J. Microbiol. Biotechnol. (2014), 24(2), 152–159
http://dx.doi.org/10.4014/jmb.1308.08019

jmb

Co-Expression of Protein Tyrosine Kinases EGFR-2 and PDGFRβ with Protein Tyrosine Phosphatase 1B in *Pichia pastoris*

Pham Ngoc Tu, Yamin Wang, Menghao Cai, Xiangshan Zhou*, and Yuanxing Zhang*

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, P. R. China

Received: August 9, 2013
Revised: October 28, 2013
Accepted: November 14, 2013

First published online
November 19, 2013

**Corresponding authors*
X.Z.
Phone: +86-21-64253025;
Fax: +86-21-64253025;
E-mail: xszhou@ecust.edu.cn
Y.Z.
Phone: +86-21-64253025;
Fax: +86-21-64253025;
E-mail: yxzhang@ecust.edu.cn

Supplementary data for this paper are available on-line only at http://jmb.or.kr.

pISSN 1017-7825, eISSN 1738-8872

The regulation of protein tyrosine phosphorylation is mediated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) and is essential for cellular homeostasis. Co-expression of PTKs with PTPs in *Pichia pastoris* was used to facilitate the expression of active PTKs by neutralizing their apparent toxicity to cells. In this study, the gene encoding phosphatase PTP1B with or without a blue fluorescent protein or peroxisomal targeting signal 1 was cloned into the expression vector pAG32 to produce four vectors. These vectors were subsequently transformed into *P. pastoris* GS115. The tyrosine kinases EGFR-2 and PDGFRβ were expressed from vector pPIC3.5K and were fused with a His-tag and green fluorescent protein at the N-terminus. The two plasmids were transformed into *P. pastoris* with or without PTP1B, resulting in 10 strains. The EGFR-2 and PDGFRβ fusion proteins were purified by Ni^{2+} affinity chromatography. In the recombinant *P. pastoris*, the PTKs co-expressed with PTP1B exhibited higher kinase catalytic activity than did those expressing the PTKs alone. The highest activities were achieved by targeting the PTKs and PTP1B into peroxisomes. Therefore, the EGFR-2 and PDGFRβ fusion proteins expressed in *P. pastoris* may be attractive drug screening targets for anticancer therapeutics.

Keywords: Protein tyrosine kinase, protein tyrosine phosphatase, peroxisome, *Pichia pastoris*

Introduction

Cancer is a serious disease, and drug therapy is currently the most efficient strategy for curing cancer. However, the low complete remission rate, and recurrence and metastasis have limited the advancement of this method [1,11], and it is necessary to develop new anticancer drugs for therapy. In cell signaling pathways, key protein kinases, particularly protein tyrosine kinases (PTKs), play an important role in cancer cell proliferation. The PTK family is classified into receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases according to structural features [8]. Signal transduction is activated by receptor autophosphorylation on tyrosine residues when PTK receptors bind their specific ligands [3]. The receptors with PTK activity are important in the control of cancer cell proliferation. Platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and kinase insert domain receptor (KDR) belong to the RTK family. Two forms of PDGFR, α and β, are cell surface tyrosine kinase receptors for platelet-derived growth factor and are closely related to vascular proliferation and cancer. Overexpression or excessive activation of PDGFRβ can stimulate tumor angiogenesis and growth. Therefore, blocking or inhibiting the overexpression of tyrosine kinase activity is a current trend in tumor therapy [2,4]. EGFR is a type of glycoprotein receptor on the cell membrane and the product of the HER-1 proto-oncogenes [6, 13]. Two strategies have been proposed to cure cancer; developing a monoclonal antibody against the extracellular domain of EGFR [9, 20], and identifying small molecule inhibitors that specifically inhibit the active domain of the kinase to disrupt autophosphorylation and signal transduction [22].

Protein tyrosine phosphorylation is important in cell signaling and is involved in many physiological activities, such as tissue differentiation and growth, sugar and fat metabolism, and the immune response [15, 17]. Protein tyrosine phosphatase 1B (PTP1B), also known as PTPN1, is

the first purified mammalian PTP and is considered as a reference prototype for following PTP studies [10]. Protein tyrosine phosphorylation is precisely regulated by the balance of PTKs and PTPs [14].

The preparation of active recombinant protein tyrosine kinases is useful for a target protein and model molecule during drug screening programs to identify anticancer therapeutics. PTK expression in recombinant *P. pastoris* is difficult and most likely occurs because the PTKs phosphorylate intracellular proteins, which disturbs cellular homeostasis and results in toxicity to the host cells [18]. In this study, the co-expression of PTKs with PTPs in *P. pastoris* was used to facilitate the expression of active PTKs by neutralizing their apparent toxicity to cells.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in Luria-Bertani medium (LB; Oxoid, Basingstoke, UK). *P. pastoris* was grown at 30°C in four different media [21]: YPD medium (1% yeast extract, 2% glucose, and 2% peptone), synthetic yeast nitrogen base (YNB) medium, MGY medium (1.34% YNB, 1% glycerol, and 0.4 mg/ml biotin), and minimal methanol (MM) medium (1.34% YNB, 0.4 mg/ml biotin, and 0.5% methanol). Antibiotics or other reagents purchased from Sigma (St. Louis, MO, USA) were selectively added (750 μg/ml hygromycin, 50 μg/ml ampicillin, 50 μg/ml kanamycin, or 300 μg/ml G418).

Plasmid and Strain Construction

General recombinant DNA techniques were performed according to standard protocols [7]. The plasmids were constructed and transformed into *P. pastoris* using electroporation [19]. All DNA sequencing and oligo syntheses (Table S1) were performed by Invitrogen Co. (Shanghai, China).

Construction of PTP1B expression strains. PTP1B and PTP1B-SKL (PTP1B-peroxisomal targeting signal 1) sequences were amplified from the plasmid pCMV6-XL5 using primers P1 and P2-1 or P2-2, respectively (Supplemental Table S1). The products were cloned into the *Bam*HI/*EcoR*I sites of pPIC3.5K to construct plasmids pPIC3.5-PTP1B and pPIC3.5-PTP1B-SKL. Primers A1 and A2 were used to amplify the resultant plasmids and produce *AOX1*-PTP1B-AOX (TT) and *AOX1*-PTP1B-SKL-AOX (TT) (Table S1). The two derivatives were ligated with the *Stu*I/*Sal*I-digested pAG32S vector to produce plasmids pAG32S-PTP1B and pAG32S-PTP1B-SKL, which were then confirmed by PCR with primers A1/A2 (Table S1) and DNA sequencing. The positive plasmids were linearized by *Bpu*1102I (*Esp*I) digestion and electroporated into *P. pastoris* GS115 to produce strains 2Y and 1Y. Using the templates pAG32S-PTP1B and pAG32S-PTP1B-SKL, PCR was performed to generate the -*Sph*I-PTP1B-pAG32S-*Kpn*I- and -*Sph*I-PTP1B-SKL-pAG32S-*Kpn*I- fragments with primer pair AGup/AGdown, respectively (Table S1). To introduce the BFP, the -*Kpn*I-BFP-*Sph*I- fragment was amplified from plasmid pDRM054 using primer pair Bup/Bdown and inserted into *Sph*I/*Kpn*I-digested -*Sph*I-PTP1B-pAG32S-*Kpn*I- and -*Sph*I-PTP1B-SKL-pAG32S-*Kpn*I- to produce pAG32S-BFP-PTP1B and pAG32S-BFP-PTP1B-SKL. The positive plasmids were linearized by *Bpu*1102I(*Esp*I) digestion and electroporated into *P. pastoris* GS115 to produce strains 4Y and 3Y.

Construction of PDGFRβ and EGFR-2 expression strains. To construct the PTK expression strains, the gene encoding PDGFRβ was amplified from pcDNA3.1-PDGFRβ using primers T71/T72, whereas the gene encoding EGFR-2 was amplified from pcDNApBluescriptIISK (+) with primers X51/X52. The two fragments were linked with *Spe*I/*Not*I-digested pPIC3.5K-His-GFP-*Spe*I-PDGFRβ-SKL-*Not*I or pPIC3.5K-His-GFP-*Spe*I-EGFR-2-SKL-*Not*I plasmids to generate pPIC3.5K-Kozak-His_{10}-linker-GFP-Linker-Thrombin site-PDGFRβ-SKL (pPIC3.5-GFP-PDGFRβ-SKL) or pPic3.5-Kozak-His_{10}-linker-GFP-Linker-Thrombin site-EGFR-2-SKL (pPIC3.5-GFP-EGFR2-SKL), respectively. These plasmids were confirmed by PCR with primers T71/T72 or X51/X52 (Table S1) and DNA sequencing. The positive plasmids pPIC3.5-GFP-PDGFRβ-SKL and pPIC3.5-GFP-EGFR2-SKL were linearized by either *Sal*I or *Pmi*I digestion and electroporated into *P. pastoris* GS115 and 1Y, 2Y, 3Y, 4Y to produce strains PD, 1PPD~4PPD, E, and 1EP~4EP, respectively.

Preparation of EGFR-2 and PDGFRβ Proteins

Strains 1EP, 2EP, 3EP, 4EP, and E were cultured in shaking flasks. Flow cytometry (FACScalibur; Becton Dickinson, Franklin Lakes, NJ, USA) was used to determine the GFP expression level in *P. pastoris*. The cell extract was prepared as described previously [21]. The protein concentration was measured according to the method of Bradford (Tiangen Ltd., Shanghai, China). Western blotting was performed to determine the EGFR-2 expression level [21]. His-tag affinity and ion-exchange chromatographies were performed on the AKTA Explorer 100 purification system (Pharmacia Bioscience) to purify the EGFR protein. The EGFR kinase activity and inhibition rate, using poly(Glu_4-Tyr) as the substrate, were assayed by ELISA using a tyrosine kinase assay kit (Millipore). The preparation of PDGFRβ was done in the same way as for EGFR-2.

Results

Construction of Phosphatase PTP1B Expression Vectors

In this study, the gene encoding phosphatase PTP1B with/without BFP or SKL was cloned into the expression vector pAG32 and successfully produced four vectors, pAG32S-PTP1B, pAG32S-PTP1B-SKL, pAG32S-BFP-PTP1B, and pAG32S-BFP-PTP1B-SKL. The sizes of the PCR amplification products using primers A1/A2 were 2,642, 2,651, 3,368, and 3,377 bp, respectively. The plasmids were linearized

Table 1. Strains and plasmids used in this study.

Strains or plasmids	Characteristics	Resource
Strains		
Escherichia coli		
Top10F′	General cloning strain	Invitrogen
DH5α	General expression strain	Sangon Biotech (Shanghai)
Pichia pastoris		
GS115	Wild type, *his4*	Invitrogen
GS115::pAG32S-PTP1B-SKL (abbr. 1Y)	GS115 derivative expressing PTP1B and SKL	This study
GS115::pAG32S-PTP1B (abbr. 2Y)	GS115 derivative expressing PTP1B	This study
GS115::pAG32S-BFP-PTP1B-SKL (abbr. 3Y)	GS115 derivative expressing BFP, PTP1B, and SKL	This study
GS115::pAG32S-BFP-PTP1B (abbr. 4Y)	GS115 derivative expressing BFP and PTP1B	This study
1Y::pPIC3.5-GFP-EGFR2-SKL (abbr. 1EP)	GS115 derivative expressing PTP1B, SKL, and EGFR-2	This study
2Y::pPIC3.5-GFP-EGFR2-SKL (abbr. 2EP)	GS115 derivative expressing PTP1B and EGFR-2	This study
3Y::pPIC3.5-GFP-EGFR2-SKL (abbr. 3EP)	GS115 derivative expressing BFP, PTP1B, SKL, and EGFR-2	This study
4Y::pPIC3.5-GFP-EGFR2-SKL (abbr. 4EP)	GS115 derivative expressing BFP, PTP1B, and EGFR-2	This study
GS115::pPIC3.5-GFP-EGFR2-SKL (abbr. E)	GS115 derivative expressing EGFR-2	This study
1Y::pPIC3.5-GFP-PDGFRβ-SKL (abbr. 1PPD)	GS115 derivative expressing PTP1B, SKL, and PDGFRβ	This study
2Y::pPIC3.5-GFP-PDGFRβ-SKL (abbr. 2PPD)	GS115 derivative expressing PTP1B and PDGFRβ	This study
3Y::pPIC3.5-GFP-PDGFRβ-SKL (abbr. 3PPD)	GS115 derivative expressing BFP, PTP1B, SKL, and PDGFRβ	This study
4Y::pPIC3.5-GFP-PDGFRβ-SKL (abbr. 4PPD)	GS115 derivative expressing BFP, PTP1B, and PDGFRβ	This study
GS115::pPIC3.5-GFP-PDGFRβ-SKL (abbr. PD)	GS115 derivative expressing PDGFRβ	This study
Plasmids		
pPIC3.5K	Ampicillin^R G418^R; P_{AOX1}-based expression vector	Invitrogen
pBluescript SK (+)	Ampicillin^R, cloning vector	Tiangen Ltd. (Shanghai)
pDRM054	Ampicillin^R bleomycin^R; vector with the P_{AOX1}-BFP-SKL transfusion gene	Prof. Suresh Subramani, UCSD
pCMV6-XL5	Provided the PTP1B template	OriGene Technologies
pAG32S	Ampicillin^R bleomycin^R; pDRM054 derivative with BFP deletion	This study
pPIC3.5-PTP1B	pPIC3.5K derivative expressing the PTP1B fusion gene	This study
pPIC3.5-PTP1B-SKL	pPIC3.5K derivative expressing the PTP1B and SKL fusion genes	This study
pPIC3.5-BFP-PTP1B	pPIC3.5K derivative expressing the BFP and PTP1B fusion genes	This study
pPIC3.5-BFP-PTP1B-SKL	pPIC3.5K derivative expressing the BFP, PTP1B, and SKL fusion genes	This study
pAG32S-PTP1B	pAG32S derivative expressing the PTP1B fusion gene	This study
pAG32S-PTP1B-SKL	pAG32S derivative expressing the PTP1B and SKL fusion genes	This study
pAG32S-BFP-PTP1B	pAG32S derivative expressing the BFP and PTP1B fusion genes	This study
pAG32S-BFP-PTP1B-SKL	pAG32S derivative expressing the BFP, PTP1B, and SKL fusion genes	This study
pcDNA3.1-PDGFRβ	Vector expressing the PDGFRβ gene	SIMM[a]
pBA3CS	Vector expressing the c-Src gene	SIMM[a]

Table 1. Continued.

Strains or plasmids	Characteristics	Resource
pcDNApBluescripIISK (+)	Vector expressing the EGFR-2 gene	Sangon Biotech (Shanghai)
pPIC3.5K-His-GFP-*Spe*I-PDGFRβ-SKL-*Not*I		Lab collected
pPIC3.5K-His-GFP-*Spe*I-EGFR-2-SKL-*Not*I		Lab collected
pPIC3.5-GFP-PDGFRβ-SKL	pPic3.5K-Kozak-His_{10}-linker-GFP-Linker-Thrombin site-PDGFRβ-SKL	This study
pPIC3.5-GFP-EGFR2-SKL	pPic3.5-Kozak-His_{10}-linker-GFP-Linker-Thrombin site-EGFR-2-SKL	This study

[a]SIMM, Shanghai Institute of Material Medica, Chinese Academy of Science.

and electroporated into *P. pastoris* GS115 under the control of an inducible *AOX1* promoter resulting in *P. pastoris* strains 2Y, 1Y, 4Y, and 3Y. The strains were grown on His-deficient plates with a hygromycin resistance gene as a marker. The positive strains were determined by PCR (Supplemental Figs. S1A and S1B) and sequencing. After 24 h methanol induction, the fluorescence in strain 3Y was not dispersed throughout the cells but showed a punctate distribution (Figs. S1C and S1D). The reason for this may be that the BFP was targeted into the *P. pastoris* peroxisomes because of the carboxyl-terminal signal peptide SKL [12]. For strain 4Y without the signal peptide SKL, the BFP-PTP1B protein was expressed and distributed in the cytoplasm, and the fluorescence was dispersed throughout the cells (Figs. S1E and S1F).

Construction of EGFR-2 and PDGFRβ Expression Vectors

Each of the EGFR-2 and PDGFRβ genes was cloned and expressed in vector pPIC3.5K fused with a His-tag and GFP at its N-terminus. The fusion plasmids were transformed into four *P. pastoris* strains with PTP1B (1Y-4Y) and the control GS115 strain to obtain 10 strains: E, 1EP~4EP, PD and 1PPD-4PPD (Table 1): all of which were under the control of an inducible *AOX1* promoter. To avoid the potential negative effect to host cells caused by PTK phosphorylation, SKL was added to the C-terminus of EGFR-2 and PDGFR to produce vectors pPIC3.5-GFP-EGFR2-SKL and pPIC3.5-GFP-PDGFRβ-SKL. The correct *P. pastoris* transformants were screened on His-deficient plates, followed by YPD-G418 plates after transforming the control strain GS115 and strains 1Y-4Y with PTP1B.

To further verify the strains, PCR amplification of strains E, 1EP, 2EP, 3EP, and 4EP was performed with primers A1/X52 or X51/X52 and a product of 1,675 bp was obtained. Using templates 1PPD, 2PPD, 3PPD, and 4PPD and primers T71/T72 or P1/P2, products of 1,572 and 1,313 bp were obtained by PCR amplification, respectively.

Co-Expression of BFP-PTP1B- and GFP-Fused RTKs in *P. pastoris*

P. pastoris strains containing pPIC3.5-GFP-PDGFRβ-SKL and pPIC3.5-GFP-EGFR2-SKL expressed GFP-SKL, whereas those with pAG32S-BFP-PTP1B or pAG32S-BFP-PTP1B-SKL expressed BFP or BFP-SKL. The BFP-SKL or GFP-SKL proteins specifically targeted the peroxisome of *P. pastoris* [12]. In the 10 recombinant *P. pastoris* strains (Table 1), the subcellular location of GFP- or BFP-fused PTP1B, EGFR-2, and PDGFRβ were investigated by fluorescence microscopy (Fig. 1). Co-expression of BFP with GFP in *P. pastoris* strains 3EP, 4EP, 3PPD, and 4PPD was detected with fluorescence microscopy, and blue or green fluorescence was observed (Figs. 1C, 1D, 1G, and 1H) indicating that PTP1B can be co-expressed with EGFR-2 and PDGFRβ in *P. pastoris*.

In strains 4EP and 4PPD (Figs. 1D and 1H), the blue fluorescence was nearly widespread in the entire cells, whereas the green fluorescence exhibited a typical punctuate pattern. Without the carboxyl-terminal SKL, the BFP-PTP1B was expressed in the cytoplasm. However, GFP-PDGFRβ-SKL and GFP-EGFR2-SKL were expressed and located in the peroxisome because of the signal peptide SKL. By contrast, for strains 3EP and 3PPD, a typical punctate blue and green fluorescence was observed and overlapped, which suggests that the fusion proteins BFP-PTP1B-SKL, GFP-PDGFRβ-SKL, and GFP-EGFR2-SKL were all sorted into the peroxisome under the control of the SKL signal (Figs. 1C and 1G). For control strains E containing pPIC3.5-GFP-PDGFRβ-SKL (Fig. 1I) and PD containing pPIC3.5-GFP-EGFR2-SKL (Fig. 1J), or PTP1B without BFP (Figs. 1A, 1B, 1E, and 1F), only green fluorescence was observed.

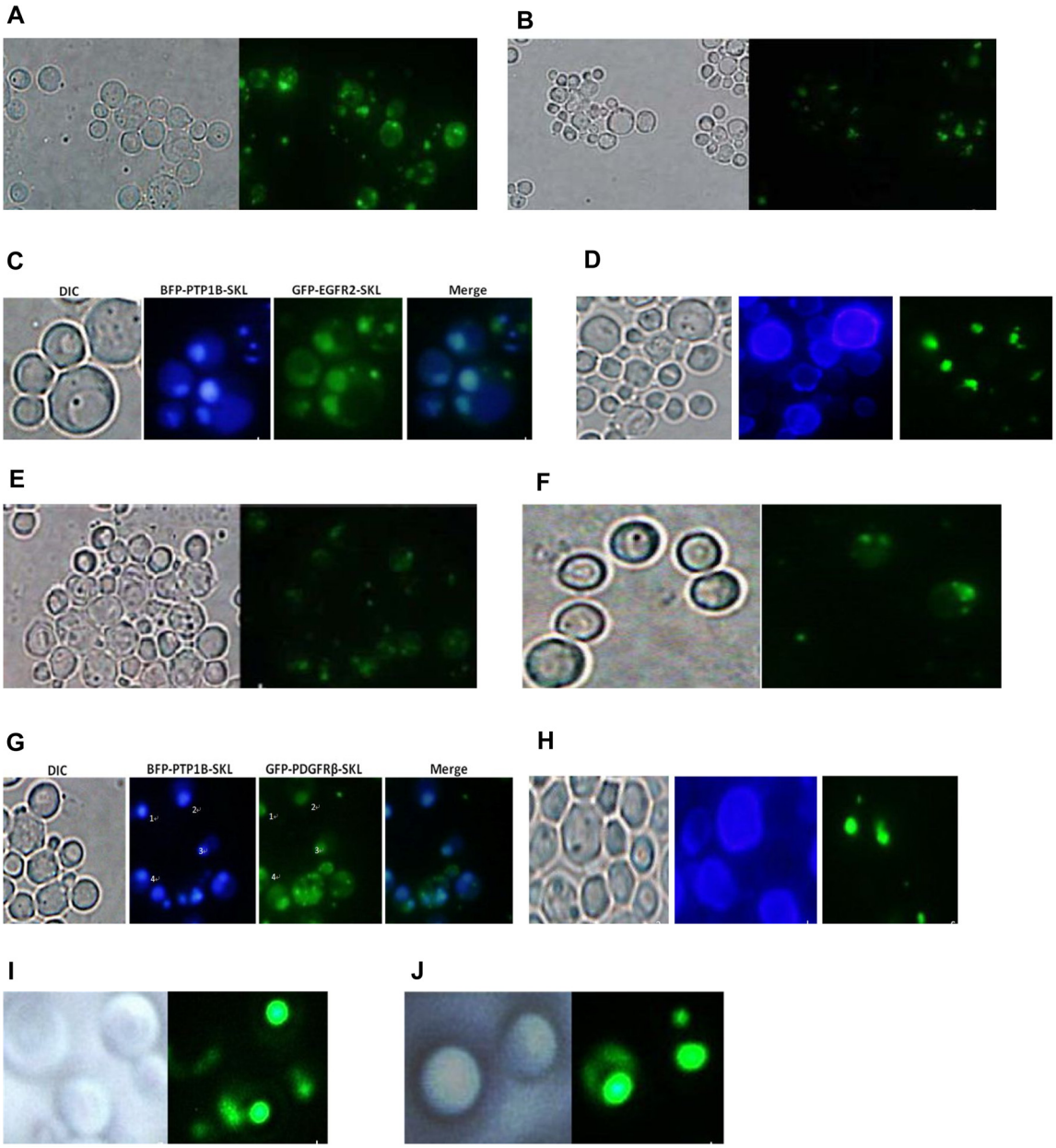

Fig. 1. Fluorescence microscopy images of *P. pastoris* cells expressing different proteins.
(**A**) Strain 1EP co-expressing GFP-EGFR2-SKL and PTP1B-SKL. (**B**) Strain 2EP co-expressing GFP-EGFR2-SKL and PTP1B. (**C**) Strain 3EP co-expressing BFP-PTP1B-SKL and GFP-EGFR2-SKL. (**D**) Strain 4EP co-expressing BFP-PTP1B and GFP-EGFR2-SKL. (**E**) Strain 1PPD co-expressing GFP-PDGFRβ-SKL and PTP1B-SKL. (**F**) Strain 2PPD co-expressing GFP-PDGFRβ-SKL and PTP1B. (**G**) Strain 3PPD co-expressing BFP-PTP1B-SKL and GFP-PDGFRβ-SKL. (**H**) Strain 4PPD co-expressing BFP-PTP1B and GFP-PDGFRβ-SKL. I. Strain E expressing GFP-EGFR2-SKL. (**J**) Strain PD expressing GFP-PDGFRβ-SKL.

Expression, Purification, and Activity Analysis of EGFR-2 and PDGFRβ Proteins

Five strains, 1EP, 2EP, 3EP, 4EP, and E, were cultured and induced with methanol in shaking flasks and sampled at 24 h intervals.

After 48 h induction, the wet cell weight (WCW) was measured. The WCW of *P. pastoris* GS115Gal, a control strain that expressed β-galactosidase intracellularly, was 4.1 g/l. However, strain E grew significantly slower than the control strain. The WCW of strain E was 1.2 g/l

Table 2. Expression and tyrosine kinase activity of EGFR-2 in different *P. pastoris* strains.

Strains	WCW[a] (g/l)	Geo-Mean GFP fluorescence intensities (GMFI)[b]				Tyrosine kinase activities of EGFR-2		
		0 h	24 h	48 h	72 h	Control	Strain cells lysate	Elute collections of 250 mM imidazole
E	1.2	5.56	158.73	103.35	67.29	0.0444	0.356	1.553
1EP	1.7	5.15	194.32	160.72	52.87	0.0573	0.482	2.687
2EP	1.4	6.12	181.44	149.12	78.41	0.0589	0.467	1.970
3EP	1.9	4.04	256.42	191.50	99.19	0.0567	0.479	2.820
4EP	1.3	3.92	241.89	184.86	87.56	0.0612	0.458	2.353

[a]Cell pellets grown in MGY medium were washed with water and then resuspended in MM medium to an OD_{600} of 1.0 for methanol induction for 48 h in shake flasks. The WCW was then measured.

[b]The Geo-Mean GFP fluorescence intensities were calculated with 10^4 cells per analysis.

(Table 2), which was only 29.3% of the control. When there was co-expression with PTP1B, strains 1EP-4EP grew more rapidly. In particular, for strain 3EP, the WCW reached 1.9 g/l, which was almost 58.3% higher than strain E.

Green fluorescence intensity was detected by flow cytometry to determine the expression of EGFR-2 in each strain according to the fused GFP expression and to select high-yield strains. Under methanol induction, GFP was expressed in strains E and 1EP-4EP, and a maximum was achieved after 24 h in strain 3EP (Table 2). The same induction and detection of GFP in strains PD and 1PPD-4PPD were also performed (data not shown).

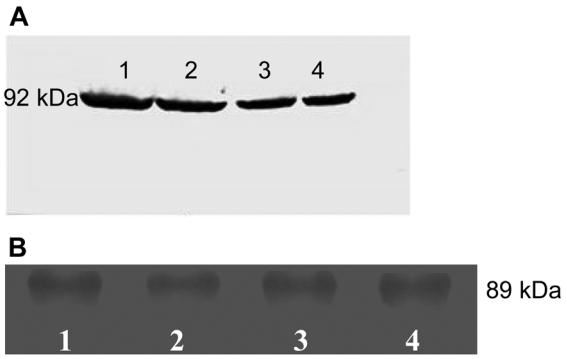

Fig. 2. Western blot analysis of the elution from Ni^{2+} affinity chromatography.

(**A**) Cell pellets grown in MM medium for 24 h were disrupted and centrifuged, and the supernatant was analyzed by western blot assay using a GFP antibody. Lane 1, strain 1EP; Lane 2, strain 2EP; Lane 3, strain 3EP; Lane 4, strain 4EP expressing the GFP-EGFR-2 fusion protein in *P. pastoris*. (**B**) Cell pellets grown in MM medium for 24 h were disrupted and centrifuged, and the supernatant was purified by Ni^{2+} affinity chromatography. The affinity elutions were evaluated by western blot analysis using a GFP antibody. Lane 1, strain 1PPD; Lane 2, strain 2PPD; Lane 3, strain 3PPD; Lane 4, strain 4PPD expressing the GFP-PDGFRβ fusion protein in *P. pastoris*.

EGFR-2 and PDGFRβ were purified using His-tag affinity and ion-exchange chromatographies. By western blotting, the EGFR-2 and PDGFRβ fusion proteins in the cell lysate were 92 and 89 kDa, respectively (Fig. 2).

The tyrosine kinase EGFR-2 is a potential target for anticancer drug screening. The tyrosine kinase activity of EGFR-2 was measured using an ELISA (Table 2, Fig. 3A). High kinase activity EGFR-2 was obtained from a His-tag affinity column with a 250 mM imidazole elution. Moreover, the highest kinase activity was achieved by strain 3EP (Table 2), which was consistent with the green fluorescence intensity of this strain.

The molecular model for screening EGFR-2 inhibitors was investigated. Tyrosine kinase substrate phosphorylation is a complex, double-substrate, including poly(Glu4-Tyr) and ATP, enzymatic process. The concentration of one substrate is first determined, and the other substrate is then changed to demonstrate its relationship with the phosphorylation reaction. When the ATP concentration was fixed in a range of 1–10 μM, substrate phosphorylation increased significantly with an increase in the ATP concentration (data not shown). The optimal concentration of poly(Glu_4-phospho-Tyr) and ATP was 10 ng/well and 5 mM, respectively. With an increase of EGFR-2 and PDGFRβ, substrate phosphorylation increased rapidly and reached a plateau when EGFR-2 was 48 ng/well (Fig. 3A) and PDGFRβ was 39 ng/well (Fig. 3B). When co-expressed with PTP1B (strains 1EP-4EP and 1PPD-4PPD), the tyrosine kinase activity was higher than when it was expressed alone (strains E and PD) (Fig. 3).

Discussion

Tyrosine kinases are the most important therapeutic target for the treatment of cancer because of their critical role in angiogenesis. However, the preparation of PTKs

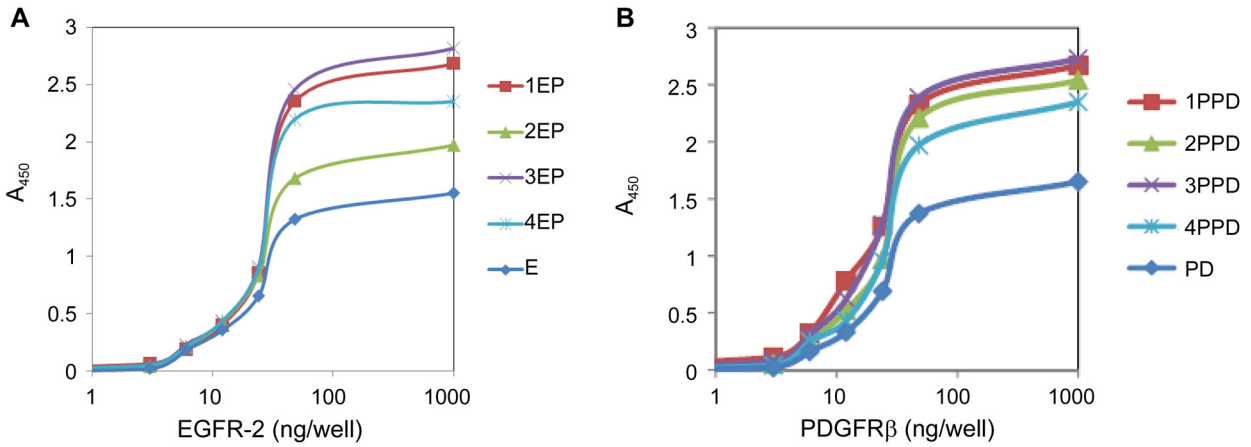

Fig. 3. ELISA to determine the phosphorylation activity of EGFR-2 and PDGFRβ.
(**A**) Plot of the A_{450} *vs.* EGFR-2 concentration shows the relationship between the phosphorylation states of the substrate and the amount of EGFR-2. In 96-well plates, 10 ng/well poly(Glu_4-Tyr) was pre-coated as the substrate. ATP solution (10 μM) diluted in 85 μl of reaction buffer (100 mM Tris-HCl (pH 7.4), 50 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM Na_3VO_4, and 0.5 mM DTT) was added to each well. The reaction was performed with the addition of 5 μl of purified EGFR-2 fusion protein at varying concentrations. (**B**) ELISA to determine the phosphorylation activity of PDGFRβ.

using recombinant *P. pastoris* is difficult primarily because PTKs phosphorylate intracellular proteins and result in serious growth inhibition and toxicity to host cells [18]. In this study, we co-expressed PTKs and PTPs in *P. pastoris* to overcome the potential negative effect caused by overexpression of PTKs.

P. pastoris can use methanol as the sole carbon and energy source because of the expression of alcohol oxidase (AOX1 and AOX2) induced by methanol. Exogenous gene expression is strictly controlled by the promoter P_{AOX} [5]. Methanol is catabolized in the peroxisome, which facilitates the separation of toxic metabolic products from the cell components [16]. To decrease the toxicity of PTK expression, the tyrosine kinases EGFR-2 and PDGFRβ were expressed and targeted to the peroxisome of *P. pastoris* by peroxisomal targeting signal 1 (SKL). Phosphatase PTP1B with/without SKL were co-expressed with EGFR-2 and PDGFRβ. The activity of the PTKs co-expressed with PTP1B was compared with that without PTP1B in *P. pastoris* after growth in medium containing methanol.

The results indicated that the co-expression of tyrosine phosphatase PTP1B and a tyrosine kinase in *P. pastoris* could partially recover the growth of *P. pastoris* and increase the expression level of tyrosine kinase. Cell growth of strain E was significantly inhibited by EGFR-2 expression, and its WCW was only 29.3% of the control (Table 2). However, when co-expressed with PTP1B, strains 1EP~4EP grew more rapidly. In particular, strain 3EP was almost 58.3% higher than strain E. Moreover, for PTK expression, the kinase activity in strains 1EP-4EP was also higher than strain E. The highest kinase activity was achieved by strain 3EP (Table 2), which was 81.5% higher than strain E. When co-expressed with PTP1B (strain 1EP-4EP, 1PPD-4PPD), the tyrosine kinase activity was higher than when expressed alone (strain E, PD) in the molecular model test (Fig. 3).

The growth and kinase activity results demonstrate that co-expression of tyrosine phosphatase PTP1B and tyrosine kinases in *P. pastoris* can partly overcome the negative effects associated with overexpression of tyrosine kinases. The establishment of a co-expression system provides a valuable tool for the production of protein tyrosine kinases, which interferes with the level of protein phosphorylation in host cells.

References

1. Adams JL, Lee D. 1999. Recent progress towards the identification of selective inhibitors of serine/threonine protein kinase. *Curr. Opin. Drug Discov. Dev*. **2:** 96-109.
2. Beckmann MP, Betsholtz C, Heldin CH, Westermark B, di Marco E, di Fiore PP, *et al*. 1988. Comparison of biological properties and transforming potential of human PDGF-A and PDGF-B chains. *Science* **241:** 1346-1349.
3. Cadena DL, Gill GN. 1992. Receptor tyrosine kinase. *FASEB J*. **6:** 2332-2337.
4. Clasesson WL. 1994. Platelet-derived growth factor receptor signals. *J. Biol. Chem*. **269:** 32023-32036.
5. Cregg JM, Cereghino L, Shi J, Higgins DR. 2000. Recombinant

protein expression in *Pichia pastoris*. *Mol. Biotechnol*. **16:** 23-52.
6. Deb TB, Su L, Wong L, Bonvini E, Wells A, David M, Johnson GR. 2001. Epidermal growth factor (EGF) receptor kinase independent signaling by EGF. *J. Biol. Chem*. **276:** 15554-15560.
7. Frederick M. 1995. *Short Protocols in Molecular Biology*, 3rd Ed. Wiley, New York.
8. Hubbard SR, Miller WT. 2007. Receptor tyrosine kinases: mechanisms of activation and signaling. *Curr. Opin. Cell Biol*. **19:** 117-123.
9. Karashima T, Sweeney P, Slaton JW, Kim SJ, Kedar D, Izawa JI, *et al*. 2002. Inhibition of angiogenesis by the antiepidermal growth factor receptor antibody ImClone C225 in androgen-independent prostate cancer growing orthotopically in nude mice. *Clin. Cancer Res*. **8:** 1253-1264.
10. Lai H, Wang H, Wang J. 2007. The function of PTP in plant MAPK path. *Chin. Bull. Bot*. **24:** 677-685.
11. Levizki A. 1996. Targeting signal transduction for disease therapy. *Curr. Opin. Cell Biol*. **8:** 239-244.
12. Monosov EZ, Wenzel TJ, Lüers GH, Heyman JA, Subramani S. 1996. Labeling of peroxisomes with green fluorescent protein in living *P. pastoris* cells. *J. Histochem. Cytochem*. **44:** 581-589.
13. Raymond E, Faivre S, Armand JP. 2000. Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. *Drugs* **60:** 15-23.
14. Stoker AW. 2005. Protein tyrosine phosphatases and signaling. *J. Endocrinol*. **185:** 19-33.
15. Tonks NK. 2006. Protein tyrosine phosphatases: from genes, to function, to disease. *Nat. Rev. Mol. Cell Biol*. **7:** 833-846.
16. van der Klei IJ, Yurimoto H, Sakai Y, Veenhuis M. 2006. The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochim. Biophys. Acta* **1763:** 1453-1462.
17. Wang WQ, Sun JP, Zhang ZY. 2003. An overview of the protein tyrosine phosphatase superfamily. *Curr. Top. Med. Chem*. **3:** 739-748.
18. Wang Y, Xuan YJ, Zhang P, Jiang X, Ni ZH, Tong LJ, *et al*. 2009. Targeting expression of the catalytic domain of kinase insert domain receptor (KDR) in the peroxisomes of *Pichia pastoris*. *FEMS Yeast Res*. **9:** 732-741.
19. Wu SX, Letchworth GJ. 2004. High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *Biotechniques* **36:** 152-154.
20. Xiong HQ, Abbruzzese JL. 2002. Epidermal growth factor receptor targeted therapy for pancreatic cancer. *Semin. Oncol*. **29:** 31-37.
21. Zhang P, Zhang WW, Zhou XS, Bai P, Cregg JM, Zhang YX. 2010. Catabolite repression of Aox in *Pichia pastoris* is dependent on hexose transporter PpHxt1 and pexophagy. *Appl. Environ. Microbiol*. **76:** 6108-6118.
22. Zwick E, Bange J, Ullrich A. 2002. Receptor tyrosine kinases as targets for anticancer drugs. *Trends Mol. Med*. **8:** 17-23.