9	5.4 ± 0.3
rCαWT-His	9.6 ± 0.5	5.3 ± 0.4
rCα(Ala^{70}Val)-His	167 ± 14	162 ± 27
rCα(Val^{104}Asp)-His	202 ± 33	220 ± 39

The phosphorylation reaction for determination of K_m MgATP contained 50 μM Kemptide, when wild-type enzyme was measured, and 250 μM Kemptide, when the mutants were measured. For measurement of K_m Kemptide the phosphorylation reaction contained 100 μM ATP, and 200 μM ATP, when the mutants were measured.

control at time points t_0 and t_{3h} in the absence of both MgATP and native Cα; lanes 20–22 show the positive control where purified protein from a coexpression experiment of rCα(Ala^{70}Val)-His and rCαWT (see the next paragraph) has been added to the Ni^{2+} chelate affinity eluate of singly expressed rCα(Ala^{70}Val)-His, demonstrating that mutant and wild-type protein can readily be purified from these mixtures on the PKI(5–24) affinity column (lane 22).

3.3. *In vivo activation*

Because it could not be excluded that Thr^{197} phosphorylation of mutant enzymes requires the conditions of the living cell, the rCαWT protein was coexpressed together with each mutant. This experiment could also provide an answer to the open question of whether Thr^{197} autophosphorylation occurs intra- or intermolecularly. If exclusively intramolecular, however, this would impair the possibilities of generating C-subunit active site mutants in *E. coli* in general. Fig. 3 shows that both mutants rCα(Ala^{70}Val)-His and rCα(Val^{104}Asp)-His could readily be purified on the PKI(5–24)-affinity column when coexpressed together with rCαWT (lanes 4,9). Furthermore, in contrast to the singly expressed enzymes (lanes 6,11), a mobility shift towards a slower migrating form was observed (lanes 5,6,10,11). Both phenomena are known to indicate phosphorylation at activation segment residue Thr^{197} [11]. The same result was achieved when the non-fusion mutant proteins (rCα(Ala^{70}Val) and rCα(Val^{104}Asp)) were coexpressed together with a wild-type His-tag fusion (not shown), indicating that the PKI(5–24) binding activation was independent from the His-tag. The rCαWT-His fusion, however, caused some proteolytic degradation at the N-terminus, indicated by small amounts of protein at ≈40 kDa (see Fig. 1, lane 2) which could not be removed by Ni^{2+} chelate affinity chromatography. As this would lead to unwanted contamination of mutant protein with traces of wild-type activity, for further analysis only the His-tagged mutant proteins were used. The possibility that mutant activation could be simply caused by using a double vector system was excluded by coexpressing the fusion and non-fusion forms rCα(Ala^{70}Val)-His and rCα(Ala^{70}Val) (Fig. 1, lane 7). None of the two proteins gained PKI(5–24) binding ability (Fig. 3, lane 8). Intermolecular phosphorylation of the mutant enzymes by the wild-type is the only plausible explanation for activation of PKI-peptide binding. The activated mutant proteins from the coexpression experiments with rCαWT were purified to homogeneity from the mixture with the wild-type enzyme on a Ni^{2+} affinity column (Fig. 3, lanes 5,10).

3.4. *Kinetic analysis of the activated mutants*

For both purified mutants from the coexpression experiments the kinetic constants were measured with MgATP and Kemptide as a substrate. Both mutants are defective in K_m for MgATP, and K_m for substrate peptide (see Table 1).

4. Discussion and conclusions

Several protein kinases are expressed in *E. coli* in an inactive or partly active form due to the absence of activation segment phosphorylation. Although the mammalian Cα-subunit of cAPK from mouse or bovine is well able to autophosphorylate and thereby activate itself during expression in *E. coli*. studies on enzyme mechanism by site-directed mutagenesis, however, are seriously hindered because the ability to autophosphorylate appears to be lost in active site mutants,

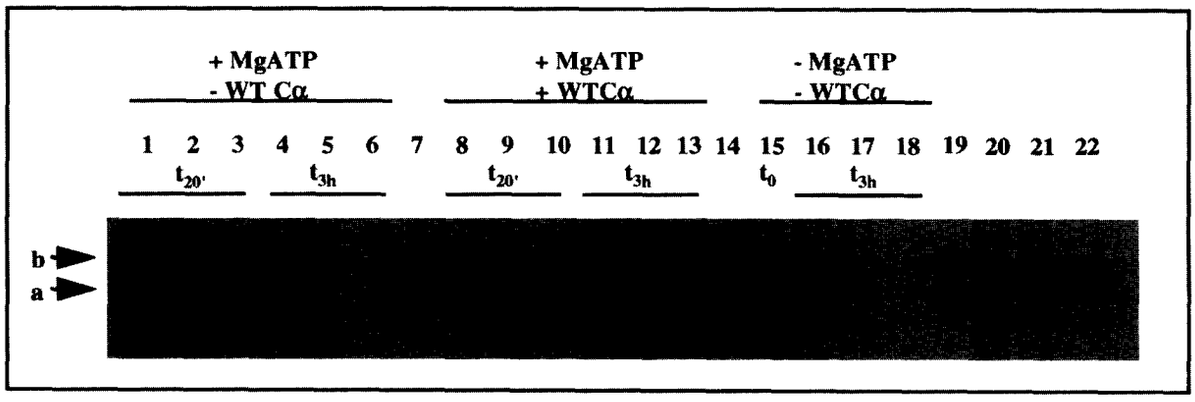

Fig. 2. In vitro activation approaches of active site mutant rCα(Ala^{70}Val)-His. Coomassie blue stained SDS polyacrylamide gel. The presence or absence of MgATP or native enzyme and the incubation time are indicated above the lanes; t_0, t_{20min}, t_{3h}, indicates that the Ni^{2+} chelate affinity enriched mutant was incubated at 30°C for O min, 20 min, and 3 h, respectively. Lanes: 7,14,19, rCαWT (a) and rCαWT-His (b), mixture of PKI(5–24) affinity purified protein; 1,4,8,11,15,16, rCα(Ala^{70}Val)-His; Ni^{2+} chelate affinity enriched; 2,5,9,12,17, material which remained unbound to the PKI(5–24) affinity columns; 3,6,10,13,18, elution from this PKI(5–24) affity columns; 20–22, control: 20, rCα(Ala^{70}Val)-His as above, to which about 100 μg/ml (each) rCα(Ala^{70}Val)-His and rCαWT, PKI(5–24) affinity purified protein from a coexpression were added; 21, material which remained unbound to the PKI(5–24) affinity column; 22, proteins eluted from this PKI(5–24) affinity column. Equivalent relative proportions of samples were loaded onto the SDS gel. Faint bands visible in the PKI(5–24) eluates (lane 3,6,10,13,18) presumably result from residual peptide binding ability of the single expressed mutant protein.

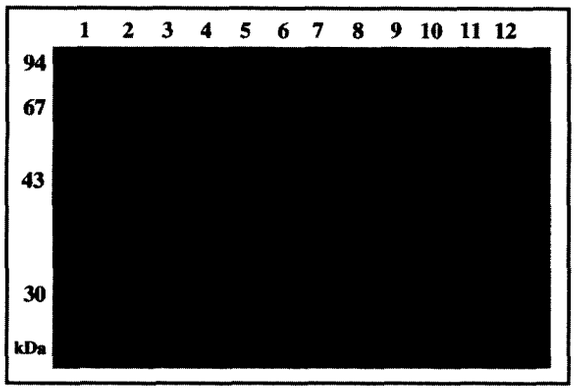

Fig. 3. Affinity chromatography of Cα active site mutants. Lanes: 1, molecular mass marker; 2, rCαWT, single expression, PKI(5–24) affinity chromatography; 3, rCαWT-His, single expression, PKI(5–24) affinity chromatography; 4, rCα(Ala^{70}Val)-His and rCαWT, coexpression, PKI(5–24) affinity chromatography; 5, rCα(Ala^{70}Val)-His, Ni^{2+} chelate affinity chromatography from rCα(Ala^{70}Val)-His and rCαWT coexpression (see lane 4); 6, rCα(Ala^{70}Val)-His, single expression, Ni^{2+} chelate affinity chromatography; 7, rCα(Ala^{70}Val)-His single expression, PKI(5–24) affinity chromatography; 8, rCα(Ala^{70}Val)-His and rCα(Ala^{70}Val), coexpression, PKI(5–24) affinity chromatography; 9, rCα(Val^{104}Asp)-His and rCαWT, coexpression, PKI(5–24) affinity chromatography; 10, rCα(Val^{104}Asp)-His, Ni^{2+} chelate affinity chromatography from rCα(Val^{104}Asp)-His and rCαWT coexpression (see lane 9); 11, rCα(Val^{104}Asp)-His, single expression, Ni^{2+} chelate affinity chromatography; 12, rCα(Val^{104}Asp)-His, single expression, PKI(5–24) affinity chromatography.

and the primary effects of the mutations introduced becomes undetectable simply due to this effect. In our studies on active site mutants of the catalytic subunit of cAPK, coexpression of the wild-type enzyme together with rCα(Ala^{70}Val) or rCα(Val^{104}Asp), or several other active site mutants which were not shown here, induced PKI peptide binding capability, and allowed kinetic analysis of the primary mutational effects. The activation, however, could not be achieved in vitro.

It has been shown previously that Thr^{197} phosphorylation affects the activity state of the cAMP-dependent protein kinase [11,12]. In the crystal structure of cAPK, the Thr^{197} phosphoryl group contacts the sidechains of His^{87}, Arg^{165}, Lys^{189}, and Thr^{195} [13,14]. The contact to Arg^{165}, a residue conserved in many kinases, may be of special significance, as it provides an interaction with the catalytic base Asp^{166}. Probably the Thr^{197} phosphoryl group affects the stereochemical orientation of the catalytic base via Arg^{165}, and thus the activation state of the enzyme.

The results strongly suggest intermolecular phosphorylation as the mechanism for the activation of the mutants. The finding that this could not be achieved in vitro in the presence of MgATP and wild-type enzyme indicates that factors in the living cell may be essential for the activation process. It is feasible that the immature conformation of the nascent polypeptide chain during translation facilitates access and phosphorylation of Thr^{197} in *trans*. For the wild-type enzyme, however, autophosphorylation in *cis*, at least in addition, cannot be excluded yet.

The data are in support of a cotranslational autophosphorylation at Thr^{197} for the active site mutants. Cotranslational intermolecular autophosphorylation of cAPK in *E. coli* may be especially sensitive to any changes in the kinetic behavior of the expressed enzyme. If the process is not under a specific control, it is possible that there are spatial and temporal limitations. Many restrictions are conceivable that require a perfect enzyme to phosphorylate the nascent peptide chain: (i) the abundance of ATP, which may fall below the K_m of an ATP-mutant (both rCα(Ala^{70}Val)-His and rCα(Val^{104}Asp)-His have K_{mATP} defects (see Table 1); (ii) the substrate recognition site, which may be accessible only for an enzyme with optimal peptide binding behavior, (iii) temporal limitations – a V_{max} mutant may simply not be fast enough to compete with the translational machinery. However, this phenomenon may be restricted to expression of cAPK or other kinases in bacteria like *E. coli*. In yeast, a great number of mutations, including those in catalytically important residues, have been successfully introduced [22]. Possibly, specific control mechanisms in the eucaryotic cell ensure the correct cotranslational modifications of the target proteins, even when the enzyme acting on it is seriously impaired. On the other hand, in eucaryotic cells activation by a foreign protein kinase cannot be excluded.

Other kinases, like Erk2, cyclin dependent kinases, protein kinase C, and most tyrosine kinases [3,23] require phosphorylation at activation segment residues for full activity. For some of these enzymes the activating kinase has not been identified yet. The idea that cotranslational phosphorylation may be required for the activation of these kinases as well provides the possibility of adopting the system to other enzymes, and may be considered not only in order to autoactivate these enzymes, but also for coexpression of those kinases which are suspected of *trans* activation, but fail in in vitro assays.

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