Evidence that the substrate backbone conformation is critical to phosphorylation by p42 MAP kinase

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Abstract The effect of prolyl bond isomers on the substrate recognition capabilities of various endoproteases may be investigated in a reaction where both *cis*/*trans* isomers co-exist. Here we address the question of whether enzyme reactions at the side chain of an amino acid preceding proline proceed through an isomer specific pathway. The proline-directed p42 mitogen-activated protein kinase (ERK2) was used to phosphorylate the serine side chain in Pro-Arg-Ser-Pro-Phe-4-nitroanilide under conditions where different amounts of *cis* prolyl isomer of the substrate were present. Initial phosphorylation rates were calculated ranging between zero at 100% *cis* isomer and around 60 pM/min at the equilibrium content of 83.5% *trans* isomer. In the presence of the peptidyl-prolyl *cis*/*trans* isomerase human hFKBP12 (500 nM), *cis*/*trans* isomerization proceeds rapidly, permitting the maximal phosphorylation rate to be observed in the dead time of the experiment. Results show that correct signature sequences are not sufficient to render potential substrates reactive to proline-directed enzymatic phosphorylations, but that the conformational state of the peptide bond following serine (threonine) is a critical determinant. Therefore, catalysis by peptidyl-prolyl *cis*/*trans* isomerases may add a new level of control to intracellular protein phosphorylations.

Key words: p42 mitogen-activated protein kinase; *Cis*/*trans* isomer specificity; Peptidyl-prolyl *cis*/*trans* isomerase; hFKBP12; *Cis*/*trans* isomer

1. Introduction

The primary substrate specificity determinant for a number of protein kinases capable of phosphorylating serine (threonine) residues is a proline following the phosphorylation site. Proline-directed protein kinases include the multigene families of cyclin-dependent protein kinases (Cdks) and mitogen-activated protein (MAP) kinases [1,2]. Cdks play an important role in control of the cell cycle. A large variety of mitogens can stimulate the activation of MAP kinases by phosphorylation on tyrosine and threonine residues causing signal transduction from the cell surface to the nucleus [3,4]. Because a proline residue represents the ultimate cause of serine (threonine) side chain phosphorylation by MAP kinases, analysis of proline-mediated substrate properties may contribute to the identification of recognition determinants associated with productive enzyme/substrate interactions.

Unlike other peptide bonds, peptidyl-prolyl bonds form *cis* and *trans* isomers of comparable thermodynamic stability. Thus, slowly interconverting prolyl isomers of native proteins [5–9], unfolded or partially folded polypeptides [10] and oligopeptides [11] offer alternatives for proteins to bind. Consequently, *cis*/*trans* isomer specific reactions have already been identified in protein–ligand and enzyme–substrate interactions [12–15] as well as in the refolding of denatured proteins [16]. Between enzymes, endoproteases often exhibit specificity for discriminating among isomeric substrates even if the isomeric bond is remote from the scissile linkage [17]. The discrimination factor among the *cis*/*trans* isomers of proline-containing substrates depends on the position of proline relative to the scissile bond. This factor tends to be high, in favor of the *trans* isomer. Obviously both critical bonds are intrinsic parts of the backbone: the peptide bond cleaved by the protease and the isomeric bond. Conversely, in proline-directed Ser(Thr) phosphorylation, the reaction center is a side chain hydroxyl group. It may be suggested that this arrangement allows single bond rotations to position the side chain correctly within the active site of the kinase regardless of the adjacent backbone conformation.

The recently determined crystal structure of a complex consisting of phospho-CDK2/cyclin A3/substrate in the presence of an inactive ATP analogue has given an impression of the substrate arrangement in the active site of a proline-directed kinase. The *trans* conformation of the prolyl bond in the bound peptide HHASPRK [18] indicates conformational specificity of substrate binding but does not provide conclusive evidence about conformational specificity of the productive pathway.

The aim of our study was to investigate, by a direct method, to which extend the proline-directed p42 MAP kinase ERK2 is able to bind productively a Ser-Pro-containing substrate in both isomeric states. Results may allow prediction of the influence of peptidyl-prolyl *cis*/*trans* isomerases on the time-course of the ERK2-mediated phosphorylations. An experimental approach to verify predictions has been developed with the human hFKBP12 as this PPIase is active toward the ERK2 substrate utilized.

2. Materials and methods

2.1. Materials

Recombinant murine p42 MAP kinase (ERK2) produced in *Escherichia coli* was purchased from New England BioLabs Inc.; recombinant eglin C was from Sigma (Deisenhofen, Germany); bovine pancreas α-chymotrypsin was from Boehringer (Mannheim, Germany); inhibitor FK506 was from Alfons Lawen (Monash University, Melbourne, Australia); [γ-^{33}P]ATP (3000 Ci/mM) was from ICN. Recombinant human hFKBP12 was kindly provided by Dr. J.U. Rahfeld (University Halle). Peptides, as Pro-Arg-Ser-Pro-Phe-NHNp and its phosphorylated analogue were synthesized according to [19].

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2.2. Preparation of cis Pro-Arg-Ser-Pro-Phe-NHNp

Pure *cis* isomer was prepared using isomer specific proteolysis by α-chymotrypsin (170 μM) applied to an equilibrated mixture of *cis* and *trans* conformer of Pro-Arg-Ser-Pro-Phe-NHNp (1.7 mM). After a reaction time of 1 min, the *trans* conformer was completely hydrolyzed at the 4-nitroanilide bond, and chymotrypsin was rapidly inactivated by adding a 1.4-fold molar excess of eglin C from a 1.16 mM stock solution in 0.5 mM HCl. The remaining *cis* conformer of Pro-Arg-Ser-Pro-Phe-NHNp is stable only transiently. Isomerization occurs according to a first-order reaction until the *cis*/*trans* equilibrium is reached. In some experiments, hFKBP12 (0.5 μM) was added to accelerate equilibration. FK506 (20 μM) was used as specific inhibitor of hFKBP12. All reactions were performed at 0°C.

For determination of the rate constant of *cis*/*trans* isomerization, aliquots were taken after different times and the *cis* content was determined by isomer specific proteolysis [20].

2.3. Kinase assay

During the re-equilibration of the *cis* peptide, aliquots (5 μl) of the mixture were subjected to p42 MAP kinase (5 μl, 10 000 U/ml) in the presence of [γ-^{33}P]ATP (15 μCi). After 3 min, phosphorylation was stopped by adding trifluoroacetic acid (TFA). To ensure initial kinetics, phosphorylation was followed only 1% toward completion. All reactions were carried out at 0°C.

Aliquots were separated by thin layer chromatography (TLC) using silica gel 60$_{F254}$ from Merck with a butanol/water/acetic acid/ethanol (2:4:1:1) mixture, and submitted to autoradiography with an intensifying screen. The signals were quantified using a Fuji BAS reader. The phosphorylated substrate Pro-Arg-Ser(OPO$_3$H$_2$)-Pro-Phe-NHNp was used as TLC reference.

3. Results

3.1. Initial enzymatic phosphorylation rates in dependence of the re-equilibration time

In order to address the issue of how the MAP kinase ERK2 recognizes a certain prolyl bond isomer in polypeptide chains, our first approach utilized a oligopeptide substrate containing a Ser-Pro motif. Derived from the consensus sequence of ERK2 sensitive phosphorylation sites, the short oligopeptide Pro-Arg-Ser-Pro-Phe-NHNp proved to be very useful in studying phosphorylation kinetics. This was reflected in both k_{cat} (0.0066 min^{-1}) and K_m (690 μM) at 0°C which ensure a specificity constant large enough for a detectable amount of substrate phosphorylation after a short period of time of ERK2 incubation.

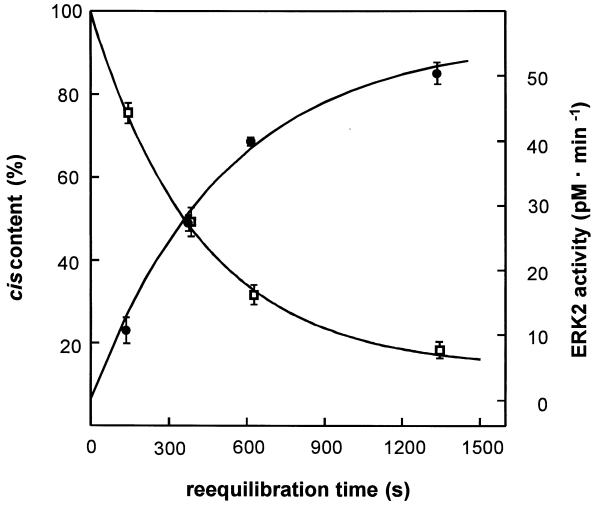

Fig. 1. Initial phosphorylation rates in dependence of the lag time that follows quenching of isomer specific proteolysis of Pro-Arg-Ser-Pro-Phe-NHNp (re-equilibration time) at 0°C. During re-equilibration, aliquots of the mixture were subjected to p42 MAP kinase (0.1 μM) in the presence of [γ-^{33}P]ATP (15 μCi). After 3 min incubation time, phosphorylation was stopped by adding TFA. Phosphorylation mixtures were separated by TLC. Autoradiographies were quantified using a Fuji BAS reader (closed circles: phosphorylation rates). *Cis* contents were determined by isomer specific proteolysis in independent measurements (open squares: percentage of *cis*). Each point represents the mean of three independent experiments.

In aqueous solution at pH 7.5, both prolyl isomers of the oligopeptide co-exist with 16.5 ± 0.2% in the *cis* conformation (Fig. 1). For evaluating *cis*/*trans* isomer specificity of ERK2, the *cis* peptide in its conformationally pure state might be prepared by isomer specific proteolysis using α-chymotrypsin. This method is based on the exclusive cleavage of the *trans* pentapeptide-4-nitroanilide at the anilide bond in a fast reaction. Subsequently, the protease was rapidly inactivated by eglin C. The remaining *cis* isomer is transiently homogeneous but re-equilibrates in a first-order rate process. To decrease the *cis*/*trans* isomerization rate, all experiments were carried out at 0°C. At this temperature, the first-order rate constant for the *cis*/*trans* isomerization of the peptide was $2.08 \pm 0.08 \times 10^{-3}$ s^{-1}.

To find out whether the isomeric composition of the peptide affects the phosphorylation rate of ERK2, aliquots of the re-equilibrating mixture were sampled over the period of the isomerization time and incubated with ERK2, followed by the analysis of phosphorylation products by TLC. When *cis*/*trans* isomer specificity occurs, the concentration of the substrate productively bound on ERK2 should depend on sampling time. Keeping in mind the rate law for initial rates, a constant magnitude for this parameter over sampling time must be lacking. As shown in Fig. 1, the increase in initial rates of the enzymatic phosphorylation correlated with the re-equilibration time, indicating *cis*/*trans* isomer specificity. Simultaneously, the content of *trans* isomer increased from 0 to 83.5%. Curve fitting of the plot resulted in a first-order rate constant of $1.95 \pm 0.13 \times 10^{-3}$ s^{-1} that fits the first-order rate constant measured directly for the *cis*/*trans* isomerization.

3.2. The effects of the peptidyl-prolyl cis/trans isomerase hFKBP12

From the above data, the dependence of the initial rate of phosphorylation on the lag time following isomer specific proteolysis indicates a crucial role of prolyl bond isomerization for ERK2 catalysis. In order to confirm the conformational specificity of ERK2, the specific acceleration of the Ser-Pro *cis*/*trans* isomerization by hFKBP12 has been utilized. This PPIase exhibits a specificity constant of $k_{cat}/K_m = 29$ mM^{-1} s^{-1} for the substrate peptide, permitting the application of catalytical amounts of hFKBP12 (substrate/hFKBP12 ratio = 560) to substantially increase the *cis*/*trans* isomerization rate. Results are shown in Fig. 2. In the presence of hFKBP12, initial phosphorylation rates are increased compared to the PPIase-free control. Moreover, the rate of phosphorylation in the hFKBP12-containing mixture reaches the level of the thermally equilibrated mixture. The effect of hFKBP12 is abolished by the hFKBP12 inhibitor FK506. To test whether the kinase is directly influenced by hFKBP12, we studied the phosphorylation of the equilibrated peptide in presence and absence of the PPIase. The initial rates of the enzymatic phosphorylation were comparable 63 ± 2 pM/min respectively 64 ± 2 pM/min (the data shown are the mean of

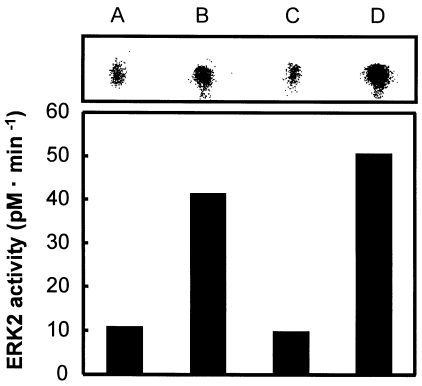

Fig. 2. Effects of the peptidyl-prolyl *cis/trans* isomerase hFKBP12 on ERK2-mediated phosphorylation. After finishing isomer specific proteolysis, phosphorylation was started by adding of 0.1 μM p42 MAP kinase. Phosphorylation of aliquots of the reaction mixture was performed as described in Section 2.3: (A) in the absence of hFKBP12, (B) with hFKBP12 (0.5 μM) and (C) in the presence of hFKBP12 (0.5 μM) and its specific inhibitor FK506 (20 μM). (D) Initial rate of phosphorylation measured for the already equilibrated substrate. The figures are representative for three independent experiments.

three independent experiments). Thus we exclude an effect of hFKBP12 on the kinase itself.

4. Discussion

The occurrence of *cis* and *trans* conformers of the peptidyl-prolyl bond in unstructured polypeptides as well as in native proteins raises a question concerning the biologically active form of the molecule. *Cis/trans* isomer specific conversions have been found directly in protein folding, proteolysis and protein/ligand interactions [8,12,13,21], all of which involve backbone linkages in the reaction. The effect of PPIase activity on the signal transduction of transmembrane receptors provides indirect evidence for *cis/trans* isomer specific processes [22,23].

The present paper describes a reliable method to evaluate the *cis/trans* isomer specificity of proline-directed protein kinases by measuring initial phosphorylation rates of oligopeptides in dependence of the lag time following a proteolytic pulse. For p42 MAP kinase, α-chymotrypsin has been used as a *cis/trans* isomer specific protease in conjunction with a Ser-Pro-Phe sequence segment forming a suitable kinase substrate. At 0°C, *cis/trans* isomerization of the substrate is slow enough to ensure a sufficient number of data points for the initial rate constants to calculate the rate constant of *cis/trans* isomerization from phosphorylation measurement. The calculated constant fits well to the rate constant determined by direct monitoring of *cis/trans* isomerization rates.

Using this method, the initial phosphorylation rates increase during the conformational re-equilibration of Pro-Arg-Ser-Pro-Phe-NHNp, where a zero phosphorylation rate occurs with the pure *cis* conformer. Phosphorylation increases up to saturation level with the emergence of *trans* conformer of the substrate. A constant initial phosphorylation rate would be expected if the *cis/trans* isomer specificity of ERK2 does not exist under the same conditions. This point was investigated further in the experiment performed in the presence of the PPIase hFKBP12. The ubiquitous FKBP12 represents just a single member of dozens of different PPIases in cells [11]. This PPIase was used as an enzyme tool to catalyze the conformational re-equilibration. Thus, the substrate was allowed to re-equilibrate in the dead time of the experiment (30 s) by catalyzing its *cis/trans* isomerization by hFKBP12. A *cis/trans* isomer specificity of ERK2 could not be seen under these conditions. Inhibition of hFKBP12 by FK506 provides evidence for the required active PPIase for the observed effect on the initial rates of phosphorylation (Fig. 2).

In conclusion, p42 MAP kinase-catalyzed phosphorylation exhibits a pronounced specificity for substrates with a *trans* prolyl bond adjacent to the reactive serine residue. This may indicate that not only the primary sequence and the accessibility of the phosphorylation site, but also the conformation of Ser(Thr)-Pro bond is important for a substrate to be recognized by a proline-directed protein kinase. For phosphorylation of oligopeptides under steady state conditions in homogeneous solution, the presence of the inert *cis* isomer only affects the K_m value. However, greater effects may occur in the phosphorylation of the conformationally homogeneous Ser(Thr)-Pro moieties in proteins, and in kinetically coupled processes where quasi-irreversibility is involved. To demonstrate the conformational specificity of a protein kinase, we used a peptide as model. Former experiments [20] using well-defined peptides already showed the isomer specific proteolysis of prolyl peptide bonds and revealed the folding helper enzymes. We used a similar approach and therefore we suggest that our data on isomer specific phosphorylation on peptides allow extension to other peptide sequences and proteins.

Regarding proteins, it has been shown by analysis of the structure database that about 5% of Ser-Pro moieties in proteins exist as *cis* prolyl bonds [24]. In both cases, complete discrimination of an otherwise phosphorylatable moiety can be suggested. Moreover, the results of this study provide the first hints that enzyme reactions at side chains of polypeptides might be affected by the backbone conformation adjacent to the reactive site. Because peptidyl-prolyl *cis/trans* isomerases of the cyclophilin and FKBP family have the potential to shift half-times of *cis/trans* isomerizations into a millisecond time-range [25], potential control of protein phosphorylation by *cis/trans* isomer specificity of proline-directed protein kinases can be specifically abolished. Provided that protein phosphatases are also able to discriminate prolyl bond isomers, the concerted action of these enzymes adds a completely new level of complexity to the phosphorylation/dephosphorylation of proteins.

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