# Sequence-specific recognition of peptide substrates by the low $M_r$ phosphotyrosine protein phosphatase isoforms

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**Abstract A number of phosphotyrosine-containing peptides derived from the PDGF receptor phosphorylation sites have been synthesised. The peptides were assayed as substrates of the two isoforms (IF1 and IF2) of the low $M_r$ PTPase. The calculated $k_{cat}$, $K_m$, and $k_{cat}/K_m$ values indicate that only one peptide is best hydrolysed by IF2 (but not IF1), whose catalytic efficiency averages those previously reported for most PTPases (except the *Yersinia* enzyme). This peptide is the only one containing a couple of no bulky hydrophobic residues at the phosphotyrosine *N*-side. The determination of the same catalytic parameters in the presence of analogues of the best hydrolysed peptide in which one or both hydrophobic residues were replaced by Asp or Lys residues confirmed the importance of the hydrophobic cluster at the phosphotyrosine *N*-side for optimal enzymatic hydrolysis. These findings are discussed in the light of the known IF2 X-ray structure.**



*Key words:* Low $M_r$ phosphotyrosine protein phosphatase isoform; Low $M_r$ phosphotyrosine protein phosphatase, substrate recognition; Platelet-derived growth factor-derived peptide; Tyrosine-phosphorylated peptide

## 1. Introduction

Phosphotyrosine protein phosphatases (PTPases) are a family of enzymes catalysing the hydrolysis of phosphotyrosine, both free and in peptides or proteins [1,2]. Due to their hydrolytic activity, PTPases counteract the action of protein tyrosine kinases (PTKs), a large group of enzymes involved in a number of cellular activities and particularly in the control of cell proliferation and differentiation. The biological importance of these enzymes is testified by the fact that some oncogenes encode growth factor receptors devoid of the regulatory subunits and hence with constitutive PTK activity. Due to the antiproliferative action of a number of PTPases, the genes encoding these enzymes have been considered tumour-suppressing genes or even anti-oncogenes [3].

At present, over 50 enzymes are classified into the PTPase family; these enzymes are grouped into several classes with poor or no homology to each other [4]. All these enzymes share only a limited sequence motif (CXXXXXRS/T) containing some of the active site residues (active site signature) and the catalytic mechanism, which proceeds through a cysteinyl-phosphate intermediate [5]. A class of PTPases comprises enzymes characterised by a reduced molecular mass (low $M_r$ PTPases) previously known as non-lysosomal acid phosphatases (EC 3.1.3.2) [6]. Low $M_r$ PTPases (EC 3.1.3.48) are present in organisms spanning the entire phylogenetic scale, from prokaryotes to mammals, where they are likely to perform different functions [7]. In mammals, three alleles (A, B and C) encoding the enzyme have been described [8]; each allele expresses two isoforms originating from alternative splicing [9], which differ from each other only in the 40–73 region [7]. These isoforms have been named fast (f) and slow (s), respectively, on the basis of the differing electrophoretic mobility or, alternatively, IF1 and IF2.

In 1994, the X-ray structure at 2.1 Å resolution of the bovine liver IF2 has been reported [10]. The enzyme is a closely packed α/β structure in which a central four-stranded open-twisted β sheet is surrounded on both sides by α helices. In the structure, the active site signature residues form a smooth phosphate binding loop (P-cradle) connecting α1 to β1. This structure differs from that of PTPases belonging to differing classes [11–13]; however, all these enzymes possess the same active site residues located at the bottom of a deep crevice lined by residues which could be involved in the differential kinetic behaviour and substrate preference of the varying PTPases [14]. Some of these residues are likely to be responsible for the differing substrate affinity as well as cGMP and purine sensitivity of IF1 and IF2; this has recently been confirmed by a study performed by using a mutagenesis approach, which underlined the molecular basis of the differing IF1 and IF2 behaviour [15].

Despite the structural and kinetic data currently available on the PTPases, little is known about the substrate specificity or preferences of these enzymes and about the molecular basis of such specificity. The bovine liver IF2 and its dominant negative mutant (C12S) appear to specifically interact, both in vitro and in vivo, with the autophosphorylated PDGF receptor; the low $M_r$ PTPase has also been reported to interact with the EGF receptor in brain and with the insulin receptor. The interaction with the wild-type enzyme leads to dephosphorylation of these receptors, which hence have been considered among the true physiological substrates of the enzyme [16–19].

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*Abbreviations:* PTPase, phosphotyrosine protein phosphatase; PTK, protein tyrosine kinase; low $M_r$ PTPase, low molecular weight phosphotyrosine protein phosphatase; IF1, low $M_r$ PTPase isoform 1; IF2, low $M_r$ PTPase isoform 2; AcP1, rat liver low $M_r$ PTPase isoform 1; AcP2, rat liver low $M_r$ PTPase isoform 2; PDGF, platelet-derived growth factor; Fmoc, fluoren-9-ylmethoxycarbonyl-; RP, reverse phase; OPfp, pentafluorophenyl ester; HOBt, hydroxybenzotriazole; BOC, *t*-butyloxycarbonyl-; Trt, trityl-; DMF, dimethylformamide; TFA, trifluoroacetic acid; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium tetrafluoroborate; TMSBr, trimethylbromosilane; TIS, triisopropylsilane; FAB, fast atom bombardment; RP-HPLC, reverse phase-high performance liquid chromatography; PNPP, *p*-nitrophenylphosphate


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The search for good substrates for PTKs and PTPases and for molecular motifs enhancing enzyme-to-substrate affinity can take advantage from the use of Tyr-phosphorylated synthetic peptides with sequences both derived from those of known proteins which physiologically undergo tyrosine phosphorylation or modified [20,21]. IF1 and IF2 exhibit differing kinetic behaviour on Tyr-phosphorylated peptides derived from the sequences of varying proteins [22]. In the present paper, we extend these findings focusing the analysis on the main phosphorylation sites of the β-PDGF receptor. We therefore synthesised Tyr-phosphorylated peptides with sequences derived from those of the PDGF receptor main phosphorylation sites as well as some peptide derivatives containing specific amino acid substitutions; the main kinetic parameters of either low $M_r$ PTPase isoform on each peptide were then determined. The substrate molecular features which are likely to ensure optimal hydrolytic activity of either isoform are also discussed.

## 2. Materials and methods

Fmoc-Tyr(PO(OBzl)OH)-OH, side chain unprotected Fmoc-Tyr($PO_3H_2$)-OH and TGA resins bound to the C-terminal Fmoc-amino acids were from Novabiochem; Fmoc-Tyr($PO_3Me_2$) was from Bachem; Fmoc-amino acids-OPfp were from Novabiochem or from Perspective Biosystems; TBTU, HOBt, dimethylformamide, diisopropylcarbodiimide, TFA and piperidine were from Perspective Biosystems. A C18 RP-HPLC column (Vydac) was used for peptide purification. All other reagents and solvents were analytical grade. Peptide sequences were taken from the SwissProt data bank.

### 2.1. Synthesis of phosphopeptides

Phosphotyrosine-containing peptides were synthesised in solid phase using a MilliGen 9050 PepSynthesizer following Fmoc chemistry. The NovaSyn TGA resin composed of a polystyrene matrix grafted with polyethylene oxide functionalised with the 4-hydroxymethylphenoxyacetic acid linker bound to the Fmoc-protected C-terminal amino acid was used; resins showed an overall substitution level of 0.10–0.20 mmol/g, depending on the resin type. Peptide synthesis was carried out by the standard Fmoc protocol using a fourfold excess (4 equiv.) of each Fmoc-amino acid OPfp-ester in the presence of 1 equiv. of HOBt. Fmoc-Tyr(PO(OBzl)OH)-OH or Fmoc-Tyr($PO_3H_2$)-OH was coupled in DMF containing 0.6 M *N*-ethylmorpholine and 1 equiv. of TBTU; Fmoc-Tyr($PO_3Me_2$)-OH was coupled with diisopropylcarbodiimide as previously reported [21]. Coupling cycles of 1 h were used throughout the synthesis; when necessary, double coupling cycles were performed. Side chain protection was accomplished by *t*-butyl ether or ester for Asp, Glu, Ser, Thr and Tyr, and by the BOC group for Lys and the Trt group for histidine. Fmoc group deprotection was carried out with 20% piperidine in DMF. All other steps were performed in DMF. Peptide cleavage and deprotection were carried out on the dried resin by incubation for 60–120 min. When using Fmoc-Tyr(PO(OBzl)OH)-OH as a synthon, the deprotection cocktail was TFA/TIS/water (90:5:5); when using Fmoc-Tyr($PO_3H_2$)-OH, the deprotection cocktail was TFA/thioanisole/anisole/ethanedithiol (90:5:3:2). Finally, when the Fmoc-Tyr($PO_3Me_2$)-OH synthon was used, peptide cleavage and deprotection were carried out as previously reported [22]. Deprotection times and cocktail compositions varied according to the amino acid content of each peptide. Each crude peptide was precipitated with cold ether, centrifuged, washed four times with an excess of cold ether and dried under vacuum. The dry powder of each peptide was resuspended in 10% acetonitrile/water and purified by semipreparative RP-HPLC on a C18 Vydac (20×250 mm) column.

### 2.2. Assessment of peptide purity

Peptide purity was assayed by both amino acid analysis and FAB mass spectrometry. The former was carried out as previously described [23]; the same method was also used to determine peptide concentrations. Values for serine and threonine were corrected for loss during hydrolysis. FAB mass spectra were recorded using a VG Analytical 70-70 EQ instrument as previously reported [24]. Phosphate analysis in each synthesised peptide was carried out on samples of known concentrations by the method of Fiske and Subbarow [25].

### 2.3. IF1 and IF2 purification and assay

Low $M_r$ PTPase IF1 and IF2 were purified from rat liver (where they are also indicated as AcP1 and AcP2, respectively) as previously described [26]. PTPase activity was assayed at pH 5.5 using PNPP or phosphotyrosine as substrates, as previously described [15]. The kinetic parameters of either PTPase isoform using each Tyr-phosphorylated peptide as a substrate were determined by following phosphate release by the malachite green assay [22]. Poorly hydrolysed peptides were incubated for longer times to enhance test sensitivity.

## 3. Results and discussion

We synthesised a number of Tyr-phosphorylated peptides whose amino acid sequences were those surrounding the main phosphorylation sites of the β-PDGF receptor. The synthesis was carried out using the Fmoc chemistry at the conditions reported in Section 2. Three differing building blocks were used to introduce the phosphotyrosine residue in each peptide: Fmoc-Tyr(PO(OBzl)OH)-OH, Fmoc-Tyr($PO_3Me_2$)-OH and Fmoc-Tyr($PO_3H_2$)-OH. The use of the latter was previously claimed to give the desired peptides in good yield without side chain modifications during cleavage and deprotection steps [27]; however, when using this synthon, we got crude peptides of poor purity, probably due to side reactions during deprotection. Similarly, the use of the Fmoc-Tyr($PO_3Me_2$)-OH synthon gave crude peptides of low purity, as previously reported [22]. Instead, we got good yields of quite pure crude peptides by using the Fmoc-Tyr(PO(OBzl)OH)-OH synthon

Table 1
Amino acid sequences of the synthetic Tyr-phosphorylated peptides[a]

| Peptide | Sequence | PDGF receptor phosphorylation site |
|---|---|---|
| PDGFRP1 | $H_2N$-ESVD**Y**VPML-COOH | Y751 |
| PDGFRP2 | $H_2N$-DSN**Y**ISKG-COOH | Y857 |
| PDGFRP3 | $H_2N$-DGG**Y**MDMSKD-COOH | Y740 |
| PDGFRP4 | $H_2N$-ESSN**Y**MAPYD-COOH | Y771 |
| PDGFRP5 | $H_2N$-DGHE**Y**IYVD-COOH | Y579 |
| PDGFRP6 | $H_2N$-EYI**Y**VDPMQL-COOH | Y581 |
| PDGFRP7 | $H_2N$-SAEL**Y**SNAL-COOH | Y716 |
| PDGFRP8 | $H_2N$-GDND**Y**IIPL-COOH | Y1021 |
| PDGFRP9 | $H_2N$-SVL**Y**TAVQ-COOH | Y1009 |
| PDGFRP9LD | $H_2N$-SDL**Y**TAVQ-COOH | Y1009 |
| PDGFRP9DD | $H_2N$-SDD**Y**TAVQ-COOH | Y1009 |
| PDGFRP9KK | $H_2N$-SKK**Y**TAVQ-COOH | Y1009 |

[a]Peptides were synthesized by Fmoc chemistry as indicated in Section 3.

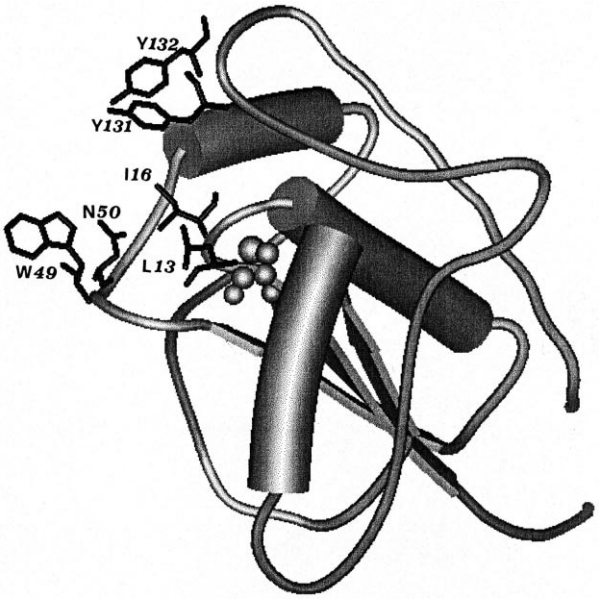


Fig. 1. Outline of the three-dimensional structure of IF2. The residues lining the border of the active site crevice are in black. The catalytic Cys residue at the bottom of the crevice is in grey.

and the deprotection protocol reported in Section 2. The crude peptides were easily purified in good final yields by RP-HPLC and their purity was checked by amino acid analysis and FAB mass spectrometry; in addition, the stoichiometry of inorganic phosphate in each peptide was quantitated by measuring it after combustion or complete enzymic hydrolysis of a known sample of the peptide. All purified peptides showed correct amino acid compositions and molecular masses without any detectable impurity, and contained an amount of inorganic phosphate corresponding to a peptide/phosphate ratio of about 1. In particular, the positive FAB mass spectra of each peptide showed a distinct molecular ion at an *m/z* value one unit higher than the calculated molecular mass of the same peptide. Table 1 reports the amino acid sequences of the synthesised peptides together with the correspondence of each peptide to the phosphorylation site in the whole β-PDGF receptor.

Each purified peptide was used as a substrate for rat liver IF1 and IF2, whose hydrolytic activities were determined by the malachite green colorimetric assay in a final volume of 0.5 ml by comparison with a calibration curve obtained as previously reported [22]. The test showed a good reproducibility and a limit of sensitivity lower than 0.5 nmol of inorganic phosphate, allowing determination of the kinetic parameters even for those peptides with the highest affinity or the lowest susceptibility to hydrolysis. By using this assay we determined the main kinetic parameters as well as the catalytic efficiencies of IF1 and IF2 in the presence of each synthetic peptide (Table 2). The following observations can be made: (i) most peptides are hydrolysed quite slowly and only in the presence of PDGFRP9 IF2 shows a rate constant higher than 25 $s^{-1}$, whereas all other peptides are hydrolysed more (or much more) slowly; (ii) the IF2 $k_{cat}$ in the presence of PDGFRP9 is comparable to those reported for a number of other PTPases (with the exception of the *Yersinia* PTPase, which shows extremely high phosphopeptide dephosphorylation rates) [20,21,28]; (iii) the apparent $K_m$ values indicate that only PDGFRP9 displays a good affinity for both IF1 and IF2 (90 and 30 μM, respectively); (iv) all other peptides display low affinities, with apparent $K_m$ values that, in some cases, are over two orders of magnitude higher. Quite low affinities were also found in an earlier study of the same enzymes in the presence of Tyr-phosphorylated peptides derived from the sequences of a number of proteins undergoing phosphorylation on tyrosine [22]. Therefore, PDGFRP9 is the first low $M_r$ PTPase substrate for which the enzyme displays an in vitro affinity comparable to those reported for a number of other PTPases [20,21,28,29]. The IF1 and IF2 $K_m/k_{cat}$ values in the presence of each phosphorylated peptide are also reported in Table 2. In the presence of PDGFRP9, IF2 shows the best kinetic efficiency. The latter is about 2–4 orders of magnitude higher than all others, in particular, it is about two orders of magnitude higher than the IF1 kinetic efficiency in the presence of the same peptide, and comparable to the values reported for a number of other PTPases except the *Yersinia* enzyme [20,21,28].

Among all synthesised peptides, PDGFRP9 is the only one containing two hydrophobic residues with no bulky side chains at positions −1 and −2 with respect to phosphotyrosine and no charged residues at both sides of phosphotyrosine. To check the importance of such features, we synthesised two analogues of this peptide in which the −2 Val and the −1 Leu −2 Val were replaced by one and two Asp residues, respectively. The kinetic behaviour of either IF1 and IF2 in the presence of these peptides (named PDGFRP9LD and PDGFRP9DD, respectively) is reported in Table 2. It can be seen that the presence of an acidic residue at −2 strongly impairs IF2 $k_{cat}$ and catalytic efficiency though maintaining unchanged $K_m$ values, whereas the presence of both Asp residues considerably lowers the affinity of either isoform for the peptide, raising the $K_m$ values by about 25 and 100 times, respectively. The presence, at the phosphotyrosine *N*-side, of one or two acidic residues replacing hydrophobic residues lowers the IF2 catalytic efficiency to values similar to those found for IF1 and for either isoform in the presence of all other peptides; this finding indicates the importance of the −1 and −2 positions for substrate recognition and hydrolysis, as reported for other PTPases [20]; instead, the presence of Pro

Table 2
Kinetic behaviour of rat liver IF1 and IF2 on the PDGF-derived Tyr-phosphorylated peptides either normal or modified[a]

| Peptide | $k_{cat}$ ($s^{-1}$) | | $K_m$ (mM) | | $k_{cat}/K_m$ ($s^{-1}$ $M^{-1}$)×$10^3$ | |
|---|---|---|---|---|---|---|
| | IF1 | IF2 | IF1 | IF2 | IF1 | IF2 |
| PDGFRP1 | 11.34 | 13.30 | 1.58 | 6.90 | 7.18 | 1.93 |
| PDGFRP2 | 0.12 | 0.05 | 0.38 | 0.36 | 0.32 | 0.14 |
| PDGFRP3 | 13.18 | 2.85 | 1.23 | 1.07 | 10.72 | 2.66 |
| PDGFRP4 | 15.23 | 14.55 | 0.60 | 0.94 | 25.38 | 15.48 |
| PDGFRP5 | 7.05 | 15.83 | 1.10 | 8.49 | 6.41 | 3.04 |
| PDGFRP6 | 4.67 | 1.40 | 0.43 | 0.22 | 10.86 | 6.36 |
| PDGFRP7 | 6.24 | 4.81 | 0.90 | 1.88 | 6.93 | 2.56 |
| PDGFRP8 | 7.14 | 1.81 | 0.60 | 5.54 | 11.90 | 0.33 |
| PDGFRP9 | 0.88 | 26.32 | 0.09 | 0.03 | 9.78 | 877.33 |
| PDGFRP9LD | 0.24 | 0.09 | 0.05 | 0.04 | 4.80 | 2.75 |
| PDGFRP9DD | 3.75 | 1.54 | 2.14 | 3.88 | 1.75 | 0.40 |
| PDGFRP9KK | 6.01 | 2.47 | 1.22 | 3.72 | 5.46 | 6.66 |

[a]The values are the average of three determinations.

at +3 (PDGFRP4, PDGFRP6 and PDGFRP8) does not seem to have any effect. PDGFRP6 was the only other peptide with two hydrophobic residues at the −1 and −2 positions, however it was a poor substrate for either isoform. This finding can be explained considering that the −2 residue in PDGFRP6 (Tyr) has a more bulky side chain than the corresponding residue in PDGFRP9 (Val); in addition, PDGFRP6 contains a charged Asp residue at −3. These features suggest that position −3 also has importance for optimal substrate binding and hydrolysis.

The reported results indicate that IF2 is more sensitive than IF1 to hydrophobic residue→acidic residue substitutions at the phosphotyrosine *N*-side in terms of substrate affinity, kinetic constant and catalytic efficiency. This result agrees with previous structural and kinetic findings indicating that in IF1 and IF2 and other PTPases, the residues lining the hydrophobic pocket are important for substrate recognition and binding as well as for the differential kinetic behaviour [10,14,15]. These residues are mainly basic in PTP1B and *Yersinia* PTPase as well as in $SH_2$-domain proteins, thus providing a possible explanation for the strong preference of a number of PTPases for substrates containing acidic residues at the phosphotyrosine *N*-side [20,29–31]. In IF1 and IF2, these residues are mainly hydrophobic: L13, I16, Y49 (W49 in IF2), E50 (N50 in IF2), Y131, Y132), thus providing a molecular basis for the strong preference of these enzymes for substrates containing hydrophobic residues at the key −1 and −2 positions in terms of either affinity (IF1) or catalytic efficiency (IF2) (Fig. 1). In particular, residues at positions 49 and 50 have been shown to be important for IF1 and IF2 differential substrate preference, kinetic behaviour and purine nucleotide activation [15]. This finding could explain the strong IF2 preference for PDGFRP9 respect to IF1; in fact, IF1 contains an acidic residue (E50) which could account for its remarkably lower catalytic efficiency on the same peptide.

The importance of position 50 for peptide recognition by either isoform was confirmed by synthesising a PDGFRP9 derivative in which two Lys residues were present at the −1 and −2 positions (PDGFRPKK). Table 2 reports the kinetic behaviour of IF1 and IF2 in the presence of this peptide. It can be seen that IF2 behaves as it does in the presence of PDGFRPDD, whereas IF1 shows only a slight rise in catalytic efficiency, whose value becomes similar to that measured in the presence of the PDGFRP9 peptide due to an increase of the $k_{cat}$ value.

From our data, it can be concluded that, apparently, PDGFRP9 is the best substrate for IF2 whereas, among all synthesised peptides, no one appears a good substrate for IF1, suggesting that IF1 and IF2 act on different physiological substrates; in fact, IF2 best hydrolyses peptides containing hydrophobic residues at the phosphotyrosine *N*-side whereas, from our study, it is not possible to assess which are the substrate molecular features allowing optimal phosphopeptide hydrolysis by IF1. The remarkable preference of IF2 for PDGFRP9 does not necessarily mean that Tyr-1009, the PDGF receptor phosphorylation site corresponding to this peptide, is the preferential site of interaction of this enzyme isoform with the receptor; rather this finding indicates that the enzyme preferential substrate(s) should possess a recognition motif in which an important role is performed by hydrophobic residues at the phosphotyrosine *N*-side, thus providing an useful information in the search of the enzyme physiological substrate(s).

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## References

[1] Charbonneau, H. and Tonks, N.K. (1992) Annu. Rev. Cell Biol. 8, 463–493.
[2] Walton, K.M. and Dixon, J.E. (1993) Annu. Rev. Biochem. 62, 101–120.
[3] LaForgia, S., Morse, B., Levy, J., Barnea, G., Cannizzaro, L.A., Ki, F., Nowell, P.C., Boghosian-Sell, L., Glick, J., Weston, A., Harris, C.C., Drabkin, H., Patterson, D., Croce, C.M., Schlessinger, J. and Huebner, K. (1991) Proc. Natl. Acad. Sci. USA 88, 5036–5040.
[4] Fauman, E.B. and Saper, M.A. (1996) Trends Biochem. Sci. 21, 413–417.
[5] Zhang, Z.-Y., Wang, Y. and Dixon, J.E. (1994) Proc. Natl. Acad. Sci. USA 91, 1624–1627.
[6] Heinrikson, R.L. (1969) J. Biol. Chem. 244, 299–307.
[7] Ramponi, G. and Stefani, M. (1997) Biochim. Biophys. Acta 1341, 137–156.
[8] Dissing, J. and Svensmark, O. (1990) Biochim. Biophys. Acta 1041, 232–242.
[9] Lazaruk, K.D.A., Dissing, J. and Sensabaugh, G.F. (1993) Biochem. Biophys. Res. Commun. 196, 440–446.
[10] Su, X.-D., Taddei, N., Stefani, M., Ramponi, G. and Nordlund, P. (1994) Nature 370, 575–578.
[11] Barford, D., Flint, A.J. and Tonks, N.K. (1994) Science 263, 1397–1404.
[12] Stuckey, J.A., Schubert, H.L., Fauman, E.B., Zhang, Z.-Y., Dixon, J.E. and Saper, M.A. (1994) Nature 370, 571–575.
[13] Yuvaniyama, J., Denu, J.M., Dixon, J.E. and Saper, M. (1996) Science 272, 1328–1331.
[14] Jia, Z., Barford, D., Flint, A.J. and Tonks, N.K. (1995) Science 268, 1754–1758.
[15] Cirri, P., Fiaschi, T., Chiarugi, P., Camici, G., Manao, G., Raugei, G. and Ramponi, G. (1996) J. Biol. Chem. 271, 2604–2607.
[16] Shimohama, S., Fujimoto, S., Taniguchi, T. and Kimura, J. (1994) Brain Res. 662, 185–188.
[17] Chiarugi, P., Cirri, P., Raugei, G., Camici, G., Dolfi, F., Berti, A. and Ramponi, G. (1995) FEBS Lett. 372, 49–53.
[18] Chiarugi, P., Cirri, P., Raugei, G., Manao, G., Taddei, L. and Ramponi, G. (1996) Biochem. Biophys. Res. Commun. 219, 21–25.
[19] Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Camici, G., Manao, G. and Ramponi, G. (1997) Biochem. Biophys. Res. Commun. 238, 676–682.
[20] Zhang, Z.-Y., Thieme-Sefler, A.M., MacLean, D., McNamara, D.J., Dobrusin, E.M., Sawyer, T. and Dixon, J.E. (1993) Proc. Natl. Acad. Sci. USA 90, 4446–4450.
[21] Pinna, L. and Donella-Deana, A. (1994) Biochim. Biophys. Acta 1222, 415–431.
[22] Stefani, M., Caselli, A., Bucciantini, M., Pazzagli, L., Dolfi, F., Camici, G., Manao, G. and Ramponi, G. (1993) FEBS Lett. 326, 131–134.
[23] Manao, G., Camici, G., Cappugi, G., Stefani, M., Liguri, G., Berti, A. and Ramponi, G. (1985) Arch. Biochem. Biophys. 241, 418–424.
[24] Camici, G., Manao, G., Cappugi, G., Berti, A., Stefani, M., Liguri, G. and Ramponi, G. (1983) Eur. J. Biochem. 137, 269–277.
[25] Fiske, H. and Subbarow, Y. (1925) J. Biol. Chem. 66, 375.
[26] Manao, G., Pazzagli, L., Cirri, P., Caselli, A., Camici, G., Cappugi, G., Saeed, A. and Ramponi, G. (1992) J. Protein Chem. 11, 333–345.

[27] Ottinger, E.A., Shekels, L.L., Bernlohr, D.A. and Barany, G. (1993) Biochemistry 32, 4354–4361.
[28] Harder, K.W., Owen, P., Wong, L.K.H., Aebersold, R., Clark-Lewis, I. and Jirik, F. (1994) Biochem. J. 298, 395–401.
[29] Hippen, K.L., Jakes, S., Richards, J., Jena, B.P., Beck, B.L., Tabatabai, L.B. and Ingebritsen, T.S. (1993) Biochemistry 32, 12405–12412.
[30] Ruzzene, M., Donella-Deana, A., Marin, O., Perich, J.W., Ruzza, P., Borin, G., Calderan, A. and Pinna, L.A. (1993) Eur. J. Biochem. 211, 289–295.
[31] Cho, H., Kishnaraj, R., Itoh, M., Kitas, E., Bannwarth, W., Saito, H. and Walsh, C.T. (1993) Protein Sci. 2, 977–984.