

ORIGINAL ARTICLE

Recombinant Expression and Purification of Mouse Nectin-like 4 Glycoprotein in 293ET Cell Line

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Objective To screen the transient and stable cell lines with high production of Nectin-like 4 (Necl-4) protein.

Methods First, cDNA sequences encoding the extracellular domain of Necls were cloned into the modified vector pAPtag at the N terminus of alkaline phosphatase (AP) for fusion expression. Next, 293ET cells stably expressed Necls-AP fusion protein and secreted it into the culture medium which were detected by the AP activity assay and Western blot analysis. Then, by adding N-glycosylation processing inhibitor kifunensine into the medium, complex glycan was inhibited to generate. The residual glycan of purified protein was removed by endoglycosidase H. Finally, AP protein was removed by using human rhinovirus protease and size exclusion chromatography. The concentration of purified Necl-4 protein was monitored by measuring the absorbance at 280 nm and analyzed by SDS-PAGE.

Results The transient and stable cell lines with high production of Necl-4 protein were screened by the color reaction with the AP-tag in the recombinant vector. The soluble and active form of purified Necl-4 protein was obtained after deglycosylation of native N-glycan protein with an expression level of 4 mg/L culture and purity of 95%.

Conclusions By using modified AP mammalian protein expression system, we can easily screen the high productive stable cell lines by using AP activity assay. By adding mannosidase inhibitor kifunensine into the medium and cutting purified protein by using endoglycosidase H, we can obtain deglycosylated Necl-4 protein in milligram quantities. Our method might throw a light on the expression and purification of glycoprotein for structural and functional studies.

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NECTIN-LIKE (Necl) molecules are a group of immunoglobulin superfamily that include four different members in human and rodents.¹⁻² All four are type I transmembrane proteins that contain three extracellular Ig-like domains, a single transmembrane domain and a short intracellular domain that interacts with protein 4.1 and PDZ-domain proteins. The Necls mediate Ca^{2+} -independent cell adhesion by binding homophilically, as well as heterophilically, to other Necls or their related nectins. Based on the previous studies, it has been reported that the Necls are mainly expressed in the nervous system and important in the organization of the plasma membrane at specific areas of cell-cell interaction.³⁻⁸

As a member of Necl family, Necl-4 (SynCAM4) plays an essential role not only in mediating Schwann cell-axon contact, but also in myelin membrane growth in the peripheral nervous system.⁹⁻¹² Genetic deletion of Necl-4 results in myelin abnormalities resembling Charcot-Marie-Tooth neuropathy.¹³ Previous works also demonstrated that Necl-4 suppresses the growth and tumorigenic ability of colon cancer cells and can cause male infertility by lack of interaction with protein 4.1G.¹⁴⁻¹⁵ Many aspects of its function point to its potential utility as an ideal structure study model and novel drug targets in the treatment of many diseases.

Expression and purification of Necl family members have been reported in a variety of expression systems for their structural study.¹⁶⁻¹⁸ Constructs expressing Necls have been reported in *Escherichia coli* (*E.coli*) and mammalian expression system from the mouse and human sources. But their studies mostly focus on the V domain and the expression of full three extracellular domain has not been reported. This is particular in light of the report that the extracellular glycosylation state of Necls is important for the maintenance of proper folding and their cis-interaction.¹⁹ In mammalian system, development and validation of stable cell lines can be high-risk, expensive, time- and labor-consuming. For improving the screening method for stable cell lines, we use a cDNA sequence encoding the extracellular domain of Necls to be in-frame fused to the alkaline phosphatase (AP) at the C terminus in pAPtag cloning vectors, the Necl-AP fusion protein, when expressed in 293ET cells can be secreted at high levels into the culture medium and thus easily detected by either the AP activity assay or Western blot analysis. The adding cleavage site of tobacco etch

potyvirus (TEV) protease or human rhinovirus 3C (HRV 3C) protease can be used completely to separate the target protein and AP tag protein. The development of fast and easy screening method is important for protein screening, and may use in pharmaceutical drug discovery and gene therapy research.

In eukaryotes, native glycosylated recombinant protein can be secreted in soluble form in mammalian expression systems. However, the conformational flexibility of glycan at the surface of the protein hampers crystal growth. Strategies have emerged to promote crystallogenesis through either shielding or reducing surface disorder. For solving the problem of glycosylation for protein crystal growth, we added *N*-glycosylation processing inhibitor kifunensine²⁰⁻²¹ to increase the sensitivity of glycoprotein Necl-4 to endoglycosidase H (Endo H), and thus, glycan can be easily removed by this combination method. The goal was to produce purified glycoprotein in sufficient quantities for further functional and structural studies.

MATERIALS AND METHODS

Bacterial strains

E.coli DH5 α strains were purchased from Transgen (Beijing, China) as host cells for cloning.

Modified pAPtag5 vector

The expression vector pAPtag5 was purchased from GenHunter (Nashville, TN, USA).²²⁻²⁴ For the purpose of transfect of mammalian cells, pAPtag5 expression vector integrates several elements necessary for mammalian transcription initiation (CMV promoter) and transcription termination (SV40 poly A signal). Besides, it contains the Igk chain signal peptide for the purpose of protein secretion into the medium and a Zeocin resistance gene for selection of stable transfectants. The essence of this vector is to allow a cDNA sequence encoding any secreted protein to be fused in-frame to human placental secreted AP. The resulting AP fusion protein, when expressed in 293ET cells, can be secreted at high levels into the culture medium and thus easily and fast detected by AP activity assay, hence its easy for protein expression detection and stable cell line screening.

The cleavage site of TEV protease (Glu-Asn-Leu-Tyr-Phe-Gln ↓ Gly) or HRV 3C protease (Leu-Glu-Val-Leu-Phe-Gln ↓ Gly-Pro) DNA sequence was inserted into the vector pAPtag5 between the *Bgl*II and *Bsp*EI in

the proper orientation and reading frame to form pAP-tag5-TEV or pAPtag5-3C vector. Restriction enzymes were all from New England Biolabs (Beverly, MA, USA).

Cell lines and cell culture

Human embryonic kidney 293ET cell line (kindly provided by Chengyu Jiang, Peking Union Medical College) was maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco Waltham, MA, USA) with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin under a humidified atmosphere of 5% CO₂ and 95% air at 37°C. 293ET cells stably expressing mouse recombinant Necls were maintained in the above medium supplemented with 500 µg/ml Zeocin (Invitrogen, Waltham, MA, USA).

Cloning and expression of Necls

cDNA fragments encoding Necl extracellular region [residues 23-328 for Necl-1 (AF195662), 48-349 for Necl-2 (NM_001025600), 34-336 for Necl-3 (NM_178721) and 25-322 Necl-4 (NM_153112)] were amplified by polymerase chain reaction (PCR) with pGEM-T vector (Promega, Madison, WI, USA). Primers used for Necls extracellular region cloning are indicated in Table 1. PCR was performed using the above primers and PrimeStar DNA polymerase (Takara, Japan).

The product of Necl-1, Necl-2 and Necl-4 were subcloned into the *HindIII* and *BglII* sites of mammalian expression vector pAPtag5, pAPtag5-TEV and pAPtag5-3C in-frame with the AP and 6xHistidine (His) tags, and Necl-3 was subcloned into *BglII* sites alone after sequencing correctly. For the establishment of stable colonies expressing Necl-AP fusion protein, 293ET cells were transfected using Lipofectamin 2000 (Invitrogen) as described by the manufacturer. Single colonies were obtained by limiting dilution in medium supplemented with 500 µg/ml Zeocin. Colonies were screened for expression level by para-nitrophenylphosphate (pNPP, Sigma Aldrich, St. Louis, MI, USA) substrate reaction and verified by Western blotting analysis.

Table 1. Primers used for Necl extracellular region cloning

Genes	5' primer	3' primer
mNecl-1	5'-CCCA AAGCTT ACATCTTTCCAGGACGATAG-3'	5'-GA AAGATCT GTGGTAGGTACTGGAGGAC-3'
mNecl-2	5'-CCCA AAGCTT ACCAGAATCTGTTTACTAAAGACGTG-3'	5'-GA AAGATCT GTGGTCCACTGCCCC-3'
mNecl-3	5'-GA AAGATCT CAGTTTCCAATACTCAGAATG-3'	5'-GA AAGATCT TATGGTCAGGGCCATTCTG-3'
mNecl-4	5'-CCCA AAGCTT ACCAGGAAGTACAGACCGAG-3'	5'-GA AAGATCT GGCGTAAGGAACCGATG-3'

mNecl: mouse Nectin-like protein gene. The boldfaced bases are restriction enzyme cutting sites.

sis of culture supernatants using the His antibody.

AP activity assay

Equilibrate pNPP to room temperature before opening. Add 100 µl of the pNPP solution and 100 µl supernatant of cell culture medium into each microplate well of 96-well plates. Shake plate 10 seconds and incubate plate at room temperature for 5 minutes or until sufficient color develops. To stop the reaction, add 50 µl of 2 mol/L NaOH to each well. Measure the absorbance at 405 nm. Each reaction is repeated three times.

Western blotting assay

The detailed Western blotting procedures have been described previously.²⁵ The protein samples was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then the gel was transferred onto nitrocellulose membrane. Protein samples were analyzed using monoclonal mouse anti-His tag (1:800, Qiagen, Duesseldorf, German) antibodies. The secondary antibody, rabbit anti-mouse immunoglobulin G conjugated horseradish peroxidase (HRP) was purchased from ZSGB-BIO (Beijing, China).

Protein deglycosylation

Kifunensine (Sigma Aldrich) was added to the DMEM medium to the final concentration of 2.5 µmol/L or 5 µmol/L. After 48 hours, take 250 µl supernatant of different kifunensine medium with 250 µl of the pNPP solution into each 1.5 ml EP tube. AP activity assay and Western blotting assay have been described above.

Necl-4 protein was purified from Necl4-AP/293ET stable cell line cultured with 5 µmol/L kifunensine. The Necl-4 protein was deglycosylated with Endo H (New England Biolabs) and peptide N-glycosidase (PNGaseF, New England Biolabs) according to the manufacturer's instructions. In brief, 10 µg of Necl-4 protein at 37°C, with 1 kU of the glycosidases in a final volume of 20 µl was used to detect the performance of Endo H and PNGaseF deglycosylation from kifunensine-free

medium. Next, 10 µg of Necl-4 protein at 37°C, was incubated with series of 0.25, 0.5 and 1 kU of Endo H enzyme and 0.5 kU of PNGaseF enzyme in a final volume of 20 µl plus 5 µmol/L kifunensine in non-reduced state. The same amount of Necl-4 protein was incubated with 1 kU Endo H and 0.5 kU PNGaseF enzyme plus 5 µmol/L kifunensine in reduced state. Incubation at 65°C for 10 minutes inactivated the glycosidases. After enzymatic reaction, the protein was subjected to 9705×g centrifugation for 30 minutes, and then analyzed by SDS-PAGE/Coomassie blue staining.

Large-scale expression and purification of Necl-4 protein using size-exclusion chromatography and analysis of yield and purity

For large-scale expression and purification of Necl-4 protein, stable clones expressing mouse Necl-4 AP fusion protein were grown to confluence in Tripleflasks (Corning, Somerset, NJ, USA) using DMEM supplemented with 2% heat-inactivated FBS, 100 µg/ml penicillin/streptomycin, 2 mmol/L L-glutamine, 500 µg/ml Zeocin and 5 µmol/L kifunensine. Supernatant containing the recombinant protein was collected 4 days post-confluence. The supernatant was spun at 2426×g at 4°C for 30 minutes. The medium was then loaded by gravity onto a 10 ml Ni-NTA column (Qiagen) which had been equilibrated with 20 mmol/L Tris-HCl (pH 8.0) and 300 mmol/L NaCl. The column was washed with 20 mmol/L imidazole and finally eluted with 250 mmol/L imidazole in the same buffer. The purified Necl-4 AP fusion protein was dialyzed and concentrated with a 15 ml Amicon Ultra centrifugal filter device with 30-kDa weight cutoff (Millipore, Darmstadt, Germany) against 20 mmol/L Tris-HCl (pH 8.0) and 300 mmol/L NaCl buffer. The protein was cleaved from the AP-tag using recombinant human HRV 3C (Sinobiological, Beijing, China) with a 6xHis tag at 4°C overnight. Following the cleavage, the protease and AP-tag was removed by adding the reaction mixture to 10 ml of Ni-NTA agarose (Qiagen) beads equilibrated with cleavage buffer. Fractions were concentrated with a 15 ml Amicon Ultra centrifugal filter device with 10-kDa weight cutoff (Millipore). Totally, 2.5 mg of the protein was digested with 100 kU of Endo H for 3 hours at 37°C. Endo H was removed by affinity chromatography with amylose resin (New England Biolabs) according to the manufacturer's instructions. The deglycosylated digested supernatant and wash solution [20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl and 250 mmol/L imidazole]

were concentrated and injected in 500 µl aliquots over a Superdex 75 (10/30) gel filtration column connected to an ÄKTA FPLC purifier (GE Healthcare, Boston, MA, USA). The flow rate was maintained at 0.5 ml/min and fractions were collected. The fractions were pooled together and concentrated with a 15 ml Amicon Ultra centrifugal filter device with 10-kDa weight cutoff (Millipore) against 20 mmol/L Tris-HCl (pH 8.0) and 150 mmol/L NaCl. Protein concentration was measured by the absorbance at 280 nm. Protein samples from each step were analyzed by SDS-PAGE. For SDS-PAGE analysis, samples containing reducing buffer were heated to 98°C for 5 minutes before loading onto 12% SDS-PAGE gels. The gels were stained with Coomassie blue staining.

RESULTS

pAPtag5 vector modification

Due to the purpose of structural study, 80 kDa AP protein need to be cut off from the fusion protein. We then constructed two pAPtag5 derived vectors, pAPtag5-TEV and pAPtag5-3C (Fig. 1). A specific protease cleavage site, TEV protease coding sequence or HRV 3C protease coding sequence, was introduced between the *Bgl*III and *Bsp*EI in-frame. After cleavage the fusion protein, AP part, containing the His tag, can be easily removed through Ni-Affinity Chromatography. Target protein can derived from flowthrough.

Expression level of different Necl-AP fusion protein

In mammalian system, development and validation of stable cell lines can be high-risk, expensive, time- and labor-consuming. For improving the screening method for stable cell lines, we used a cDNA sequence encoding the extracellular domain of Necls to be in-frame fused to AP at the C terminus in pAPtag cloning vectors, the Necl-AP fusion protein, when expressed in 293ET cells can be secreted at high levels into the culture medium and thus easily detected by either the AP activity assay or Western blot analysis. As indicated in Fig. 2A, color reaction showed the expression level of Necls-AP and AP alone were generally high, Necls-AP with TEV cleavage site except Necl3-AP fusion proteins exhibited moderate expression level, Necl4-AP with 3C cleavage site appeared moderate expression level, but not for other Necls-AP proteins. AP activity assay revealed the absorbance value of Necls-AP fusion protein at 405 nm was

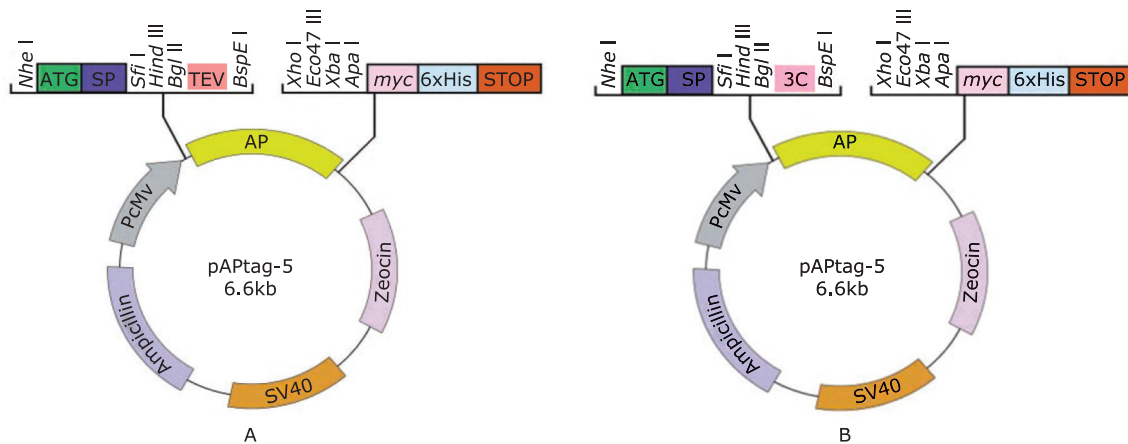


Figure 1. Schematic diagram of pAPtag5-TEV (A) and pAPtag5-3C (B) modified from mammalian expression vector pAPtag5. The vector contains the Igk chain singal peptide, followed by three enzyme restriction sites indicated in italicized that are available for cloning. SP: signal peptide; P_{CMV}: cytomagalovirus immediate/early promoter and enhancer element; TEV: tobacco etch potyvirus coding sequence; 3C: rhinovirus 3C protease coding sequence; AP: alkaline phosphatase coding sequence; SV40: SV40 DNA replication.

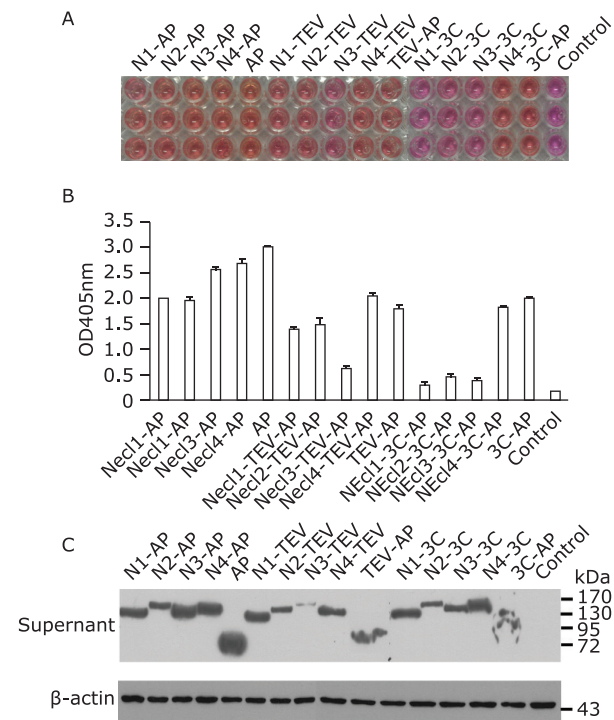


Figure 2. Fast and easy screening the expression level of Necl-AP fusion protein with pAPtag5, pAPtag5-TEV or pAPtag5-3C recombinant vectors in 293ET cells. A. Color reaction results of the pNPP and AP fusion protein. Orange color means high level expression, pink color means low level expression. B. The column chart above shows the results of absorbance of Necl-AP fusion protein at 405 nm. Each reaction is repeated three times. C. Immunoblot analysis of protein expression in 293ET cells transduced with pAPtag5-Necl, pAPtag5-TEV-Necl or pAPtag5-3C-Necl recombinant vectors. Necl: Nectin-like protein; AP: alkaline phosphatase; pNPP: para-nitrophenylphosphate.

coincided with the results of color reaction (Fig. 2B).

To further confirm if the color reaction can properly reveal the expression level of protein, Western blot analysis of the samples was conducted (Fig. 2C). The results confirmed the expression level of Necl-AP, Necl-AP with TEV cleavage site and Necl-AP with 3C cleavage in 293ET cells was stable. It indicated that this fast and easy methods could be applied in the screening and comparison of Necl-AP fusion protein with pAPtag5, pAPtag5-TEV or pAPtag5-3C recombinant vectors in 293ET cells.

Protein deglycosylation

Necl-4 plays critical role in axonal myelination in the peripheral nervous system and several diseases. In addition, Necl4-AP with 3C cleavage site exhibited favorable expression level. Therefore, we picked Necl-4 with 3C cleavage site for further deglycosylation study.

Kifunensine is an alkaloid compound, originally isolated from the actinomycete *Kitasporia kifunensis*, that exhibits potent and selective inhibition of class I α -mannosidases. Kifunensine is used to suppress endoplasmic reticulum-associated degradation (ERAD) via the inhibition of endoplasmic reticulum-associated mannosidase activity. By adding 2.5 or 5 μ mol/L kifunensine into the culture medium, we found that the AP activity assay showed there were no significant color changes (Fig. 3A). But the results of immunoblot analysis showed that after adding kifunensine the Necl4-AP fusion protein exhibited slightly lower molecular weight than normal one (Fig. 3B).

After the glycosidases treatment, molecular weight of purified Necl-4 had no change without adding kifunensine in the culture medium (Fig. 3C). It means Necl-4 showed Endo H and PNGaseF resistance without kifunensine. On the other hand, Necl-4 in the presence of 5 $\mu\text{mol/L}$ kifunensine yielded essentially glycan-free material at the various concentrations of Endo H (Fig. 3D, Lanes 2-4), while N-glycans of Necl-4 still showed PNGaseF resistance in the presence of kifunensine (Fig. 3D, Lane 5). Deglycosylation of Necl-4 protein was optimal under denaturing conditions (using SDS and heat) by Endo H (Fig. 3D, Lane 6) or PNGaseF (Fig. 3D, Lane 7). That means glycan structure hampered the recognition site for PNGaseF under non-reducing condition.

Large-scale expression and purification of Necl-4 protein

For large-scale expression and purification of Necl-4 protein, stable clones expressing mouse Necl-4 AP fusion protein were grown to confluence in Tripleflasks with 5 $\mu\text{mol/L}$ kifunensine. The supernatant was purified using Ni-NTA column. The purified Necl-4 AP fusion protein is shown as Fig. 4A lane 1. The protein

was then cut by HRV 3C protease to remove AP-tag (Fig. 4A, lane 2). Since AP-tag and HRV 3C protease had His-tag, we can easily removed them by purified using Ni-NTA column again (Fig. 4A, lane 3). Next, 2.5 mg glycan-Necl4 protein was digested with 100 kU of Endo H for 3 hours at 37°C (Fig. 4A, lane 4). Endo H which fused to Myelin Basic Protein (MBP) was removed by affinity chromatography with amylose resin (Fig. 4A, lane 5). Finally, the deglycosylated Necl4 protein fractions were concentrated (Fig. 4A, lane 6) and injected in 500 μl aliquots over a Superdex 75 (10/30) gel filtration column connected to an ÄKTA FPLC purifier. The final yield is about 4 mg/L culture with at least 95% purity.

DISCUSSION

Mammalian expression system can be prepared for expression of more complex proteins which also need proper post-translational modifications. Cell lines can be used for transient or stable cell line expression, which offers higher productivity and less variation if long-term production of a target protein is required. However, development and validation of stable cell lines can be high-risk, expensive, time- and labor-con-

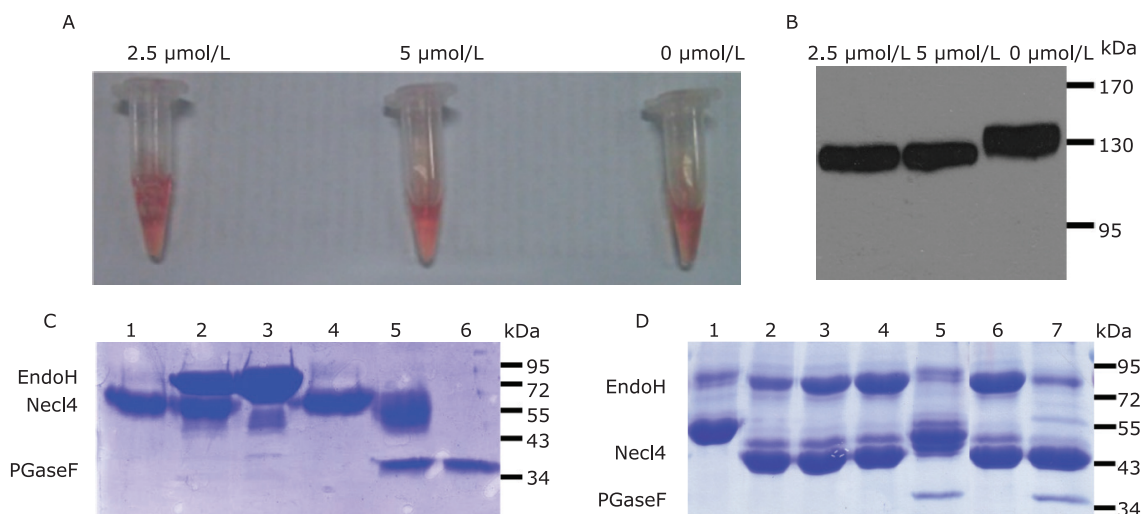


Figure 3. Endo H and PNGaseF digestion of Necl-4 produced in 293ET cells before and after adding kifunensine under various conditions. A. Color reaction results of pNPP and Necl4-AP fusion protein secreted from Necl4-AP/293ET stable cell line subjected to different concentrations of kifunensine. B. Immunoblot analysis of protein expression in Necl4-AP/293ET stable cell line cultured with kifunensine. C. Endo H and PNGaseF digested Necl-4 without adding kifunensine in the culture medium. Lane 1: purified Necl-4 protein; Lane 2: Necl-4 cut by 1 kU Endo H after 6 hours; Lane 3: Endo H protein; Lane 4: purified Necl-4 protein; Lane 5: Necl-4 cut by 1 kU PNGaseF after 6 hours; Lane 6: PNGaseF protein. D. Endo H and PNGaseF digested purified Necl-4 by adding kifunensine in the culture medium. Lane 1-5 purified Necl-4 protein was digested in non-reduced state. Lane 1: Necl-4 protein; Lane 2-4: Necl-4 cut by 0.25, 0.5 and 1 kU Endo H after 1 hour; Lane 5: Necl-4 cut by 0.5 kU PNGaseF after 1 hour. Lane 6-7 Necl-4 protein was digested in reduced state. Lane 6: Necl-4 cut by 1 kU Endo H after 6 hours; Lane 7: Necl-4 cut by 0.5 kU PNGaseF after 6 hours. Molecular weight of protein (Endo H: 80 kDa; Necl-4: 55 kDa; PNGaseF: 36 kDa). Endo H: endoglycosidase H. PNGaseF: peptide N-glycosidase.

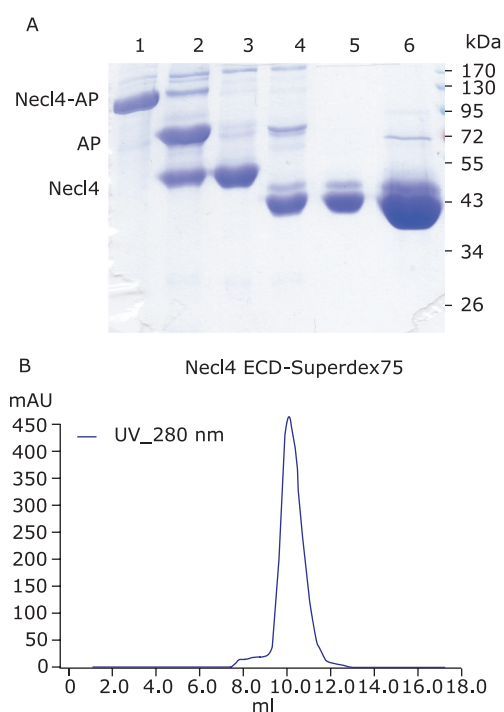


Figure 4. The large-scale purification process of deglycosylated Necl4 protein. A. Purification process of mouse Necl-4 protein in 293ET cell line by using SDS-PAGE analysis with Coomassie blue staining. Lane 1: purified Necl4-AP fusion protein from cell supernant when clone was treated with 5 μ mol/L kifunensine; Lane 2: after cleavage with 3C protease; Lane 3: after 3C protease and AP-tag were removed; Lane 4: after glycan chain of Necl-4 cut with Endo H enzyme; Lane 5: the purified Necl-4 after amylose resin; Lane 6: the concentrated Necl-4 protein after dialysis. Molecular weight of protein (Necl4-AP fusion protein: 130 kDa; AP: 80 kDa; Necl-4 before cutting glycan chain: 50 kDa; Necl-4 after cutting glycan chain: 43 kDa). B. Necl-4 extracellular coding domain purified by superdex 75 gel filtration column connected to an ÄKTA FPLC purifier. The main panel shows the elution profile of Necl-4 extracellular coding domain from the column. The X-coordinate shows the elution volume of protein, the Y-coordinate shows the absorb of protein at 280 nm.

suming. Here we use AP protein as a tag, the detection and analysis of secreted protein of stable cell lines can be performed by reaction of AP fusion protein with its substrate pNPP, a yellow water-soluble reaction product can be formed within 5 minutes. Protein expression level can be compared quantify through detection of the absorbs light at 405 nm by spectrophotometer. The adding cleavage site of TEV protease or HRV 3C protease can be used to completely separate the target protein and AP tag protein. The development of this method is an important research tool in protein screening, and may use

in high-throughput expression and purification in pharmaceutical drug discovery and gene therapy research.

In eukaryotes, glycosylation is one of the most common posttranslational modifications occurred in protein biosynthesis and serves a variety of structural and functional roles in membrane and secreted proteins. Heterologous protein production in mammalian expression systems has the advantage that such proteins are able to fold under native condition and can be purified from the medium if they can be expressed in secreted soluble forms, while most proteins in this class would otherwise have to be refolded from denatured precipitates produced in bacteria. At the same time, it is troublesome to crystallography because it prevents the formation of reproducible lattice contacts and therefore forming crystals. Strategies have emerged to promote crystallogenes through either shielding or reducing surface disorder. Here we show that glycoprotein Necl-4 stably expressed in 293ET cells in the presence of N-glycosylation processing inhibitor kifunensine is highly sensitive to Endo H, while there is no effect without adding inhibitor or use other glycosidase under non-reducing condition. Moreover, treatment with kifunensine leads to a much more homogeneous product that may help the crystallizability of the protein. Therefore, adding inhibitors could be trialed for the crystallization of recalcitrant glycosylation proteins.

In summary, by using modified AP mammalian protein expression system, we can easily screen the high productive stable cell lines from AP activity assay. By adding mannosidase inhibitor kifunensine into the medium and cutting purified protein by using Endo H, we can obtain deglycosylated Necl-4 protein in milligram quantities. Our method might throw a light on the expression and purification of glycoprotein for structural and functional studies.

Conflicts of interest statement

The authors have no conflicts of interest to disclose.

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