EXPRESSION AND PURIFICATION PROTEIN EPIDERMENT GROWTH FACTOR RECEPTOR-2 TYROSINE KINASE WITH PROTEIN TYROSINE PHOSPHATASE-1B INTO PEROXISOME OF *Pichia pastoris*

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ABSTRACT

Mediated by the balanced action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs), the appropriate control of protein tyrosine phosphorylation is essential for cellular homeostasis. A strategy of co-expressing PTKs with PTPs was developed to overcome the apparent toxicity associated with expressing PTKs, and to allow the expression and preparation of active PTKs from P. pastoris. To test the hypothesis that the difficulty of expressing PTKs in P. pastoris is due to PTKs phosphorylating P. pastoris proteins, thus resulting in P. pastoris toxicity, we tried to introduce a protein tyrosine phosphatase activity to overcome this apparent kinase activity-triggered problem. The gene encoding recombinant PTP1B was cloned into vector pRDM054 with blue fluorescent protein (BFP) and peroxisomal targeting signal 1 (SKL) to the C-terminus of the PTP1B, and it was cloned into vector pAG32 with the four vectors pRDM054-PTP1B, pRDM054-PTP1B-SKL, pRDM054-BFP-PTP1B and pRDM054-BFP-PTP1B-SKL to form the expression vectors by strain GS115 in Pichia pastoris under the control of inducible AOX1 promoter. The tyrosine kinases EGFR-2 is important with signal-transducing kinase activity. They are the most important therapeutic target for the treatment of cancer because of their crucial role in angiogenesis, which is fundamental to the malignancy of tumors. The tyrosine kinase EGFR-2 gene was cloned and expressed in vector pPIC3.5K and fused with His-tag and green fluorescent protein (GFP) at its N-terminus, added SKL to the C-terminus of the EGFR-2 and the resultant vector was pPIC3.5-GFP-EGFR2-SKL, in Pichia pastoris with PTP1B gene and the control GS115 strain to get 5 trains, under the control of inducible AOX1 promoter. The right P. pastoris transformants were screened on His-deficient plates and YPD-G418 plates by turns after electroporation of strains GS115 with and without of PTP1B, and high yield strains were selected. The EGFR-2 fusion protein was purified by Ni^{2+} Sepharose. The activity of tyrosine kinases co-expressed with the PTP1B was compared to that produced without PTP1B in P. pastoris after growing in medium containing methanol. Fusion proteins of EGFR-2 was 92 kDa, by western blot, and their tyrosine kinase activity were measured by ELISA. In the recombinant P. pastoris, compared with expressing tyrosine kinases alone, the tyrosine kinases co-expressed with PTP1B exhibited high kinase catalytic activity, the highest kinase catalytic activities were achieved by targeting the tyrosine kinases and PTP1B into peroxisomes. Further results shows that the EGFR-2 fusion protein expressed in P. pastoris can be used as an attractive target for future anti-cancer therapeutics.

Keywords: *EGFR-2; Expression; Peroxisome; Pichia pastoris; Protein tyrosine kinase; Protein tyrosine phosphatase*

INTRODUCTION

Protein kinases are a large family of cell signaling mediators undergoing intensive research to identify inhibitors or modulators useful for medicine. As one strategy, small-molecule compounds that bind the active site with high affinity can be used to inhibit the enzyme activity. Many of protein kinase genes expressed in man is already known to play important roles in biology, and all could potentially be important as targets for pharmaceutical intervention in medicine. Structural biology offers valuable information useful in the design of new inhibitors (Card *et al.*, 2005), but a limitation in its application to kinases can often be the inability to produce highly purified proteins in amounts suitable for cocrystallography. Inhibitor binding sometimes can be sensitive to the specific conformation state of a kinase (Nagar *et al.*, 2002), or to changes in the kinase sequence caused by mutations, such as those occurring during cancer progression (Carter *et al.*, 2005; Deininger *et al.*, 2005; Berthou *et al.*, 2004; Morotti *et al.*, 2002).

The epidermal growth factor receptor (EGFR), that is ERBB (alias ERBB1) reclassified as EGFR, EGFR family of tyrosine kinase have four members; ERBB1, ERBB2 (alias EGFR-2, NEU or HER2), ERBB3 (alias EGFR3, HER3), and ERBB4 (alias EGFR4, HER4), while EGFR-2 is an orphan tyrosine kinase receptor (Schlessinger, 2002). Amplification and overexpression of EGFRs are frequently found in a variety of epithelial cancers, such as breast, lung, colon, ovarian, and brain tumors, and play a central role in the etiology and progression of these tumors (Alroy *et al.*, 2002; Jorissen *et al.*, 2003). The frequency of EGFR amplification is directly associated with malignant tumor. In addition, amplified EGFR levels indicate a bad prognosis and shorter overall survival (Jaros *et al.*, 1992). Recent analysis of the EGFR gene in tumors has shown that regions of this gene frequently undergo alteration. Hence, not only amplification but also mutation may be the cause of the increased malignancy in EGFR overexpressing cells (Akbasak and Sunar-Akbasak, 1992). Several studies have shown that mutations in the tyrosine kinase (TK) domain of the EGFR gene are present in a subset of non-small cell lung cancers, and those tumors with the EGFR mutations have been reported to be highly sensitive to gefitinib, an EGFR TK inhibitor (Paez *et al.*, 2004; Lynch *et al.*, 2004; Shigematsu *et al.*, 2005). Signaling through EGFR is intricately involved in human cancer, and therefore serves as a target for cancer therapy. Several strategies exist to target EGFR including monoclonal antibodies (mAbs) directly towards the extracellular domain of EGFR such as Cetuximab (Erbitux), that is the most extensively studied and clinically approved chimeric mAb designed to specifically inhibit EGFR (Azemar *et al.*, 2000). The prototypic member of the protein tyrosine phosphatase (PTP) superfamily, as a positive mediator of the ErbB2 induced signals that trigger tumorigenesis and metastasis (Betires-Alj and Neel 2007; Julien *et al.*, 2007). Nevertheless, the function of PTP1B is not restricted to metabolic regulation and a complex mixture of positive and negative effects of the phosphatase on various tyrosine phosphorylation dependent signaling pathways have been reported. Those substrates largely conforming to the criteria discussed above, where substrate-trapping and/or overexpression approaches have been combined with validating under-expression or knockdown strategies, include receptor PTKs such as EGFR (Flint *et al.*, 1997; Haj *et al.*, 2003), PDGFR (Haj *et al.*, 2003; Haj *et al.*, 2002; Lammers *et al.*, 1993), CSF-1 (colony-stimulating factor 1) receptor (Heinonen *et al.*, 2006), IR (Cicirelli *et al.*, 1990; Kenner *et al.*, 1996; Walchli *et al.*, 2000; Elchebly *et al.*, 1999; Zabolotny *et al.*, 2004; Haj *et al.*, 2005) and IGF-1 (insulin-like growth factor-1) receptor (Kenner *et al.*, 1996; Buckley *et al.*, 2002), as well as cytoplasmic PTKs such as c-Src (Liang *et al.*, 2005; Cheng *et al.*, 2001; Bjorge *et al.*, 2000). However, the link between PTPase inhibition and EGFR activation has not been made for particulate exposures. Here we show that DEP exposure induces EGFR-2 dependent phosphorylation through a mechanism involving the inactivation of EGFR directed PTPase activity

in primary human airway epithelial cells, a principal target cell of inhaled PM (Tal *et al.*, 2008). Weijland, 1996 have previously described a yeast expression system for the catalytic domain of *Src*, which utilizes the coexpression of the phosphatase, PEST-PTPase, but due to proteolysis and time requirement for yeast expression is not the most convenient procedure in practice. In order to increase the yield of active and soluble protein from bacteria it was necessary to coexpress the kinases with a phosphatase (Zhang *et al.*, 1992). Nevertheless, the beneficial effect of protein phosphatase tyrosine kinase coexpression on the expression of protein kinases might generally be adaptable and help overcome toxic effects of protein tyrosine kinase activity in *P. pastoris*. For this concept, we have developed a strategy to co-express EGFR-2 as a fusion protein with a protein tyrosine phosphatase. This strategy helps overcome the apparent toxicity associated with expressing EGFR-2 alone, and allows the expression and purification of active EGFR-2 from *P. pastoris*. To test the hypothesis that the difficulty of expressing EGFR-2 in *P. pastoris* has been due to EGFR-2 phosphorylating *P. pastoris* proteins, thus resulting in *P. pastoris* toxicity, we determine if introducing a protein tyrosine phosphatase (PTP) activity will overcome this apparent kinase activity-triggered problem. It was hoped that the PTP would dephosphorylate any *P. pastoris* protein that may be phosphorylated by EGFR-2 and prevent any toxicity caused by EGFR-2 kinase activity. Methylotrophic yeasts also provide a new approach to express the recombinant protein in the specific organelles, namely peroxisomes. Methylotrophic yeasts can grow in medium containing methanol as the sole carbon and energy source. Peroxisomes occupy only a small cell volume fraction when growing in glucose medium. However, when cells are transferred to methanol, these yeasts synthesize high levels of peroxisomes and cytosolic enzymes such as alcohol oxidase (*AOX*) that are necessary for the metabolism of methanol. Morphologically, clusters of large peroxisomes may occupy up to 80% of the total cellular volume and *AOX* compartmentalized in peroxisomes can accumulate up to 30% of the total cell protein (Veenhuis *et al.*, 1983; Gould *et al.*, 1992; Van der Klei *et al.*, 2006). Expression and compartmentalization of the recombinant protein in peroxisomes will prevent undesirable modifications such as proteolytic degradation or glycosylation, and is also in particular advantageous when proteins produced are toxic or harmful for the host (Van Dijk *et al.*, 2000). To eliminate the potential negative phosphorylation effect of tyrosine kinase to the host cells as mentioned in *S. pombe*, we expressed and targeted the recombinant EGFR-2 and PTP1B in peroxisomes of *P. pastoris* via adding a peroxisome targeting signal Ser-Lys-Leu (SKL) at the *C*-terminus of EGFR-2 and PTP1B and then purified and characterized an EGFR-2. In this study, the EGFR-2 tyrosine kinase is produced with high yields in *P. pastoris* by using a pAG32 vector encoding the PTP1B tyrosine phosphatase, compared to the phosphorylated form produced without PTP1B, consistent with the known selectivity of this inhibitor for the unactivated conformation of the enzyme.

MATERIALS AND METHODS

Strain, vector and culture medium

Pichia pastoris host strain GS115(His$^-$), *Escherichia coli* host strain TOP10 and intracellular expression vector pPIC3.5K were purchased from Invitrogen. The control strain *P. pastoris* GS115rljGH(His$^+$, Mut$^+$) under *AOX1* promotor was from our lab[44]. Plasmid pRDM054 was kindly provided by Prof. Suresh Subramani, University of California, San Diego. Plasmid pP3.5-HIS10-tag-GFP-ERB-SKL and pP KDR-Fus were from our lab (Wang *et al.*, 2009). Plasmid pCMV6-XL5 was purchased from OriGene Technologies. Plasmid pBlueScript II SK (+) was purchased from Sangon (Shanghai, China). MD/MM medium (1.34% yeast nitrogen base, 400 µg/L biotin, 2% agar, 2% dextrose or 0.5% methanol) was used for selecting transformants with Mut$^+$ or Muts methanol utilization phenotype. YPD-Hygromycine medium (1% yeast extract, 2%

peptone, 2% dextrose, 2% agar, and 750 mg/L Hygromycine) and YPD- Hygromycine medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, and 2 mg/mL G418) were used for selecting transformants. The *P. pastoris* cells were cultured in MGY medium (1% yeast extract, 2% peptone, 1% glycol, 400 µg/L biotin, and 0.1 mol/L potassium phosphate, pH 6.0) for growth and in MMY medium (1% yeast extract, 2% peptone, 400 µg/L biotin, 1% methanol, and 0.1 mol/L potassium phosphate, pH 6.0) for induction.

Construction of the expression vectors

Construction of human PTP1B expression into pRDM054 plasmid, the scheme of construction of the expression plasmid was shown in Fig. 1. Construction of wild type human PTP1B expression plasmid was achieved in three steps. First, the human PTP1B was amplified by PCR from a PTP1B-containing pCMV6-XL5 vector. The PTP1B fragment, containing a *Stu* I and *Sal* I cloning sites, was cloned into pCMV6-XL5 expression vector (OriGene Technologies). Second, the DNA fragment containing Kozak sequence (ACC ATG GCT) (formed by annealing of two DNA oligos: P1 5'-A AGGCCT ACC ATG GCT ATG GAG ATG GAA AAG GAG T-3'; P2-1 5'-G GAATTC CTA CAACTTGGA TGT GTT GCT GTT GAA CAG G-3' and P2-2 5'-G GAATTC CTA TGT GTT GCT GTT GAA CAG G-3') was introduced into this plasmid, through the *Stu* I site at the 5' end and *Sal* I end. Third, the human PTP1B encoding the region of the stop codon was amplified and cloned into this plasmid through *Sal* I site.

Construction of human EGFR-2 expression plasmids, the scheme of construction of the expression plasmid was shown in Fig. 2. The DNA fragment encoding the EGFR-2 was cloned by the pBlueScript II SK (+) vector between the *Spe* I site at the 5' end and a *Avr* II site at the 3' end with primers X51 (5'-CAT ACTAGT ATG CGG ATC CTG AAA GAG ACG-3') and X52 (5'-CATTA GCGGCCGC CCTAGG CTA TTA CAACTTGGA CAC TGG CAC GTC CAG ACC CAG GTA-3'). Which introduced a thrombin cleavage site (formed by annealing of two DNA oligos:

5'-G GAATTC TTG GTA CCT CGT GGT TCC ACTAGT CAT-3' and 5'-ATG ACTAGT GGA ACC ACG AGG TAC CAA GAATTC C-3') between the *Eco*R I site at the 5' end and a *Spe* I site at the 3' end, immediately upstream of EGFR-2-SKL.

EGFR-2 and PTP1B were coexpression and purification. The EGFR-2 expression in pPIC3.5K plasmid and PTP1B expression in pRDM054 plasmid were transformed into *P. pastoris* genome. Fistly, PTP1B expression in pRDM054 plasmid was linearized with *Bpu*1102I(*Esp*I) (to allow the integration at the *AOXI* locus of the *P. pastoris* genome) and transformed into competent cells of *P. pastoris* strain GS115 by electroporation, according to the Invitrogen's protocol. Transformants were resuspended plated on YPD agar (1% yeast extract, 2% tryptone, 2% dextrose, and 2% agar) containing Hygromycine B 750 ng/ml for selection. Integration of BFP-PTP1B in the recombinant *P. pastoris* genome was confirmed by genomic PCR using the primers of 5' *AOX1* and 3' *AOX1* according to the Invitrogen's protocol. The PCR products were sequenced further. *S*econdly, EGFR-2 expression in pPIC3.5K plasmid was linearized with *Sal* I (to allow the integration at the *AOXI* locus of the *P. pastoris* genome) and transformed into competent cells of *P. pastoris* strain GS115 and *P. pastoris* strain GS115 has PTP1B plasmid by electroporation, according to the Invitrogen's protocol. His+ transformants were selected on MGY plates (1.34% YNB, 4×10^{-5}% biotin, 1% glycerol, and 2% agar). His^{+} transformants were resuspended plated on YPD agar (1% yeast extract, 2% tryptone, 2% dextrose, and 2% agar) containing G418 2.0 mg/ml for multi-copy selection. Integration of His-GFP-EGFR2-SKL in the recombinant *P. pastoris* genome was confirmed by genomic PCR using the primers of X51 and X52. The PCR products were sequenced further. Expression analysis was taken by the flow cytometry method (Hohenblum *et al.*, 2003), the expression of EGFR-2 fusion protein was induced by MM medium (1.34% YNB, 4×10^{-5}% biotin, and

0.5% methanol) at 28°C, after a 24h induction, as GFP could exhibit fluorescence with excitation at 488 nm and an emission at 510nm. After diluting to 1×10^7 cells mL-1 with phosphate-buffer saline (PBS), 1 mL sample was analyzed on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) with a 488 nm argon laser. 104 cells were measured per analysis. The ordinate FL1H refers to the green fluorescence intensity for GFP, while the abscissa FL3H refers to the red fluorescence intensity for PI.

The expression of EGFR-2 fusion protein was induced by 0.5% methanol at 28°C, after a 24h induction, the *P. pastoris* strain were harvested by centrifugation at 5000 g for 2-3 minutes at room temperature, resuspended in lysis buffer (50 mmol/L NaH_2PO_4,1 mmol/L PMSF 1% NP-40, pH 8.0), and lysed by sonication. After the resin was packed into a Ni^{2+} chelating affinity chromatography column which was linked to an automated AKTA Explorer 100 (Amersham Pharmacia Biotech), and pre-equilibrated with buffer A (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 20 mmol/L Imidazole, pH8.0). The column thoroughly washed by with buffer B (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 250 mM Imidazole, pH8.0), and buffer C (50 mmol/L NaH2PO4, 300 mmol/L NaCl, 1 mol/L Imidazole, pH8.0). The eluted proteins were collected and stored at -80°C until ready to assay.

All purification steps were performed at 4°C. The two EGFR-2, EGFR-2 with PTP1B and EGFR-2 without PTP1B, were expressed and purified using the same procedure. The eluted proteins were analyzed by SDS-PAGE. The content of protein in the band was measured by densitometric scanning. After electrophoresis, gels were stained with Coomassie brilliant blue R-250. Western blot was performed by transferring the proteins to a PVDF membrane using electrophoretic transfer method, with affinity purified rabbit anti-GFP antibody (Proteintech Group Inc., USA) as the primary antibody and peroxidase-conjugated goat anti rabbit IgG (Jackson ImmunoResearch, USA) as the secondary antibody. Tyrosine kinase activities of purified EGFR-2 were tested using Protein tyrosine kinase Assay Kit (Millipore, Lot: DAM1692714). The PTK kit assay system for the *in vitro* determination of protein tyrosine kinase activity is based on an ELISA assay using a PTK-specific polymer substrate-coated microtiter plate (Zhong *et al.*, 2005. The precoated substrate is a synthetic random polymer substrate poly-Glu-Tyr (PGT) containing multiple tyrosine residues. The phosphorylation reaction is initiated by the addition of a protein tyrosine kinase in the reaction buffer contains Mg^{2+}, Mn^{2+} and ATP. The phosphorylated polymer substrate is probed with a purified phosphotyrosine specific monoclonal antibody conjugated to horseradish peroxidase (HRP). Color is developed with HRP chromogenic substrate (OPD). A_{450} ismeasured by spectrophotometry (ELISA reader) and reflects the relative amount of tyrosine kinase activity in the sample.

RESULTS AND DISCUSSION

The difficulty of expressing EGFR-2 in *P. pastoris* was due to EGFR-2 phosphorylating *P. pastoris* proteins, thus resulting in *P. pastoris* toxicity, we determined if introducing a protein tyrosine phosphatase (PTP) activity would overcome this apparent kinase activity-triggered problem. It was hoped that the PTP would dephosphorylate any *P. pastoris* protein that might be phosphorylated by EGFR-2 and prevent any toxicity caused by EGFR-2 kinase activity. There were several options to introduce the phosphatase activity. The PTP and EGFR-2 genes can be introduced by chromosomal insertion by different plasmids, and a fusion protein with *P. pastoris*.