High Level Production of human Protein Tyrosine Kinase-6 in Insect Cells Using *Drosophila* Peptidoglycan Recognition Protein-LB as a fusion protein

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PTK6, an intracellular protein tyrosine kinase, is significantly overexpressed in a majority of breast cancers and has a role in promoting the proliferation of the cancer cells, but not of normal cells. Here, we report high-level production of the catalytic unit of PTK6 fused with *Drosophila* peptidoglycan recognition protein (PGRP)-LB, in the baculovirus system. We first found that the PGRP-LB was potentially useful as a fusion partner to increase the yield of heterologous protein in the baculovirus system. The purified recombinant protein exhibited a 1.5-fold activity with much higher yield than the bacterially-expressed protein. The protein expressed in the baculovirus system will be useful for the crystallization to determine its crystal structure helping understand the molecular mechanism of PTK6 and design its inhibitors.

Key words – PTK6, overexpression, recombinant protein, baculovirus

Introduction

PTK6, also known as Brk, is a human intracellular non-receptor tyrosine kinase with a similar structure to the well-studied Src family of kinases containing SH3, SH2, and a kinase domain [3]. However, PTK6 is distinguished from the Src family of kinases by the lack of the N-terminal consensus sequence for myristoylation [3]. Moreover, the kinase domain of PTK6 needs the linker region between SH2 and the kinase domain for activity [2], while that of the Src family shows enzymatic activity without the linker region. PTK6 is significantly overexpressed in 65% of breast tumors, but not in normal mammary tissue or benign lesions, suggesting that a PTK6 inhibitor could be an antiproliferative breast cancer drug with few side-effects [1]. However, the three-dimensional structure of PTK6 and the molecular mechanism whereby this kinase participates in tumorigenesis still remain poorly understood [4].

Recently, the catalytic unit of the PTK6 protein (amino acids 180 - 451), consisting of the kinase domain and the linker region followed by the kinase domain, was identi-

fied and produced as a GST-fusion protein using a bacterial expression system [2]. In order to better understand the molecular mechanism of the PTK6 and design its specific inhibitor for an anti-cancer drug, detailed biochemical information including a high resolution structure is required. However, we found that the bacterially expressed protein is not suitable for the structural studies because of its low yield, although it may be useful to screen inhibitors of PTK6 *in vitro*. To overcome the problems of the bacterial expression system, we focused on the baculovirus/insect cell system. Although this baculovirus/insect cell system has many advantages for recombinant protein production, it often produces a very low yield. The primary strategy we have employed to induce high-level production is to express the PTK6 protein fused to the C-terminus of a protein that is naturally expressed in a cytosolic form at high levels in insect cell. Fortunately, we have known that *Drosophila* peptidoglycan recognition protein (PGRP)-LB [6] could be chosen for this purpose since PGRP-LB protein was overexpressed in the baculovirus/insect cell system with a high yield of approximately 10 mg per 1 liter culture (Oh BH; personal communication), which is much higher than expected yield. In fact, PGRPs have been shown to participate in peptidoglycan-medicated activation

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of innate immune responses by recognizing or cleaving peptidoglcyan from bacteria. Among twelve PGRP members expressed in *Drosophila*, only PGRP-LB has been well-expressed in cytosolic form although some isoforms of PGRP-LB is recently reported to be secreted into hemolymph [7].

Here, we describe the expression of the catalytic unit of PTK6 in the eukaryotic baculovirus/insect cell system using a PGRP-LB fusion partner. The recombinant protein was expressed in a soluble form with a higher activity and yield compared with the bacterially expressed protein. Therefore, PGRP-LB protein can be used as a fusion partner to increase the expression level of other proteins in the baculovirus/insect cell system.

Materials and methods

Vector constructions

We modified pFASTBAC-HTc (Invitrogen, USA) to generate a vector expressing *Drosophila* PGRP-LB-fusion protein. Prior to the introduction of the *Drosophila* PGRP-LB gene, the hexa-histidine tag region in pFASTBAC-HTc was substituted with deca-histidine tag plus the SmaI site using a PCR technique, resulting in the vector pFASTBAC- 10HTc. Then, the *PGRP-LB* gene (amino acid residue number 11 - 183), which was amplified by PCR, was introduced into the SmaI site of pFASTBAC-10HTc, generating the vector pFASTBAC-HTc-PGRP. We inserted the PTK6 (amino acids 180 - 451) fragment using NcoI and HindIII sites in the pFASTBAC-HTc and pFASTBAC-HTc-PGRP vectors, resulting in pFASTBAC-HTc-PTK6 (180 - 451) and pFASTBAC-HTc-PGRP-PTK6 (180-451), respectively.

Cell culture and transfection

The plasmids, pFASTBAC-HTc-PTK6 (180 - 451) and pFASTBAC-HTc-PGRP-PTK6(180 - 451) were transfected into SF-9 cells using CELLFECTIN reagent (Life Technology, USA) according to the manufacturer's manual (Bac-to-Bac Baculovirus expression system, Life Technology, USA). The recombinant viruses were amplified using SF-9 cells in SF-900II serum-free medium (Life Technology, USA). To produce the recombinant protein, SF-9 cells were maintained and cultured in 2 liter of Grace medium supplemented with 20% SFM medium and 0.1% Pluronic F-68 reagent (Life Technology, USA) as previously described [5]. Once the cells reached a density of 3×10^6 cell/ml, they were infected at a multiplicity of infection of 1-5 and then harvested by centrifugation at 48 h post-infection. The cells were stored at -70℃ until use.

Protein purification

The cells were resuspended in 50 ml of a resuspension buffer [20 mM Tris buffer (pH 8.0), 200 mM NaCl, 2 mM 2-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. For isolation of the soluble proteins, the cells were sonicated briefly and the cellular debris was removed by centrifugation at 12,000 rpm for 30 min at 4℃. For the purification of histidine-tagged and PGRP-LB-fused proteins, approximately 2 ml of Ni-NTA resin (Qiagen) was added into the supernatant in an empty column (Biorad) and the mixture was incubated for 1 hr at 4℃ on a rolling mixer. The resin was recovered by flowing the slurry through the column, and washed with the resuspension buffer containing 20 mM imidazole. The bound fusion protein was eluted from the resin with the buffer containing 200 mM imidazole. Fractions containing the fusion proteins were analyzed on SDS-PAGE to examine expression levels and purity. Fusion protein-containing fractions eluted from the Ni-NTA column were pooled. Recombinant TEV protease was then added at 0.1 mg TEV per milligram of the fused protein, and the cleavage reaction was incubated in the presence of 5 mM 2-mercaptoethanol overnight at room temperature. Progress of the reaction was monitored by SDS-PAGE. The cleavage mixture was concentrated to 0.5 ml using an ultrafiltration device (Millipore, USA). The protein mixture was loaded onto Superdex 200HR 16/60 (Pharmacia). The fractions containing the PTK6 (amino acids 180 - 451) protein were pooled and applied to a His-Trap column (1 ml, Pharmacia, USA) to remove the PGRP-LB tag which was co-eluted with the PTK6 protein from the column. PTK6 (amino acids 180 - 451) was isolated from the column with a linear gradient against the buffer supplemented with 100 mM imidazole. The pure PTK6 protein was dialyzed against a 20 mM MES buffer (pH 6.0) containing 150 mM NaCl and 0.1 mM EDTA, and then concentrated to 8 mg/ml.

In vitro kinase assay

The catalytic activities of PTK6 proteins were determined with a random copolymer of glutamate and tyrosine [poly(Glu,Tyr), Sigma, USA] as a substrate, as described previously [2].

Results and Discussion

Expression of the recombinant PTK6 catalytic unit

We chose the pFASTBAC-HTc vector since the vector is for the Bac-to-Bac expression system, a fast method to construct recombinant baculovius. The resulting pFASTBAC-HTc-PGRP-LB vector contains a deca-histidine-affinity tag, PGRP-LB (11 - 183), a TEV protease recognition site, and an extensive multiple cloning site. Fig. 1 shows the vector structure along with a schematic diagram of expressed proteins and the sequence between the deca-hisitidine tag and the MCS. The deca-histidine tag at the N-terminus of PGRP-LB is used in affinity purification using Ni-NTA chromatography.

The DNA fragment spanning amino acids 180 - 451 of PTK6 was ligated into the vectors pFASTBAC-HTc and pFASTBAC-HTc-PGRP-LB, generating pFASTBAC-HTc-PTK6 (180 - 451) and pFATBAC-HTc-PGRP-LB-PTK-6 (180 - 451), respectively. The two recombinant baculoviruses were generated and amplified according to the Bac-to-Bac method. High-five cells were cultured and infected by each amplified baculovirus. To examine the initial expression level, the recombinant proteins were purified initially using Ni-NTA chromatography from the cell fraction. A high purity of both the proteins was achieved by the one-step metal affinity chromatography, as visualized by coomassie-stained SDS-PAGE gels (Fig. 2). Of note, the PGRP-LB-fused PTK6 protein showed approximately 10-fold higher intensity than His-tagged PTK6. The estimated yields are 2 mg/liter and 0.2 mg/liter culture for PGRP-LB-fused and histidine-tagged proteins, respectively. The PGRP-LB-fused protein was then purified further.

Purification of the recombinant PTK6 catalytic unit

To release the PTK6 protein from the fusion partner, a TEV protease recognition site is located between PGRP-LB and PTK6. Overnight incubation with a recombinant TEV protease resulted in the complete digestion at the TEV protease recognition site as judged by SDS-PAGE (data not shown). The reaction mixture was concentrated and then loaded into a size-exclusion chromatographic column. The size-exclusion chromatographic column was used to remove imidazole from the reaction mixture to prepare the sample for a second round of Ni-NTA chromatography, although it was not very effective to separate the PTK6 protein from the fusion partner because of the similarity in the molecular size between them (Fig. 3A). The observed molecular weight of the PTK6 protein on the size exclusion column was matched with a calculated molecular weight of a monomeric species, ruling out a possibility that the purified PTK6 is a large molecular aggregate. Subsequently, the fractions containing PTK6 (amino acids 180 - 451) were pooled and applied to a second round of Ni-NTA column to remove the fusion protein PGRP-LB which was co-eluted with the PTK6 protein from the size-exclusion column. PTK6 (amino acids 180 - 451) protein was isolated from the column with a linear gradient against the buffer containing 100 mM imidazole, since the PTK6 fragment is bound to Ni-NTA resin with a relatively high affinity even in the absence of a histidine affinity tag (Fig. 3B). The purity of the final PTK6 product was ~95 % as determined by SDS-PAGE. To facilitate X-ray crystallographic study of the PTK6 protein, a high concentration of target protein is required. The solubility of the PTK6 protein was optimized in a MES buffer (pH 6.0) containing EDTA. The final yield

Fig. 1. Schematic drawing of vector map. *Top,* a bar diagram of the protein expressed from pFASTBAC-HTc-PGRP vector are shown. *Bottom,* the DNA and protein sequences from the start codon to the MCS are shown. The TEV protease recognition site is underlined with the site of cleavage indicated by an arrow. The MCS is as it is in pFASTBAC-HTc (Invitrogen).

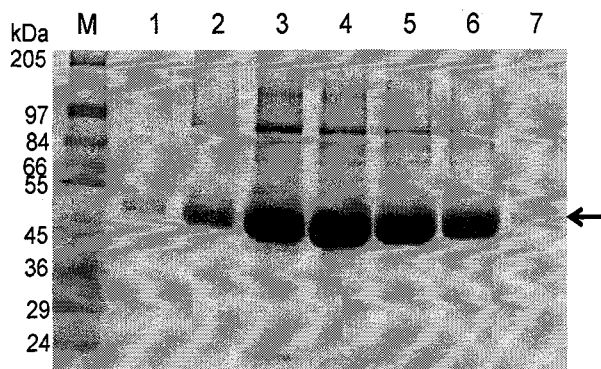was about 0.5 mg purified PTK6 protein per liter culture (Table 1).

Fig. 2. SDS-PAGE gel showing the eluted fractions from the Ni-NTA column. The arrow indicates the PGRP-LB-fused PTK6 protein band (~ 50 kDa). Fifteen μl was taken from each fraction (1 ml) and loaded onto the 15% SDS-PAGE gel for analysis (Lanes 1 - 7). The gel was stained with coomassie blue. Protein molecular weight standards are shown in lane M.

PTK6 expressed in the insect cell shows higher activity

As mentioned above, the bacterially-expressed PTK6 protein [2] is not suitable for structural analysis due to the low yield, although it might be useful for assaying its activity. We assessed the purified PTK6 protein in the baculovirus system by measuring the kinase activity. The PTK6 protein in the baculovirus system showed approximately 1.5 fold higher activity than the bacterially-expressed PTK6 protein (Fig. 4). The PTK6 protein expressed in the baculovirus system may have undergone post-translational modifications such as phosphorylation which enhance the kinase activity, although in vivo post-translational modification of PTK6 has not been known. Alternatively, the bacterially-expressed PTK6 sample could be contaminated with misfolded PTK6 proteins, implying that the PTK6 sample in the baculovirus/insect cell system is more homogenous.

In conclusion, we have presented an efficient procedure for the production of a large quantity of the catalytic unit
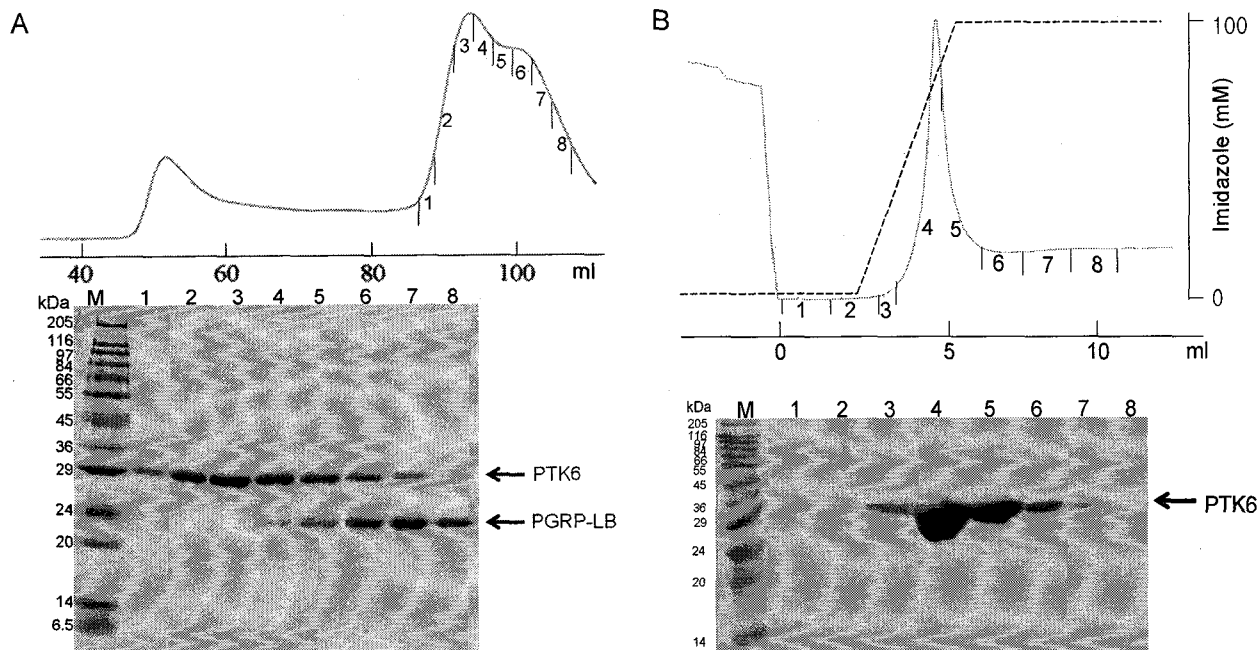

Fig. 3. Purification of recombinant PTK6. (A) Size exclusion chromatography of the cleaved proteins by TEV protease. The PGRP-LB-fused PTK6 protein eluted from the Ni-NTA column was treated with TEV protease, resulting in cleavage between PGRP-LB and PTK6 (data not shown). The reaction mixture was concentrated up to 0.5 ml and loaded into Superdex S-200 HR 16/60 in 20 mM Tris (pH 8.0) buffer containing 200 mM NaCl and 2 mM 2-meracptoethanol. Twenty μl from each fraction (1 ml) was analyzed by 15% SDS-PAGE (bottom). Each lane number (1 - 8) corresponds to the fractions indicated on the chromatogram. (B) Further purification by a second round of Ni-NTA affinity chromatography. The fractions (1 - 4) from the size-exclusion column were pooled and applied to a Ni-NTA column. The absorbance at 280 nm is shown in a solid line, and imidazole gradient referring to the right y axis is shown in a dashed line. Each lane number (1 - 8) in the gel corresponds to the fractions indicated on the chromatogram. Protein molecular weight standards are shown in lane M.

Table 1. Purification of PTK6 (amino acids 180 - 451) protein using two independent fusion proteins in the baculovirus/insect cell system.

Purification step	PGRP-LB-fused PTK6 (180 - 451)	His-tagged PTK6 (180 - 451)
Ni-NTA	2 mg	< 0.2 mg
Size-exclusion	1 mg	ND
Second round of Ni-NTA	0.5 mg	ND

The quantities of the proteins from 1 liter culture of the insect cell were estimated by coomassie-stained SDS-PAGE gels. Histidine-tagged PTK6 (amino acids 180 - 451) was poorly expressed, which did not allow for further purification.
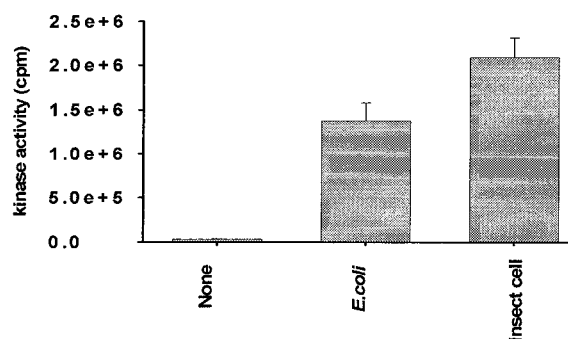
Fig. 4. Activity of PTK-6 in the baculovirus/insect cell system. Phosphorylation of an artificial peptide substrate was monitored by measuring radioactivity from ^{32}P incorporated substrate. The activity of the PTK6 protein in the baculovirus/insect cell system is approximately 1.5 fold higher than that of PTK6 protein expressed in *E. coli*. The data shown are mean ± standard deviation of triplicate experiments.

of PTK6 with a higher activity than bacterial expression. In particular, the use of PGRP-LB as a fusion protein greatly increased the production yield in the baculovirus/insect cell system. Our approach can be applied to the large-scale production of other eukaryotic or human kinases or intracellular proteins. The purified PTK6 protein is now being used for initial crystallization screening, the first step for the determination of a three-dimensional structure.

Acknowledgements

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초록 : 곤충세포에서 새로운 퓨전 단백질인 초파리 유래 PGRP-LB를 이용한 인간 PTK6의 과발현 및 생산

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단백질 티로신 kinase인 PTK6는 대부분의 유방암에서 과발현되며, 암세포의 증식만을 촉진하는데 역할을 한다. 이 연구에서 PTK6의 활성도메인을 초파리의 peptidoglycan recognition protein (PGRP) -LB 단백질을 퓨전 파트너로 사용하여 바큘로바이러스 시스템을에서 과발현하는데 성공하였다. 우리는 PGRP-LB가 바큘로바이러스 시스템에서 잠재적으로 퓨전 단백질로 사용될 수 있는 가능성을 처음으로 발견하였다. 정제된 PTK6단백질은 기존의 박테리아에서 발현된 단백질보다 1.5배 높은 활성을 지녔다. 이 단백질은 PTK6의 분자기전 및 그것의 저해제 개발에 필수적인 결정 구조를 규명하는데 사용될 것이다.