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Heterologous high yield expression and purification of neurotensin and its functional fragment in *Escherichia coli*

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ABSTRACT

Peptide synthesis is widely used for the production of small proteins and peptides, but producing uniformly isotopically labelled peptides for NMR and other biophysical studies could be limited for economic reasons. Here, we propose a use of a modified pGEV-1 plasmid to express neurotensin (NT_{1-13}), pGlu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]-OH, as a C-terminal fusion protein with the GB1 domain of streptococcal protein G. The free carboxyl-terminus is important for the function of several peptide hormones, including neurotensin. Therefore, for the pGEV-NT_{1-13} construct, the C-terminal pGEV-encoded 6×His tag was removed and an N-terminal 8×His tag was introduced for affinity purification. To facilitate removal of tags using CNBr cleavage, a methionine was introduced at the N-terminal of the peptide. Furthermore, this pGEV-NT_{1-13} plasmid was used as a template to include a Pro-7 to Met mutation for CNBr cleavage, giving NT_{8-13}, the sub-fragment crucial for the biological activity of this peptide. These two constructs are being used to produce uniformly labelled NT_{1-13} and NT_{8-13} in high yield and in a cost effective way, using cheap ^{15}N and/or ^{13}C source. The modification proposed here using the pGEV-1 plasmid could be an alternative option for the high expression of other isotopically labelled and unlabelled short peptides, including hormones and hydrophobic membrane peptides.

Introduction

Neurotensin (NT_{1-13})[1], first isolated from bovine hypothalamic extracts [1], is a tridecapeptide (pGlu[1]-Leu[2]-Tyr[3]-Glu[4]-Asn[5]-Lys[6]-Pro[7]-Arg[8]-Arg[9]-Pro[10]-Tyr[11]-Ile[12]-Leu[13]-OH). It acts as a neuromodulator in the brain and as a local hormone in the gastrointestinal tract [2]. Only amino acids 8–13 of NT_{1-13} (NT_{8-13}) are required for biological functions including analgesia, hypothermia, and increased locomotive activity [3,4]. The binding of [^{3}H]-NT_{1-13} and NT_{8-13} to the NTS1 receptor gives dissociation constants (K_d) of 2.1 nM (±12%) and 0.63 nM (±11%), respectively [5]. The function of the seven N-terminal amino acids of NT_{1-13} (NT_{1-7}) is still not understood.

Currently, there are a very limited number of crystal structures of diffusing-ligand activated GPCRs, and molecular details of the binding sites for agonist-binding GPCRs are not known from directed studies [6–8]. So far, a crystal structure of neurotensin has not been reported. NMR, which is a powerful method for structural determination, could be an option to determine the structure of bound neurotensin at high resolution. Solution-state NMR studies (NOEs and J-couplings) of NT_{1-13} in the absence of the receptor have revealed that no preferred conformation exists in solution [9]. A series of double-quantum filtering 2D correlation experiments using solid-state NMR (SSNMR) exhibited a disordered state of the NT_{8-13} in the absence of receptor and a β-strand conformation of the ligand in the presence of its receptor NTS1 [10]. Mutagenesis and structure–activity studies combined with modelling techniques [11,12] were also utilised to predict the receptor binding site and the conformation of bound NT_{8-13}. The model suggests that the ligand adopts a β-turn conformation, in agreement with predictions for over 100 GPCR-activating peptides [13], which contradicts the SSNMR data presented for NT_{8-13} [10]. SSNMR experiments by Williamson et al. [14] have indicated that both tyrosine side chain and the C-terminal end of NT_{8-13} interact strongly with the receptor upon binding. Although many attempts have been made to resolve the structure of the bound NT_{8-13} forms, its bound conformation is still under dispute.

Here, we propose an expression system for the production of neurotensin in a bacterial system using the pGEV-1 vector [15] encoding the immunoglobulin-binding domain of streptococcal protein G (GB1 domain), target peptide and 6×His. The approach has the potential for efficient uniform or selective isotopic labelling for NMR studies. The GB1 domain is small, has a stable fold and high bacterial expression capability and therefore has been widely

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[1] *Abbreviations used:* CNBr, cyanogen bromide; ESI, electrospray ionisation; GB1, immunoglobulin-binding domain of streptococcal protein G; GEV-NT_{1-13}, vector for expression of the fusion protein GB1 linked to NT_{1-13}; GEV-NT_{8-13}, vector for expression of the fusion protein GB1 linked to NT_{8-13}; GPCR, GTP-binding protein-coupled receptor; IPTG, isopropyl β-D-1-thiogalactopyranoside; NT_{1-13}, neurotensin; NT_{1-7}, seven N-terminal amino acids of NT_{1-13}; NT_{8-13}, residues 8–13 of NT_{1-13}; NTS1, neurotensin receptor type 1; PMSF, phenylmethanesulfonyl fluoride; YNB, yeast nitrogenous base.

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used as a fusion tag to produce several peptides as soluble proteins at high yield [16–18]. The sequence encoding the NT_{1-13} peptide with an N-terminal 8×His-Met and C-terminal stop codons was inserted into the pGEV-1 vector. The resulting plasmid encodes the fusion protein GB1–8×His-Met-NT_{1-13}. The inclusion of C-terminal stop codons prevents the addition of the vector-encoded C-terminal 6×His tag, which would block the functionally important C-terminus of NT_{1-13}. Here, by extension of the strategy described above, the small peptide, neurotensin (8–13) was also successfully expressed in *Escherichia coli* for the first time with good yield by mutating the Pro-7 of the NT_{1-13} construct to Met to provide the required CNBr cleavage site.

Materials and methods

Construction of pGEV-NT_{1-13}

Oligonucleotides (MWG-Biotech) coding for 8×His for affinity purification, followed by a CNBr cleavage site (Met) and the NT_{1-13} sequence (Fig. 1) were synthesised using optimal codons for *E. coli* (http://www.kazusa.or.jp/codon). The oligonucleotides were designed to provide an overhanging *NheI* site on the 5′ end of the sense strand, and an overhanging *XhoI* site on the 5′ end of the antisense strand for cloning into the pGEV-1 vector [15]. Sense and antisense oligonucleotides were annealed (95 °C; 1 h), followed by cooling (65 °C; 30 min) prior to ligation to the vector. The plasmids were transformed into *E. coli* XL1 Blue (Stratagene) and plated onto LB-agar plus 100 μg/mL ampicillin. Presence of the correct insertion was verified by DNA sequencing. For expression, plasmids were transformed into *E. coli* BL21(DE3) (Novagen).

Construction of pGEV-NT_{8-13}

The recombinant plasmid pGEV-NT_{1-13} was used as a template for the generation of Pro-7 to Met (P7M) mutation. The mutant plasmid was designed to provide another CNBr cleavage site to obtain the NT_{8-13} peptide. A pair of complementary mutagenic oligonucleotide primers (MWG-Biotech) (Fig. 1) was used to replace Pro-7 with Met using site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene). The mutant clones were verified by DNA sequencing.

Expression and purification of GB1 fusion proteins

E. coli cells strain BL21(DE3) harbouring the pGEV-NT_{1-13} and pGEV-NT_{8-13} plasmids were grown at 37 °C in 50 mL M9 minimal media plus 100 μg/mL ampicillin containing yeast nitrogenous base (YNB, BD) overnight with 200 rpm shaking. One-litre cultures of M9 minimal media containing YNB were inoculated with 50 mL of overnight culture of the fusion protein construct, grown at 37 °C with shaking to an OD_{600} 0.8, and induced with 1 mM isopropanyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Cells were harvested by centrifugation (6000*g*; 20 min; 4 °C), resuspended in cold 15–20 mL 50 mM Tris–HCl (pH 8.0), 500 mM NaCl, 5 mM imidazole and 1 mM phenylmethanesulfonyl fluoride (PMSF). The cell suspension was disrupted by using a French Press Cell three times at 10,000 psi. The crude lysate was centrifuged (70,000*g*; 90 min), and the supernatant fraction was passed through 0.40 and then 0.22 μm filters (Millipore). A 5 mL His-Trap column was charged with 50 mM $NiCl_2$ and equilibrated in 5 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl (pH 8.0) before the supernatant fraction was loaded onto the column. The column was washed with 40 mM imidazole, 500 mM NaCl, and 10 mM Tris–HCl (pH 8.0) and the GB1 fusion proteins were eluted with 500 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl (pH 8.0). The fraction containing the target fusion protein was lyophilised, resuspended in water, desalted using two 5-mL Sephadex G25 superfine columns (10 mM NH_4HCO_3 at pH 8.0 for equilibrium and buffer exchange), and lyophilised.

Cleavage of GB1 fusion proteins and purification of peptides

Neurotensin peptides were cleaved from the GB1 fusion proteins by CNBr. The lyophilised proteins were dissolved in 0.1 M

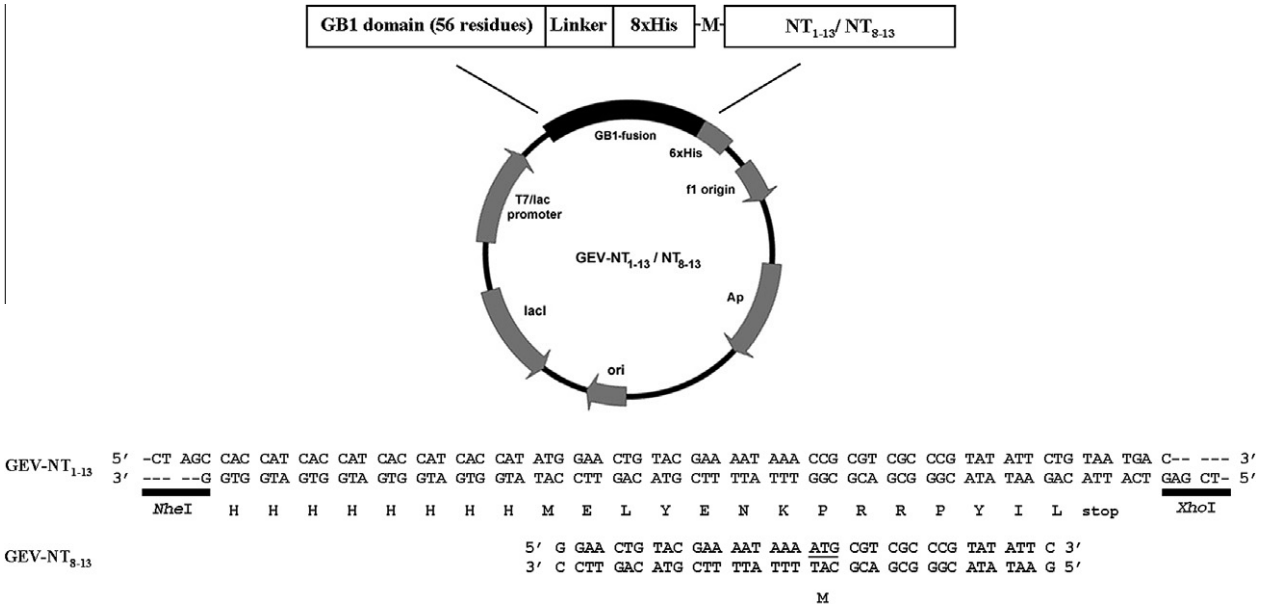

Fig. 1. Scheme of GEV-NT_{1-13} and GEV-NT_{8-13} (P7M) plasmids. A pGEV-1 plasmid map is shown with a schematic of the insertion. Oligonucleotide sequences used for cloning the full length peptide (NT_{1-13}) and site-directed mutagenesis to give pGEV-NT_{8-13} are given. In the pGEV-1 plasmid, a noncleavable linker and 6×His tag is encoded (plasmid map). By the inclusion of stop codons downstream of the NT_{1-13} coding sequence, this tag is not included in the translated product. A sequence encoding 8×His-Met-NT_{1-13} was inserted into this plasmid using *NheI* and *XhoI* cloning sites. The inclusion of Met generates a CNBr-cleavable site. By placing stop codons between the C-terminal of NT_{1-13} and the vector-encoded 6×His tag, a free C-terminus can be generated (introducing a Met site at the 3′ end of an insert is not a viable strategy, as it would yield a C-terminal homoserine upon CNBr cleavage of the product). The GEV-NT_{8-13} vector was constructed by using pGEV-NT_{1-13} as a template for a P7M substitution using site-directed mutagenesis. Underlined letters indicate mutated nucleotide residues.

HCl at the final concentration of 5 mg/mL. Solid CNBr was then added to a final molar ratio of 100:1 (peptide:CNBr). The sample was sealed under a nitrogen atmosphere and incubated at room temperature in the dark for 24 h. The reaction was terminated by diluting the sample 10-fold with dH_2O and lyophilised. Samples were subjected to a preparative C18 reverse-phase HPLC column (Hichrom) with a linear gradient of acetonitrile from 20% to 40% (v/v) over 45 min. Individual fractions were collected and verified by electrospray ionisation (ESI) mass spectrometry. Fractions containing pure peptide were pooled and twice lyophilised to remove all organic solvents. To monitor purity, analytical HPLC chromatography (C18 reverse-phase column, Hichrom) was performed.

Results and discussion

To enable the expression of NT_{1-13} and the 8–13 active sub-fragment in *E. coli*, two vectors were constructed using the pGEV-1 expression vector (Fig. 1). Firstly, 8×His-Met-NT_{1-13} was fused in-frame with the GB1 domain by the ligation of a synthetic double-stranded oligonucleotide into the *NheI*/*XhoI* cloning site (Fig. 1). Stop codons at the 3′ end of NT_{1-13} were included to disrupt the addition of the vector-encoded C-terminal 6×His tag. The resulting pGEV-NT_{1-13} plasmid was used as a template for site-directed mutagenesis. The replacement of Pro-7 of NT_{1-13} with Met provides a CNBr cleavage site to yield the pGEV-NT_{8-13} expression construct (Fig. 1).

pGEV-NT_{1-13} and pGEV-NT_{8-13} were transformed into BL21(DE3) cells and expression was induced with 1 mM IPTG for 4 h at 37 °C with shaking at 200 rpm. Analysis of soluble fractions by SDS–PAGE shows induced protein bands migrating between the 6 and 14 kDa markers, agreeing with the predicted molecular weight of the fusion proteins of 9.2 kDa (Fig. 2A). The GB1–8×His-Met-NT(P7M) fusion protein was expressed as soluble protein at high level, similar to GB1–8×His-Met-NT_{1-13}. Expression of GB1 fusion proteins in M9 culture was achieved at up to 12.8 mg/10^8 cells, approximately 80% of the yield achieved with LB media (data not shown), suggesting that expression of uniformly isotopically labelled peptides using minimal media should be high.

The fusion proteins were purified from the soluble cell fraction using Ni^{2+}-affinity purification. The fusion proteins were eluted using a step gradient using 500 mM imidazole. The eluant yielded highly pure (>95%) fusion proteins (Fig. 2B).

CNBr cleaves N-terminally to Met residues [19], and can be used to remove the GB1–8×His-Met and GB1–8×His-Met-NT_{1-7} fusions from GB1–8×His-Met-NT_{1-13} and GB1–8×His-Met-NT(P7M) proteins, yielding the NT_{1-13} and NT_{8-13} peptides, respectively. Cleavage was performed with CNBr under acidic conditions (0.1 M HCl) with >70% cleavage efficiency (Fig. 2B). The cleaved samples were purified by preparative reverse-phase HPLC. The linear gradient of acetonitrile from 20% to 40% (v/v) over 45 min separated NT_{1-13} and NT_{8-13} from the fusion tag and uncleaved products (Fig. 3). Both neurotensin peptides were eluted as a single peak at approximately 26.5% (v/v) acetonitrile. ESI mass spectrometry was used to analyse peaks from HPLC. The results confirmed that the purified peptides were cleaved at the specific methionine sites, yielding products of 1690 and 817 Da for NT_{1-13} and NT_{8-13}, respectively (Fig. 3). Functional characterisation of neurotensin peptides have been reported previously [5,20].

The expression of NT_{1-13} was previously performed using GST as a tag protein, yielding 1.8 mg per litre of LB [21]. Here, using GB1 as a fusion protein, NT_{1-13} and NT_{8-13}, yields of 9.2 mg (5.4 μmol) and 4.2 mg (5.1 μmol) per litre of M9 are achieved, respectively (Table 1). This improvement supports the observation of Hammarstrom et al. [22] for over 45 human proteins, that the GB1 + His fusion gives significantly higher yields of protein than the GST + His fusion. To produce a final yield of about 10 mg (5.6 μmol) of U–^{13}C, ^{15}N-labelled NT_{1-13}, suitable for NMR studies, only 4.4 g of ^{13}C-glucose and 1.7 g of $^{15}NH_4Cl$ would be required with a significant cost saving when compared with individual protected labelled amino acids using solid phase peptide synthesis.

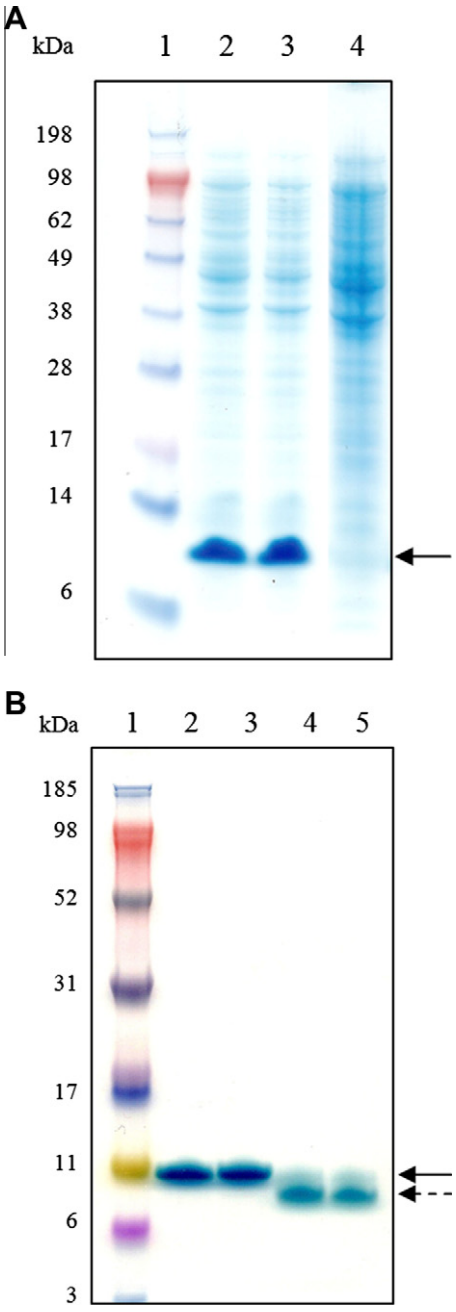

Fig. 2. SDS–PAGE analysis of expressed fusion proteins. GB1-NT_{1-13} and GB1-NT(P7M) were expressed in BL21(DE3) cells for 4 h with 1 mM IPTG at 37 °C. Soluble fractions were separated on a 12% NuPAGE gel and visualised using Coomassie Brilliant Blue staining (A). Induced bands indicated by a solid arrow migrating below the 14-kDa marker (lane 1 see blue from Invitrogen) are seen. The bacterial proteins from uninduced cells are shown in lane 4. The predicted molecular weight of the expressed proteins is 9.2 kDa. Ni^{2+}-affinity purification was used to isolate the fusion proteins (B). Lane 1, molecular weight markers MultiMark (Invitrogen); lanes 2 and 3, Ni^{2+}-affinity column-purified GB1-NT_{1-13} and GB1-NT(P7M), respectively, indicating the high degree of purification. Following CNBr cleavage, a small increase mobility as a product after the cleavage of GB1-NT_{1-13} (lane 4) and GB1-NT(P7M) (lane 5) indicated by a dashed arrow is observed. The efficiency of CNBr cleavage determined by gel densitometry is >70%. A solid arrow indicates the position of GB1 fusion proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

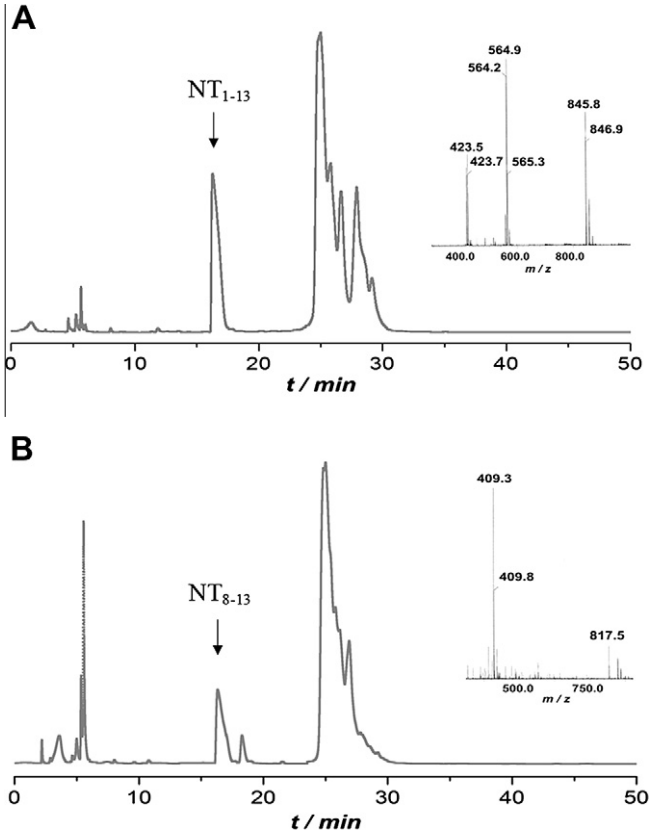

Fig. 3. Reverse-phase HPLC purification of neurotensin peptides. Reverse-phase HPLC traces (280 nm) and ESI mass spectra (insert) of purified peptides showing the separation of NT_{1-13} (A) and NT_{8-13} (B) from the fusion tag and uncleaved products. Mass observed of NT_{1-13}: 845.8 Da $(M+2H)^{2+}$, 564.9 Da $(M+3H)^{3+}$ and 423.5 Da $(M+4H)^{4+}$ and mass observed of NT_{8-13}: 817.5 Da (M+H) and 409.3 Da $(M+2H)^{2+}$. Theoretical isotope mass number of NT_{1-13} and NT_{8-13} are 1689.9 and 816.5, respectively.

Table 1
Purification of NT_{1-13} and NT_{8-13} from *E. coli* BL21(DE3) harbouring pGEV-NT_{1-13} and pGEV-NT_{8-13} plasmids. The results were derived from 1 L M9 minimal media cultures of *E. coli* expressing each recombinant protein.

Construct	pGEV-NT_{1-13}	pGEV-NT_{8-13}
Cell pellet[a] (g)	4.4	4.4
GB1 fusion protein[b] (mg)	65.8	64.7
Fusion protein purity[c] (%)	>95	>95
Purified peptide[d] (mg)	9.2	4.2
Peptide purity[e] (%)	>95	>95

[a] The wet weight of cells after 1 mM IPTG induction for 4 h at 37 °C.
[b] Fusion protein after Ni^{2+}-affinity purification.
[c] Purity of fusion protein determined by densitometry of gel shown in Fig. 2B (lanes 2 and 3).
[d] Target peptide after HPLC purification.
[e] Purity of peptide determined by HPLC chromatogram.

In conclusion, we constructed a system, pGEV-NT_{1-13}, for the expression of NT_{1-13} using a GB1 tag. The 8×His, followed by a CNBr cleavage site was inserted between the fusion tag and peptide gene, resulting in the target peptide with free C-terminal end, which is necessary for ligand binding [12,23]. This construct facilitates the production of a six amino acid peptide, NT_{8-13} by single mutation (P7M), introducing a CNBr cleavage site just before the desired product. Both constructs produce the target peptides with high yield and low cost for potential use as uniformly isotopically labelled peptides for NMR and other spectroscopic and biophysical studies. In addition, the expression of peptides with a free C-terminus using GEV-1 plasmid at high yield in solution could be useful for other peptide hormone and ligand expression.

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