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Alessandra Modesti[1]
Luca Bini[2]
Laura Carraresi[1]
Francesca Magherini[1]
Sabrina Liberatori[2]
Vitaliano Pallini[2]
Giampaolo Manao[1]
Lorenzo A. Pinna[3]
Giovanni Raugei[1]
Giampietro Ramponi[1]

[1]Dipartimento di Scienze Biochimiche, Università di Firenze, Italy
[2]Dipartimento di Biologia Molecolare, Università di Siena, Italy
[3]Dipartimento di Chimica Biologica and Centro CNR per lo Studio delle Biomembrane, Università di Padova, Padova, Italy

Expression of the small tyrosine phosphatase (Stp1) in *Saccharomyces cerevisiae*: A study on protein tyrosine phosphorylation

Small tyrosine phoshatase 1 (Stp1) is a *Schizosaccharomyces pombe* low-molecular-mass phosphotyrosine-phosphatase 50% identical to *Saccharomyces cerevisiae* Ltp1. In order to investigate the role of Stp1 in yeast, a mutant was generated having the characteristic of a dominant negative molecule. Changes in protein tyrosine phosphorylation in *S. cerevisiae* proteome in response to Stp1 or its dominant negative mutant expression were analyzed by high-resolution two-dimensional (2-D) electrophoresis. The most remarkable result is the modification by phosphorylation on tyrosine of several proteins involved in carbohydrate metabolism. Twelve proteins were identified on the basis of their positions in the anti-phosphotyrosine immunoblot of the 2-D electrophoresis. Ten of these present tyrosyl residues that are within the consensus sequence for protein kinase CK2 (casein kinase-2). These data open the possibility for the identification of Stp1 substrates in yeast and provide hints about the nature of tyrosine phosphorylating agents in yeast and in other organisms where *bona fide* tyrosine kinases are lacking.

Keywords: *Saccharomyces cerevisiae* / Two-dimensional electrophoresis / Tyrosine phosphorylation / Casein kinase-2 / Low-molecular-weight phosphotyrosine phosphatase EL 4229

1 Introduction

The regulation of cell proliferation and differentiation involves cascades of reversible protein phosphorylation events induced by extra- and intracellular signals and occurring on tyrosyl and/or seryl/threonyl residues. The level of protein phosphorylation is controlled by the concerted action of protein kinases and protein phosphatases. Phosphotyrosine protein-phosphatases (PTPs) take part in such regulation, either directly *via* the dephosphorylation of one or more target proteins, or by counteracting the activities of signal transducing tyrosine kinase [1]. In *Saccharomyces cerevisiae*, tyrosine-specific kinases have not been identified. However, a number of genes encoding putative PTPs have been reported, including both phosphotyrosine-specific and dual-specific enzymes. Two of the *S. cerevisiae* PTPs appear to be MAP kinase phosphatases: the dual-specific PTP encoded by the MSG5 gene contributes to the reversal of pheromone arrest [2], and the PTP2 protein involved in osmoregulation [3]. Other *S. cerevisiae* PTPs are involved in the cell cycle control required for progression through S-phase or in the dephosphorylation of the Cdc28 kinase [4]. Ptp1 appears to be the first phosphotyrosine-specific phosphatase reported in budding yeast [5]. Overexpression of PTP1 in *S. cerevisiae* rescues the synthetic lethality resulting from disruption of both PTC1 and PTP2 [6] but its physiological role is still unclear. In addition, when overexpressed in *Schizosaccharomyces pombe*, either *S. cerevisiae* PTP1 or mammalian PTP1B can complement a mutation in an endogenous PTP, Cdc 25, and activate the cell cycle regulator, Cdc2 [7]. Wilson *et al.* [8] have shown that in yeast cells lacking PTP1 tyrosine phosphatase, a yeast nucleolar protein, the immunophilin Fpr3, exhibits enhanced levels of tyrosine phosphorylation. The FPR3 gene product, a nucleolarly localized proline rotamase of the FK506- and rapamycin-binding family, is identical to a phosphotyrosil protein which is increased in ptp1 mutant cells (p70) and is also dephosphorylated *in vitro* by recombinant Ptp1. The yeast immunophillin Fpr3 is the first identified tyrosine-phosphorylated protein that is not itself a protein kinase and is the only known physiological substrate of Ptp1. The kinase responsible for Fpr3 phosphorylation has been shown to be a *bona fide* Ser/Thr protein kinase, termed "casein kinase-2" (CK2) which is especially abundant in nucleoli [9]. CK2 phosphorylates Fpr3 at a tyrosyl residue (Tyr184) specified by acidic residues (IYDSE), among which the

Correspondence: Prof. Giampietro Ramponi, Dipartimento di Scienze Biochimiche, Università di Firenze, Viale Morgagni 50, 50134 Firenze, Italy
E-mail: modesti@scibio.unifi.it
Fax: +39-55-4222725

Abbreviations: CK2, casein kinase-2; **LMW-PTP**, low molecular weight phosphotyrosine-phosphatase; **PDGF**, platelet-derived growth factor; **Stp1**, small tyrosine phosphatase-1

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aspartic acid adjacent to the *C*-terminal side of tyrosine, has been shown to play a crucial role: in fact, a peptide substrate reproducing this sequence was also phosphorylated by CK2 kinase on Tyr 184 [10].

Low-molecular-weight PTPs (LMW-PTPs) are ubiquitously present in mammalian tissues (for a review see [11]). LMW-PTPs possess the active site motif (H/V)C(X)5R(S/T), and share the same catalytic mechanism of classical PTPs: the signature motif includes a cysteine, involved in the formation of a phosphocysteinyl intermediate, and an arginine, responsible for binding the phosphoryl group of the substrate and for the stabilization of the transition state [12]. The three-dimensional structure and catalytic mechanism of the LMW-PTP has been clarified [13, 14]. Recently some insights about its physiological role have been achieved: in mammalians, LMW-PTP is involved in the negative regulation of cell proliferation induced by platelet-derived growth factor (PDGF). LMW-PTP interacts with and dephosphorylates the PDGF receptor [15]. Moreover, it is involved in cell adhesion modulation acting on p190 Rho-GAP [16]. It is very likely, however, that PDGF receptor is not the unique receptor substrate, since LMW-PTP is probably involved also in the modulation of the activated insulin receptor [17]. LMW-PTPs are present not only in mammals but also in phylogenetically distant organisms. Screening of an *S. pombe* cDNA expression library led to the identification of a gene encoding an LMW-PTP [18] named small tyrosine phosphatase (Stp1). Its overexpression is able to rescue the yeast temperature-sensitive mutations of cdc-25. This fact is probably due to the ability of Stp1 to dephosphorylate Tyr15 of cdc-2. On the other hand, disruption of Stp1 causes no visible phenotype. This phenomenon could be due to the fact that protein phosphatases in fission yeast are generally encoded by multigene families having redundant functions [19]. It is likely that LMW-PTPs are not an exception. The Stp1 protein shows 42% of identity with respect to the mammalian isoforms. Determination of the main kinetic constants of the recombinant Stp1 protein indicates that its enzymatic activity is very similar to that of mammalian LMW-PTP [20]. We have also obtained a Cys11 to Ser mutant that, similarly to the C12S mammalian LMW-PTP mutant [21], completely abolishes the enzymatic activity but can still bind the substrate, representing for this reason a dominant negative molecule. The purpose of this study was to investigate the possible role of Stp1 in protein tyrosine dephosphorylation in yeast. Recombinant *S. cerevisiae* strains were obtained expressing either Stp1 or its dominant negative molecule. Analysis of the phosphotyrosine protein profiles and of the silver staining patterns in the two different strains was performed with the use of high-resolution 2-D electrophoresis.

2 Materials and methods

2.1 Construction of PAAStp1 and PAAStp1C11S yeast expression plasmids

The fragments corresponding to Stp1 and to Stp1C11S mutant coding sequences were obtained from the pGEX-Stp1 and pGEX-Stp1C11S bacterial plasmids [20], and were separately cloned into pAAH5 yeast expression vector [22] in order to generate PAAStp1 and PAAStp1C11S recombinant plasmids. The identity of the constructs was verified by DNA sequencing.

2.2 Transformation and expression of recombinant Stp1 and Stp1C11S in *S. cerevisiae*

S. cerevisiae strain W303 (Ade^- Ura^- His^- Trp^- Leu^-) was separately transformed, by the lithium acetate procedure [23] with the PAAStp1 (PAStp1 strain) and with the PAAStp1C11S plasmids (strain PAStpC11S). PAAH5 strain, transformed with the pAAH5 empty plasmid was used as control strain. Expression of the active Stp1 was assayed for its activity on *p*-nitrophenylphosphate (pNPP) [24]. Western blot were performed as previously described [25] using rabbit polyclonal antibodies against Stp1.

2.3 Evaluation of plasmid stability

The three yeast strains PAStP1, PASTPC11S and PAAH5 were grown at 30°C in YDP complete medium (1% yeast extract, 2% Bacto peptone (Difco, Detroit, MI, USA), 2% glucose) up to the stationary phase. Plasmid stability was evaluated as % of conservation of the Leu^- phenotype after prolonged growth in nonselective medium: the same number of cells were plated on selective and nonselective media at different times during 80 h of continuous growth (about 25 generations) under nonselective conditions. The ratio of the number of colonies on the two media gave the percentage of cells still harboring the recombinant plasmids.

2.4 Evaluation of growth rate

The growth rate of the three recombinant strains was evaluated in 10 mL of a synthetic medium containing 0.67% Difco-Yeast Nitrogen Base with appropriate supplements (ADE, URA, L-His, L-Trp, each 50 mg/L; Sigma, St. Louis, MO, USA) and 2% w/v glucose, at 30°C, starting from 0.3 OD_{660}. Cultures were grown up to the stationary phase and at different times, 1 mL of culture was collected to estimate the cell density.

Table 1. PTPase activity of clear lysates from the two recombinant strains (PASTP1 and PASTPC11S) in comparison to the control one (PAAH5)

Recombinant strains	U/mL[a)]	mg/mL[b)]	U/mg
PASTP1	1.5	1.01	1.48
PASTPC11S	0.25	0.85	0.29
PAAH5	0.18	0.78	0.23

a) On pNPP as substrate

2.5 High-resolution 2-D electrophoresis, immunoblotting, and preliminary spot assignment

2-D electrophoresis was performed using the Immobiline-polyacrylamide system as described [26]. The IEF was carried out on a commercial sigmoidal IPG, from pH 3 to 10 (18 cm long IPG strips; Amersham Pharmacia-Biotech, Uppsala, Sweden) [27]. The second dimension was carried out on 9–16% polyacrylamide linear gradient gels (18 cm × 20 cm × 1.5 mm). Gels were stained with ammoniacal silver nitrate as described [28]. For each silver nitrate gel, 45 μg of total yeast proteins were applied. The 2-D polyacrylamide images were obtained with a computing densitometer (Molecular Dynamics 300S, 4000 × 5000 pixels, 12 bits/pixel; Sunnyvale, CA, USA) and processed with the Melanie II computer system (Bio-Rad, Hercules, CA, USA) on a SUN SPARC station; gel calibration was carried out on the basis of internal standards. For figure presentation, images were transferred to a Macintosh computer and completed with arrows and numbers using the Adobe PhotoShop 2.5 and Persuasion 2.1 programs. After 2-D electrophoresis, the polyacrylamide gels were electroblotted onto nitrocellulose membranes [29]. Anti-phosphotyrosine antibodies (PY20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:2500 in blocking solution and incubated for 2 h as previously described [25]. After three washes in phosphate-buffered saline solution with 0.5% Tween-20, the blots were developed using a chemiluminescence detection system (Amersham-Pharmacia-Biotech). The electrophoretic coordinates of spots stained with anti-phosphotyrosine antibodies were determined using, as “anchors”, spots stained with Ponceau S on the nitrocellulose replica [30, 31]. Subsequently, the pattern of immunoreactive spots in the replica was matched to the pattern of spots in the silver-stained gel. In this way, a silver-stained pattern annotated with anti-phosphotyrosine reactive spots was obtained and used to perform computer matching with on-line reference maps for spot assignment. The immunoreactive spot volume was determined by densitometry of X-ray film values (Melanie 2 computer system). Volume ratios of the spots observed expressing Stp1 or Stp1C11S with those observed expressing the wild-type proteins were obtained using differential analysis tool of Melanie II. Matching our images with the Yeast Swiss 2D-PAGE reference map using the Melanie II system was carried out for protein identification. More precisely, electropherograms from wild-type (PAAH5) strain were employed in matching in order to avoid possible interference from altered phosphorylation occurring in our mutant strains. Protein sequences were aligned using the Insight II package (Molecular Stimulation Inc. Cans, Urbana, IL, USA) with the protein homology tools.

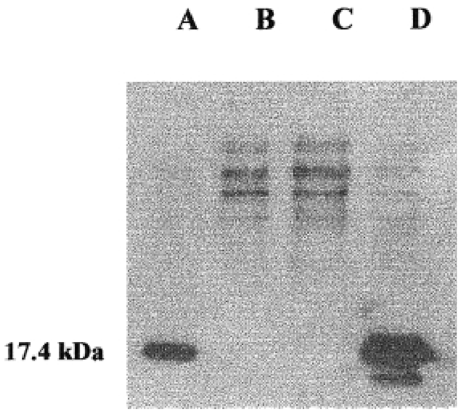

Figure 1. Western blot analysis of an extract of (A) PASTPC11S strain and (D) PASTP1 strain. (B), (C) extracts from the control strain PAAH5. Immunodetection using antiserum to the Stp1 was performed as described in Section 2.5.

3 Results

3.1 Cloning and expression of stp1 and stp1C11S genes

In order to obtain two yeast strains expressing the Stp1 or the C11S mutated Stp1 protein, respectively, we separately cloned the corresponding coding sequences into the yeast expression vector pAAH5 that allows constitutive expression. The two recombinant plasmids, PAAStp1 and PAAStp1C11S, were used to transform the W303 yeast strain. Clear lysates of the recombinant strains were assayed for their enzymatic activity using pNPP as substrate. The results are shown in Table 1. The recombinant strain overexpressing Stp1 showed a 5-fold enzymatic activity increase in comparison with the control, indicating the overexpression of the Stp1 protein. Western blot analysis using polyclonal antibodies against Stp1 (Fig. 1) revealed a sharp band corresponding to the normal and the dominant negative protein, in the PASTP1 and PASTPC11S strains, respectively. In Table 1, clear lysates obtained

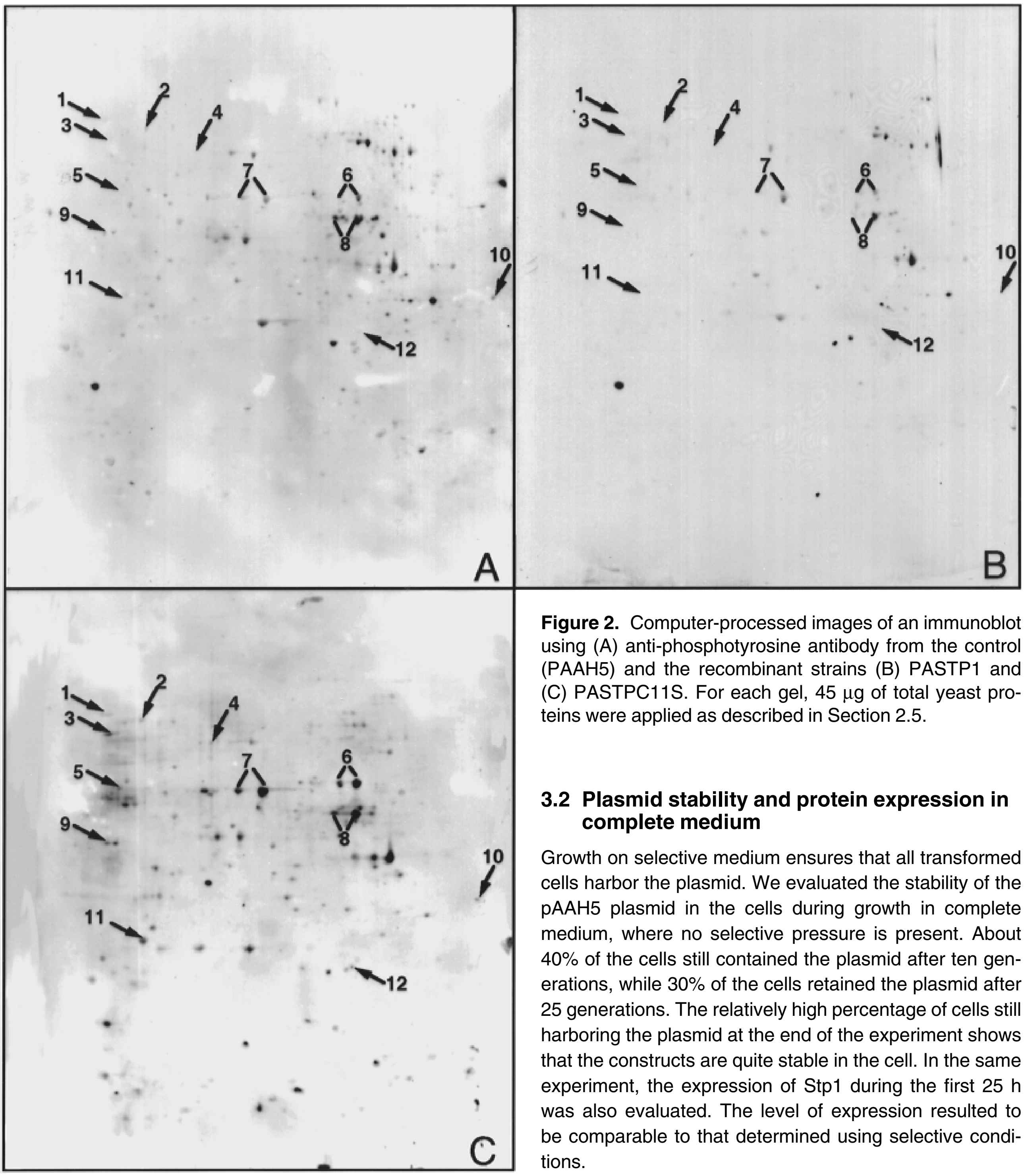

Figure 2. Computer-processed images of an immunoblot using (A) anti-phosphotyrosine antibody from the control (PAAH5) and the recombinant strains (B) PASTP1 and (C) PASTPC11S. For each gel, 45 µg of total yeast proteins were applied as described in Section 2.5.

from a strain overexpressing the dominant negative molecule show the same specific activity with respect to the control strain. This is obviously due to the fact that this *in vitro* assay on pNPP is performed with an excess of substrate.

3.2 Plasmid stability and protein expression in complete medium

Growth on selective medium ensures that all transformed cells harbor the plasmid. We evaluated the stability of the pAAH5 plasmid in the cells during growth in complete medium, where no selective pressure is present. About 40% of the cells still contained the plasmid after ten generations, while 30% of the cells retained the plasmid after 25 generations. The relatively high percentage of cells still harboring the plasmid at the end of the experiment shows that the constructs are quite stable in the cell. In the same experiment, the expression of Stp1 during the first 25 h was also evaluated. The level of expression resulted to be comparable to that determined using selective conditions.

3.3 Evaluation of the growth rate

In order to obtain a first indication on the physiological role of the Stp1 in *S. cerevisiae*, we explored the possible effects on the yeast growth rate due to the expression of either Stp1 or its dominant negative mutant Stp1C11S

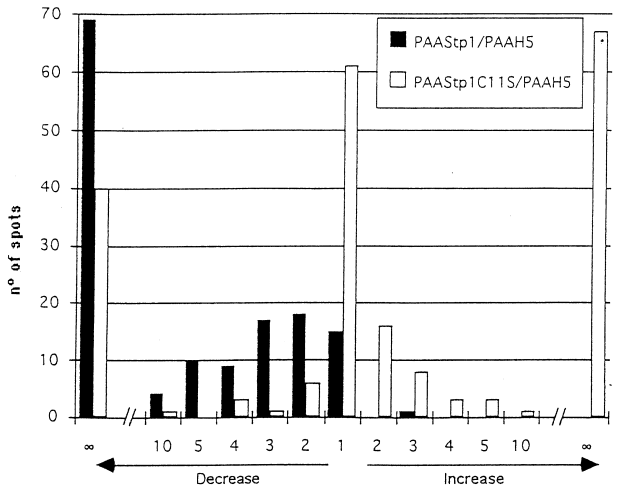

Figure 3. Summary of immunoreactive spot intensity data related to the images of Fig. 2. The *y*-axis indicates the number of spots; the *x*-axis indicates variation classes, induced by expression of Stp1 (PASTP1, black bars) or of its dominant negative mutant (PASTPC11S, white bars). Classes are defined by the several fold increase or decrease of single spots determined with the differential analysis tool of Melanie II. Quantitative variations (presence/absence of the spots) are grouped in class ∞.

protein. The two strains, PASTP1, PASTPC11S, together with the control PAAH5 strain, were grown in complete medium as indicated in Section 2.3. The growth rate of the three strains shows no significant difference, thus demonstrating that STP1, at least under these growth conditions, has no influence on this parameter.

3.4 Influence of Stp1 on tyrosine phosphorylation

Tyrosine-phosphorylated proteins in wild-type and recombinant strains have been monitored by 2-D electrophoresis and immunoblotting (Fig. 2A–C), using anti-phosphotyrosine antibodies. Previous works from our laboratory indicate that anti-phosphotyrosine antibodies (PY20) are able to reveal phosphorylation on tyrosine in a specific way, as shown by Western blot analysis of activated PDGF and insulin receptors [32, 33]. The wild-type immunoreactivity pattern shows about 140 positive spots, as determined with the Melanie II software. Upon simple inspection, it is already evident that the number and the intensity of immunoreactive spots in PASTP1 is lower than in the PAAH5 control strain pattern. On the contrary, expression of PASTPC11S shows opposite results with respect to the control strain. The number of positive spots present in PASTP1 and PASTPC11S is 74 and 170, respectively.

The intensity ratio between every immunoreactive spot of the PASTP1 or PASTPC11S strain and the corresponding ones of the control strain has been determined. The quantitative results of this analysis are reported in Fig. 3. The single spots were grouped with respect to the magnitude of their severalfold increase or decrease in immunoreactivity, considering their intensity in the PASTP1 (black bars) or PASTPC11S (white bars) strain with respect to the intensity in the PAAH5 control strain. The histogram indicates that expression of either normal Stp1 or C11S dominant negative mutant has marked effects on the overall tyrosine phosphorylation state of yeast proteins. Overexpression of Stp1 reduces considerably the intensity of 58 spots and renders as many as 69 spots undetectable (black bars). Moreover, in this strain only 15 proteins appear to be unaffected by Stp1 overexpression and only one shows an increase. On the other hand, the expression of the dominant negative mutant (empty bars) causes an intensity increase in tyrosine phosphorylation reactivity of 31 spots relatively to the wild-type strain and the appearance of 67 spots, which were undetectable in the wild-type strain and that become immunoreactive in PASTPC11S. In the same strain, the expression of Stp1C11S has no effect on as many as 61 spots. Several spots show also a decrease in intensity but their total number (11) is lower than that of the spots showing an increase. Forty of those spots, which are immunoreactive in the control strain, become undetectable in the PAStpC11S strain. In order to achieve a preliminary assignment of spots, experimental electrophoretic patterns, including silver-stained and antibody-stained results (see Section 2.5), were matched to the SWISS-2DPAGE *S. cerevisiae* reference map. In this reference map, among 1940 distinct spots, 101 correspond to known proteins.

Among the PY20 anti-P-Tyr-antibody reactive spots showing variations in the amount of their phosphorylation in response to Stp1 or Stp1C11S expression, 18 were assigned. Twelve of them (Table 2) clearly show opposite behavior depending of the Stp1 or Stp1C11S expression. Their tyrosine phosphorylation level decreases upon active enzyme expression (PASTP1 strain, Fig. 2B), or increases in the strain expressing the dominant negative protein (PASTPC11S strain, Fig. 2C). For this reason, they could be good candidates as Stp1 substrates. Among them, there are enzymes involved in glucose or amino acid metabolism, protein synthesis initiation factor, heat shock proteins, a membrane ATP synthase, and a 45.8 kDa component whose function is not yet known.

3.5 Influence of Stp1 on silver-stained 2-D polyacrylamide gels

Protein expression patterns are also influenced by the overexpression of Stp1 and of its dominant negative

Table 2. Phosphotyrosine proteins identified by matching with *S. cerevisiae* reference map present on SWISS-2DPAGE

SWISS-PROT AC	Spot No.	Protein	Stp1C11S	Stp1
P10591	1	Heat shock protein SSA1	+	– –
P11484	2	Heat shock protein SSB1	+	– –
P19882	3	HSP 60 precursor	+ +	– –
P06169	4	Pyruvate carboxy-lase isoz.1	+	–
P10081	5	EIF4A	+	– –
P00924	6	Enolase 1	+	–
P00925	7	Enolase 2	+	–
P00330	8	Alcohol dehydrogenase I	+	–
P38344	9	45.8 kDa in SHM1-MRPL37 int.reg.	+	– –
P00950	10	Phosphoglycerate mutase1	+	– –
P22203	11	Vacuolar ATP synthase (sub. E)	+	–
P00358	12	Glyceraldehyde 3P dehydr. 2	+ +	– –

C11S mutant. Figure 4 shows the computer-processed images of silver-stained proteins in control (PAAH5, Fig. 4A) and recombinant strains (PASTP1, Fig. 4B; PASTPC11S, Fig. 4C). The total number of spots per gel, obtained from three electrophoretic runs for each strain, was about 1400. Variations in spot intensity at defined coordinates were observed when the 2-D polyacrylamide gels from recombinant strains were matched with the control gel. In many positions, the appearance or disappearance of specific spots was also observed. Figure 5 demonstrates that expression of the Stp1 (PASTP1 strain, black bars) markedly affects the yeast global protein profile. About 850 spots are not remarkably altered whereas qualitative variations (appearance/disappearance) involve about 450 spots. On the other hand, expression of the dominant negative mutant has a more limited effect, leaving as many as 1180 spots unaffected and showing qualitative variations for 310 spots.

4 Discussion

In order to investigate the possible role of Stp1 on protein tyrosine dephosphorylation in yeast, we expressed the active enzyme and its dominant negative mutant in *S. cerevisiae*. Expression of both protein species does not have any detectable influence on yeast growth rate in complete medium (2% carbon source). It is also possible that the tyrosine phosphatase activity in Stp1 could be redundant in yeast, as already suggested by Mondesert *et al.* [18] on the basis of gene disruption experiments. The effects on the yeast proteome of this genetic manipulation were monitored by 2-D electrophoretic protein separation. Spot assignment was preliminarily carried out with a procedure widely used in proteome research, namely by matching experimental electropherograms with a reference, annotated electrophoretic images accessible on the Internet (*cf.* Section 2.5). Electrophoretic spots were detected by general staining with silver salts or by selective staining with anti-phosphotyrosine antibodies. The latter procedure is a convenient, reliable method to study protein Tyr-phosphorylation. As has been demonstrated by recent mass spectrometric investigations, anti-phosphotyrosine immunoreactive spots correspond to proteins known to be phosphorylated on tyrosine [34].

In this way, we could identify twelve proteins as tyrosine-phosphorylated, corresponding to spots numbers 1–12 in Figs. 2 and 4. Some of these proteins (enolase, alcohol dehydrogenase, phosphoglycerate mutase, 3GAPDH) are known to be tyrosine-phosphorylated in several organisms [35–38], this phosphorylation being very important in metabolic regulation (see below). In addition, an analysis of their sequences in yeast indicated the occurrence of tyrosine phosphorylation sites recognized by CK-II (Table 3) in ten of them (heat shock protein SSB1, heat shock protein 60, pyruvate decarboxylase, eukaryotic initiation factor 4A, enolase1, enolase2, alcohol dehydrogenase I, 34.7 kDa in SHM1-MRPL37 intergenic region, vacuolar ATP synthase subunit E, 3GAPDH). In heat shock protein SSA1 and phosphoglycerate mutase, potential tyrosine phosphorylation sites have been identified by NetPhos expert system (http://www.cbs.dtu.dk/services/NetPhos/). On this basis, we can envisage that proteins identified by matching in silver-stained gels are also reactive with the anti-phosphotyrosine antibody and that the variations in their phosphorylation in the wild-type and recombinant yeast strains are reflected in the Western blots. Although sequencing of the tyrosine phosphorylation sites is mandatory for a conclusive demonstration, we propose that tyrosine phosphorylation widely occurs in some housekeeping yeast proteins and that some of these proteins are substrates for Stp1. As a further technical comment, we employed a 3–10 pH gradient in order to achieve the widest possible electrophoretic window. This choice prevented us from focusing on tyrosine-phosphorylation-induced pH shifts.

The 2-D silver-staining profile is modified by expression of either Stp1 or its dominant negative mutant. As expected, the electrophoretic pattern obtained is very complex, but, nevertheless, it indicates that Stp1 deeply modifies the

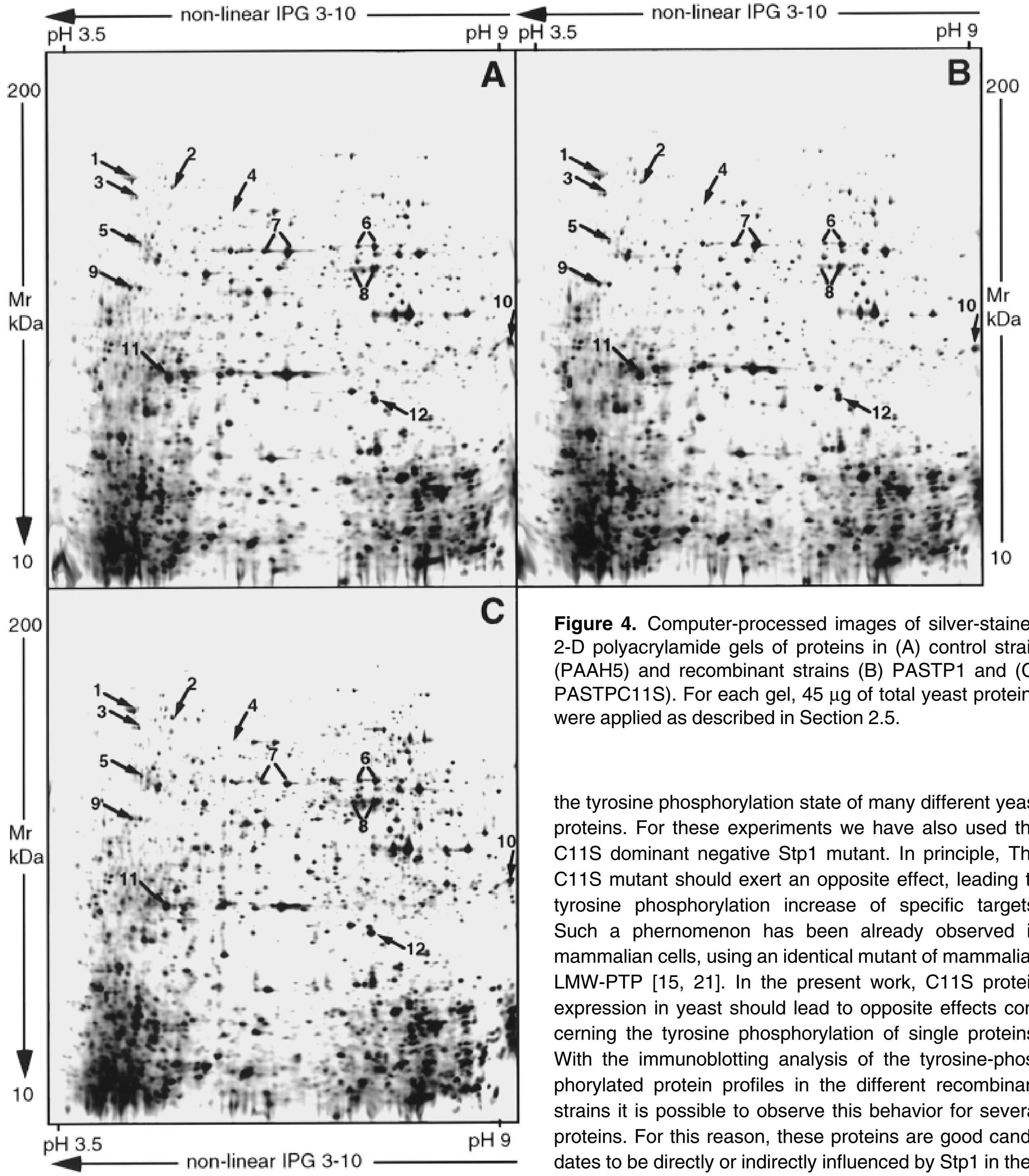

Figure 4. Computer-processed images of silver-stained 2-D polyacrylamide gels of proteins in (A) control strain (PAAH5) and recombinant strains (B) PASTP1 and (C) PASTPC11S). For each gel, 45 μg of total yeast proteins were applied as described in Section 2.5.

level of expression of many proteins: four of them (three heat shock proteins and an eukaryotic initiation factor) are involved in protein translation and maturation. This fact could indicate an influence of this small tyrosine phosphatase on global protein biosynthesis. Moreover, our experiments show that Stp1 has a strong influence on the tyrosine phosphorylation state of many different yeast proteins. For these experiments we have also used the C11S dominant negative Stp1 mutant. In principle, The C11S mutant should exert an opposite effect, leading to tyrosine phosphorylation increase of specific targets. Such a phernomenon has been already observed in mammalian cells, using an identical mutant of mammalian LMW-PTP [15, 21]. In the present work, C11S protein expression in yeast should lead to opposite effects concerning the tyrosine phosphorylation of single proteins. With the immunoblotting analysis of the tyrosine-phosphorylated protein profiles in the different recombinant strains it is possible to observe this behavior for several proteins. For this reason, these proteins are good candidates to be directly or indirectly influenced by Stp1 in their phosphorylated state. Among them, twelve proteins have been identified by matching their position to reference yeast protein 2-D gel in a database accessible *via* Internet. Many of these proteins are known to be regulated, in mammals, by tyrosine phosphorylation such as some glycolytic enzymes (3GAPDH, phosphoglycerate mutase, enolase) [35–37]. These observations suggest that the modulation of the glycolytic flux could be controlled by phosphorylation and dephosphorylation on tyrosine residues also in yeast.

Glucose homeostasis is one of the biological effects of insulin in mammalian cells. Recently, we demonstrated that LMW-PTP (the Stp1 mammalian enzyme) associates and dephosphorylates the insulin receptor, leading to a modulation of the signal starting at the hormone-activated receptor, and in this way negatively influencing both the mitogenic and metabolic effects induced by insulin. On the other hand, in a recent study Muller *et al.* [39], describes a metabolic effect of insulin on carbohydrate metabolism in *S. cerevisiae* mediated by tyrosine phosphorylation. Moreover, a lower eukaryote, *Neurospora crassa*, produces an insulin-like molecule, detectable by radioimmunoassay, and able to induce an insulin-like effect in mammalian adipocites. These findings suggest that mechanisms of hormonal control of glucose metabolism (involving tyrosine phosphorylations) could exist also in yeast. All these data stimulate the investigation about the role of Stp1 in carbohydrate metabolism pathways in yeast. Stp1 could be active on substrates that become tyrosine-phosphorylated in response to hormone treatment: similarly to the findings already demonstrated for LMW-PTP, Stp1 could have a negative role in modulating the metabolic effects of hormone stimulation.

Table 3. Potential Tyr phosphoacceptor sites for CK2 in yeast Tyr phosphorylated proteins displaying opposite behaviors upon Stp1 *vs.* Stp1C11S expression

Protein	Residue	Sequence
Heat shock protein SSA1	–	–
Heat shock protein SSB1	Tyr23	CVATYESSVE
HSP 60 precursor	Tyr495	LIDEYGDDFA
Pyruvate carboxylase isoz.1	Tyr37	LLK**IYE**VEGM
EIFA4	Tyr254	LTD**LYD**SISV
Enolase 1	Tyr11	ARS**VYD**SRGN
Enolase 2	Tyr11	ARS**VYD**SRGN
Alcohol dehydrogenase I	Tyr12	GVI**FYE**ESHGH
	Tyr257	ISE**FYD**FTSS
45.8 kDa in SHM1-MRPL37 int.reg.	Tyr244	CKI**PYE**SEDE
	Tyr257	ISE**FYD**FTSS
Phosphoglycerate mutase1	–	–
Vacuolar ATP synthase (sub. E)	Tyr43	ADQEYETEKT
Glyceraldehyde 3P dehydr. 2	Tyr311	LVS**WYD**NEYG

The proteins are the same as in Table 2. The tyrosine and acidic and/or phosphorylatable residues that could act as specificity determinants are underlined. Whenever present, the optimal motif hydrophobic-Tyr-acidic (see text) is bold typed.

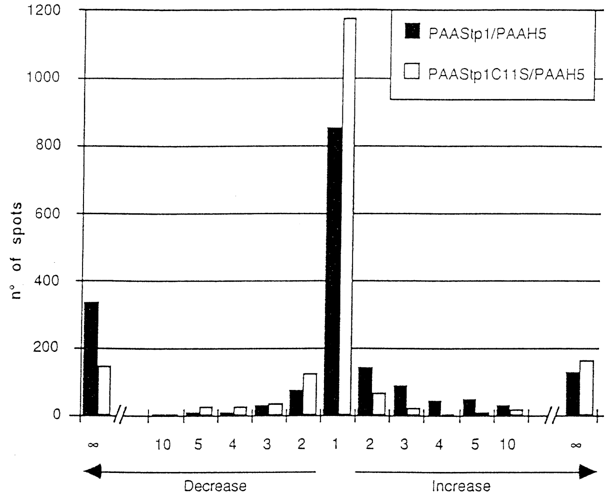

Figure 5. Summary of silver-stained spot intensity data related to Fig. 4. Description as for Fig. 3.

The data presented also shed new light on the controversial issue of tyrosine phosphorylation in yeast as well as in other organisms where *bona fide* protein tyrosine kinases are absent [40]. Here, we show that indeed a striking multitude of tyrosine-phosphorylated proteins are detectable in *S. cerevisiae*, whose phosphorylation level is in several cases critically dependent on the activity of yeast LMW-PTP. This finding, together with the known toxic effect of yeast transformation with *bona fide* tyrosine kinases [41], is strongly consistent with a physiological role of tyrosine phosphorylation in yeast. This in turn raises the question about the nature of protein kinase(s) involved in this process. Given the number and the variety of the tyrosine-phosphorylated proteins identified in this work, their targeting by the dedicated dual specificity kinases present in yeast (Swe1 and MAPKKs), responsible for the phosphorylation of T×Y motifs in Cdc28 and MAP kinases, respectively [40], is hardly conceivable. The implication of pleiotropic Ser/Thr protein kinases present in yeast, a few of which have been shown to display significant tyrosine phosphorylating activity, at least *in vitro* [42], seems more likely. In this respect, a first choice candidate would be protein kinase CK2 (formerly misnamed "casein kinase-2"), a ubiquitous and constitutively active enzyme endowed with amazing pleiotropicity [43–45]. In *S. cerevisiae*, CK2 is essential for viability and is suggested to be implicated in a variety of cell functions [46]. Targeting of canonical Ser/Thr sites by CK2 is specified by multiple acidic residues, mainly located downstream from the phosphorylatable amino acid. Surprisingly, a tyrosyl residue which is phosphorylated in yeast immunophilin Fpr3 (Tyr184) also displays these features and it has been shown to be actually phosphorylated by CK2 both *in vivo* and *in vitro* [8, 9]. By using a series of peptides derived from this site, the demonstration was provided that targeting of tyrosyl residues by CK2 obeys rules that are partially similar but in some respects distinct from those determining the phosphorylation of seryl residues [10]. In particular, the "hydrophobic-Tyr-acidic" motif appears to be even more important than an acidic residue at position *n*+3, which is the crucial determinant of serine phoshorylation. Based on this information, we have analyzed the tyrosine-phosphorylated proteins fished out by comparative 2-D analysis (see Table 2) for their content of tyrosyl residues, which are potential targets for CK2. As shown in Table 3, in 10 out of the 12 identified phosphoproteins one or more such sites are present. Moreover, in 9 cases out of 12, these sites display the "optimal hydrophobic-Tyr-acidic" motif. In contrast, the same motif was found just in two (P04806 and P05694) out of 12 proteins (the two aforementioned proteins plus P00942, P02579, P07283, P11412, P14540, P23301, P32288, P32835, and P35176) randomly analyzed among the proteins identified in the reference map used in this study. These observations corroborate the view that CK2 is exploited in yeast as an ancillary tyrosine kinase committed with the phosphorylation of numerous proteins, besides immunophilin Fpr3. They also disclose the possibility that CK2 and Stp1 play a concerted role in tuning the phosphorylation level of a pool of tyrosyl residues whose targeting is specified by multiple acidic determinants.

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5 References

[1] Camici, G., Manao, G., Cappugi, G., Modesti, A., Stefani, M., Ramponi, G., *J. Biol. Chem.* 1989, *264*, 2560–2567.

[2] Doi, D., Gartner, A., Ammerer, G., Errede, B., Shinkawa, H., Sugimoto, D., Matsumoto, D., *EMBO J.* 1994, *13*, 61–70.

[3] Maeda, T., Wurgler-Murphy, S. M., Saito, H., *Nature* 1994, *369*, 242–245.

[4] Russell, P., Moreno, S., Reed, S. I., *Cell* 1989, *57*, 295–303.

[5] Guan, K., Deschenes, R. J., Qiu, H., Dixon, J. E., *J. Biol. Chem.* 1991, *266*, 12964–12970.

[6] Maeda, T., Tsai, A. Y. M., Saito, H., *Mol. Cell Biol.* 1993, *13*, 5408–5417.

[7] Hannig, G., Ottilie, S., Schievella, A. R., Erikson, R. L., *Yeast* 1993, *9*, 1039–1052.

[8] Wilson, L. K., Benton, B. M., Zhou, S., Thorner, J., Martin, G. S., *J. Biol. Chem.* 1995, *270*, 25185–25193.

[9] Wilson, L. K., Dillon, N., Thorner, J., Martin, G. S., *J. Biol. Chem.* 1997, *272*, 12961–12967.

[10] Marin, O., Meggio, F., Sarno, S., Cesaro, L., Pagano, M. A., Pinna, L. A., *J. Biol. Chem.* 1999, *274*, 29260–29265.

[11] Ramponi, G., Stefani, M., *Biochem. Biophys. Res. Commun.* 1997, *1341*, 137–156.

[12] Cirri, P., Chiarugi, P., Camici, G., Manao, G., Raugei, G., Cappugi, G., Ramponi, G., *Eur. J. Biochem.* 1993, *214*, 647–657.

[13] Su, X. D., Taddei, N., Stefani, M., Ramponi, G., Nordlund, P., *Nature* 1994, *370*, 575–578.

[14] Zhang, M., Van Etten, R. L., Stauffacher, C. V., *Biochemistry* 1994, *33*, 11097–11105.

[15] Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Fiaschi, T., Camici, G., Manao, G., Romanelli, R. G., Ramponi, G., *J. Biol. Chem.* 1998, *273*, 6776–6785.

[16] Chiarugi, P., Cirri, P., Taddei, L., Giannoni, E., Camici, G., Manao, G., Raugei, G., Ramponi, G., *J. Biol. Chem.* 2000, in press.

[17] Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Camici, G., Manao, G., Ramponi, G., *Biochem. Biophys. Res. Commun.* 1997, *238*, 676–682.

[18] Mondesert, O., Moreno, S., Russell, P., *J. Biol. Chem.* 1994, *269*, 27996–27999.

[19] Shiozaki, K., Russell, P., *Cell Mol. Biol. Res.* 1994, *40*, 241–243.

[20] Modesti, A., Cirri, P., Raugei, G., Carraresi, L., Magherini, F., Manao, G., Camici, G., Ramponi, G., *FEBS Lett.* 1995, *375*, 235–238.

[21] Chiarugi, P., Marzocchini, R., Raugei, G., Pazzagli, C., Berti, A., Camici, G., Manao, G., Cappugi, G., Ramponi, G., *FEBS Lett.* 1992, *310*, 9–12.

[22] Ammerer, G., *Methods Enzymol.* 1993, *101*, 192–201.

[23] Ito, H., Fukuda, Y., Murata, K., Kimura, A., *J. Bacteriol.* 1983, *153*, 163–168.

[24] Heinrikson, R. L., *J. Biol. Chem.* 1969, *244*, 299–307.

[25] Chiarugi, P., Cirri, P., Raugei, G., Manao, G., Taddei, L., Ramponi, G., *Biochem. Biophys. Res. Commun.* 1996, *219*, 21–25.

[26] Bjellqvist, B., Hughes, G. J., Pasquali, C., Paquet, N., Ravier, F., Sanchez, J. C., Frutiger, S., Hochstrasser, D. F., *Electrophoresis* 1993, *14*, 1357–1365.

[27] Righetti, P. G., Tonani, C., *Electrophoresis* 1991, *12*, 1021–1027.

[28] Oakley, B. R., Kirsch, D. R., Morris, R., *Anal. Biochem.* 1981, *105*, 361–363.

[29] Towbin, H., Staehelin, T., Gordon, J., *Biotechnology* 1992, *24*, 145–149.

[30] Magi, B., Bini, L., Marzocchi, B., Liberatori, S., Raggiaschi, R., Pallini, V., *Methods Mol. Biol.* 1999, *112*, 431–443.

[31] Bini, L., Liberatori, S., Magi, B., Marzocchi, B., Raggiaschi, R., Pallini, V., in: Rabilloud, T. (Ed.), *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods*, Springer, Berlin, Heidelberg, New York 2000, pp. 127–141.

[32] Cirri, P., Chiarugi, P., Taddei, L., Raugei, G., Camici, G., Manao, G., Ramponi, G., *J. Biol. Chem.* 1998, *273*, 32522–32527.

[33] Taddei, M. L., Chiarugi, P., Cirri, P., Talini, D., Camici, G., Manao, G., Raugei, G., Ramponi, G., *Biochem. Biophys. Res. Commun.* 2000, *270*, 564–569.

[34] Soskic, V., Gorlach, M., Poznanovic, S., Boehmer, F. D., Godovac-Zimmermann, J., *Biochemistry* 1999, *38*, 1757–1764.

[35] Sergienko, E. A., Kharitonenkov, A. I., Bulargina, T. V., Muronetz, V. V., Nagradova, N. K., *FEBS Lett.* 1992, *304*, 21–23.

[36] Sale, E. M., White, M. F., Kahan, C. R., *J. Cell Biochem.* 1987, *33*, 15–26.

[37] Reiss, N., Kanety, H., Schlessinger, J., *Biochem. J.* 1986, *239*, 691–700.

[38] Reuter, C., Findik, D., Presek, P., *Eur. J. Biochem.* 1990, *190*, 343–350.

[39] Muller, G., Rouveyre, N., Crecelius, A., Bandlow, W., *Biochemistry* 1998, *37*, 8683–8695.

[40] Hunter, T., Plowman, G. D., *Trends Biochem. Sci.* 1997, *22*, 18–22.

[41] Superti-Furga, G., Fumagalli, S., Koegl, M., Courtneidge, S. A., Draetta, G., *EMBO J.* 1993, *12*, 2625–2634.

[42] Pinna, L. A., Ruzzene, M., *Biochim. Biophys. Acta* 1996, *1314*, 191–225.

[43] Pinna, L. A., *Biochim. Biophys. Acta* 1990, *1054*, 267–284.

[44] Pinna, L. A., Meggio, F., *Progr. Cell Cycle Res.* 1997, *3*, 77–97.

[45] Guerra, B., Issinger, O. G., *Electrophoresis* 1999, *20*, 391–408.

[46] Glover III, C. V. C., *Progr. Nucleic Acids Res.* 1998, *59*, 95–133.