Nuclear export of opioid growth factor receptor is CRM1 dependent

Nancy P Kren, Ian S Zagon and Patricia J McLaughlin

Department of Neural & Behavioral Sciences, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033, PA, USA

Corresponding author: Patricia J McLaughlin. Email: pxm9@psu.edu

Abstract

Opioid growth factor receptor (OGFr) facilitates growth inhibition in the presence of its specific ligand opioid growth factor (OGF), chemically termed [Met⁵]-enkephalin. The function of the OGF-OGFr axis requires the receptor to translocate to the nucleus. However, the mechanism of nuclear export of OGFr is unknown. In this study, endogenous OGFr, as well as exogenously expressed OGFr-EGFP, demonstrated significant nuclear accumulation in response to leptomycin B (LMB), an inhibitor of CRM1-dependent nuclear export, suggesting that OGFr is exported in a CRM1-dependent manner. One consensus sequence for a nuclear export signal (NES) was identified. Mutation of the associated leucines, L₂₁₇ L₂₂₀ L₂₂₃ and L₂₂₅, to alanine resulted in decreased nuclear accumulation. NES-EGFP responded to LMB, indicating that this sequence is capable of functioning as an export signal in isolation. To determine why the sequence functions differently in isolation than as a full length protein, the localization of subNES was evaluated in the presence and absence of MG132, a potent inhibitor of proteasomal degradation. MG132 had no effect of subNES localization. The role of tandem repeats located at the C-terminus of OGFr was examined for their role in nuclear trafficking. Six of seven tandem repeats were removed to form deltaTR. DeltaTR localized exclusively to the nucleus indicating that the tandem repeats may contribute to the localization of the receptor. Similar to the loss of cellular proliferation activity (i.e. inhibition) recorded with subNES, deltaTR also demonstrated a significant loss of inhibitory activity indicating that the repeats may be integral to receptor function. These experiments reveal that OGFr contains one functional NES, L₂₁₇ L₂₂₀ L₂₂₃ and L₂₂₅ and can be exported from the nucleus in a CRM1-dependent manner.

Keywords: OGFr, nuclear export signaling, CRM1, leptomycin B

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Introduction

Chemically termed [Met⁵]-enkephalin, opioid growth factor (OGF) is a native opioid peptide that plays an important role in regulating cell growth.¹ OGF is a constitutively expressed endogenous opioid that is autocrine produced and secreted² and acts to inhibit the proliferation of normal¹ and neoplastic cells.³ The inhibitory action of OGF is mediated by interfacing with the opioid growth factor receptor (OGFr).⁴ OGFr has pharmacological/biochemical characteristics consistent with classical opioid receptors in that the receptor binds to an opioid peptide and binding can be blocked by opioid antagonists such as naloxone and naltrexone. However, OGFr shares no homology with the classical opioid receptors at the amino acid or nucleic acid level.² OGFr is associated with the outer nuclear envelope⁴ and does not resemble a G protein-coupled receptor. The OGF-OGFr axis is ubiquitously expressed and has been demonstrated to be a determinant

in the growth of numerous cancer cell lines.⁵ The mechanism of action of the OGF-OGFr axis is related to DNA synthesis, targeting the p16/p21 cyclin-dependent kinase inhibitory pathway and delaying the G1-S phase of the cell cycle.^{1,6}

OGFr has three nuclear localization signals (NLS), NLS_{267–296}, NLS_{383–386}, and NLS_{456–460}.⁷ Two of the three NLSs, NLS_{383–386}, and NLS_{456–460} are essential for nuclear localization of the receptor.⁷ Translocation into the nucleus is required for the receptor to function.⁷ However, there is little information on how OGFr is exported from the nucleus and whether alterations to nuclear export or sequence alterations affect the receptor's function.

Nuclear export is often mediated by a nuclear export signal (NES). One well-characterized NES is the loosely conserved leucine rich sequence.⁸ This consensus sequence has been identified as Leu-X_{2–3}-Leu-X_{2–3}-Leu-X-Leu,⁹ with variations being acceptable.¹⁰ The NES is recognized by the

eukaryotic protein known as chromosomal maintenance 1 (CRM1), or exportin 1.¹¹ Utilizing the NetNES 1.1,^{12,13} a NES prediction program, OGFr is predicted to have one NES with leucines at residues 217, 220, 223, and 225. In order to determine the mechanism of nuclear export for OGFr, an OGFr-EGFP fusion protein was transfected into COS-7 cells to investigate nuclear-to-cytoplasmic trafficking and localization of the receptor. In addition, site-directed mutagenesis studies were undertaken to evaluate how alterations in protein sequence altered localization of OGFr. Finally, localization of OGFr in the presence of MG132 or LMB was conducted to determine the relationship between nuclear localization and proteosomal degradation or receptor dimerization. OGFr contains one NES that was identified as a sequence capable of functioning as a NES in isolation. Thus, OGFr is exported from the nucleus in a CRM1 dependent manner.

Materials and methods

Cell culture and molecular constructs

COS-7 monkey kidney cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco's modified Eagles media supplemented with 10% fetal bovine serum, 1.2% sodium bicarbonate with 20 units/mL penicillin and 20 µg/mL streptomycin. Approximately 2×10^5 cells were seeded and allowed to attach overnight. Cells were transfected with 5 µg of DNA per well, using 5 µL of Lipofectamine 2000 (Invitrogen). OGFr-EGFP plasmid was constructed as previously described;⁷ leucines associated with the predicted nuclear

export signal were mutated to alanines using the QuikChange kit (Agilent, 200519). SubNLS was previously generated.⁷ DeltaTR was generated using site-directed mutagenesis removing amino acids S516 to A646. NES-EGFP was generated by PCR amplification of the NES and four amino acids on either side. The NES was subsequently cut with EcoRI and BamHI and ligated into EGFP-N1. All primers are listed in Table 1.

Imaging

For imaging, approximately 2×10^5 cells were seeded onto coverslips, transfected, treated (described below), counterstained with Hoechst 33342 (Invitrogen, H3570) and fixed in 4% paraformaldehyde. At least two coverslips per group were imaged using epifluorescent microscopy, with at least 30 images collected per group. Images were batch normalized, exported as .tiff files, and analyzed using Cell Profiler (www.cellprofiler.org) to quantitate the nuclear to cytoplasmic (NC) ratio. Images were converted to gray and split by channel. Nuclei were identified as primary objects by intensity of Hoechst staining. Based on the identified primary objects, the intensity of EGFP in the nucleus was analyzed and used to set a threshold for EGFP positive cells. For the cells that were identified as positive, a fixed cytoplasm of 25 pixels around the nucleus was measured for EGFP intensity. The nuclear to cytoplasmic ratios were calculated by dividing average EGFP intensity of the nucleus by the average EGFP intensity of the defined cytoplasm. Additionally, the average intensity for the cell (nucleus and cytoplasm) was collected.

Table 1 Primers utilized to generate altered OGFr plasmids

Mutation		Primer	Template	Protocol
L217A	F	CGC ATC ACA CGC ATC GCC AAG TCG CTG GGT GA	OGFr-EGFP	Site directed mutagenesis
	R	TCA CCC AGC GAC TTG GCG ATG CGT GTG ATG CG		
L220A	F	GCA TCC TCA AGT CGG CGG GTG AGC TGG GCC	OGFr-EGFP	Site directed mutagenesis
	R	GGC CCA GCT CAC CCG CCG ACT TGA GGA TGC		
L223A	F	GTC GCT GGG TGA GGC GGG CCT CGA GCA C	OGFr-EGFP	Site directed mutagenesis
	R	GTG CTC GAG GCC CGC CTC ACC CAG CGA C		
L225A	F	GGG TGA GCT GGG CGC CGA GCA CTT CCA G	OGFr-EGFP	Site directed mutagenesis
	R	CTG GAA GTG CTC GGC GCC CAG CTC ACC C		
L217-220 A	F	CAT CGC CAA GTC GGC GGG TGA GGC GGG C	L217A	Site directed mutagenesis
	R	GCC CGC CTC ACC CGC CGA CTT GGC GAT G		
L223-225 A	F	GGG TGA GGC GGG CGC CGA GCA CTT CCA G	L223A	Site directed mutagenesis
	R	CTG GAA GTG CTC GGC GCC CGC CTC ACC C		
L217-220-223 A	F	GTC GCT GGG TGA GGC GGG CCT CGA GCA C	L217-220 A	Site directed mutagenesis
	R	GTG CTC GAG GCC CGC CTC ACC CAG CGA C		
subNES	F	GGG TGA GGC GGG CGC CGA GCA CTT CCA G	L217-220-223 A	Site directed mutagenesis
	R	CTG GAA GTG CTC GGC GCC CGC CTC ACC C		
NES-EGFP	F	GGC GCC GAA TTC TAA TGA TCA CAC GCA TCC TCA ACT CG	OGFr-EGFP	PCR amplification
	R	GGC GCC GGA TCC TGG AAG TGC TCG AGG CCC AG		
deltaTR	F	AAA GAA GGT ACC CCT GGG GGA CCT ACA AGG GAT GAG CCA GCC AAG	OGFr-EGFP	Site directed mutagenesis
	R	CTT GGC TGG CTC ATC CCT TGT AGG TCC CCC AGG GGT ACC TTC TTT GG		

Note: The template that was used with each set of primers as well as the protocol is noted.

For leptomycin B experiments, the cells were treated with 20 ng/ml of Leptomycin B (Sigma L2913) or equivalent volumes of 70% methanol for 3 h in the absence of serum. For degradation experiments, cells were treated with 25 μ M MG312 (Calbiochem, 474791) or equivalent volumes of dimethyl sulfoxide for 5 h. Cycloheximide was used at 28 μ g/mL where indicated. For DNA synthesis studies, cells were pulsed with 30 μ M 5-bromo-2'-deoxyuridine (BrdU) 3 h prior to fixing, and stained with anti-BrdU (B35132, Invitrogen) as well as anti-GFP (A21311, Invitrogen) following previous protocols.^{1,5}

Protein detection by Western blotting

Cells were trypsinized, collected, and lysed in RIPA buffer plus protease inhibitors (Complete mini EDTA-free, 11836170001) and phosphatase inhibitors (PhosStop, 04906837001). Equal volumes were loaded onto SDS-PAGE gels, electrophoresed at 100 volts, and transferred onto nitrocellulose. Membranes were probed with anti-ubiquitin antibody at 1:1000 (Cell Signaling, 3936), and stripped and re-probed with GAPDH at 1:20,000 in order to normalize protein expression.

Data analyses

All data (nuclear/cytoplasmic ratios, and BrdU indexes) were averaged across groups and compared using one-way analysis of variance (ANOVA) with Newman-Keuls post hoc tests.

Results

The fusion protein OGFr-EGFP was localized throughout the nucleus and the cytoplasm (Figure 1b), in a manner similar to that observed for endogenous OGFr and human cancer cells transfected with OGFr-EGFP.⁷ COS-7 cells exposed to LMB for 3 h and immunostained for endogenous OGFr demonstrated nuclear accumulation of endogenous OGFr (Figure 1a). Because LMB covalently binds to the NES binding domain of CRM1 and blocks CRM1 dependent nuclear export,^{14,15} these observations suggested that endogenous OGFr is exported in a CRM1-dependent manner. Additionally, when COS-7 cells were transfected with OGFr-EGFP fusion protein and exposed to LMB, OGFr-EGFP accumulated in the nucleus. These data support the hypothesis that OGFr is exported from the nucleus in a CRM1-dependent manner. To identify the NES recognized by CRM1, the protein sequence for OGFr was submitted to the NetNES database¹² and one nuclear export signaling sequence was predicted to be comprised of residues L₂₁₇ L₂₂₀ L₂₂₃ and L₂₂₅. The identified export sequence in OGFr matched the consensus sequence for a NES. This region was shown to be evolutionarily conserved (Figure 1c).

To further characterize the predicted NES in OGFr, the leucines associated with the predicted NES were mutated to alanine, in isolation as well as in combination. A schematic of the mutations is presented in Figure 2(a) and the primers that were utilized are listed in Table 1. It was hypothesized that mutations of the functional NES would result in an accumulation of OGFr in a manner similar to that when

Figure 1 Nuclear accumulation of OGFr in response to LMB. (a) COS-7 cells were treated with LMB or 70% methanol and stained for endogenous OGFr. Scale bar represents 10 μ m. (b) COS-7 cells transfected with OGFr-EGFP were treated with LMB or 70% methanol, fixed and imaged for GFP localization. (c) The evolutionary conservation of the proposed NES sequence. (A color version of this figure is available in the online journal.)

cells are exposed to LMB. COS-7 cells were transfected with each of the various NES mutants and the cytoplasmic–nuclear localization assayed. Representative images for wildtype OGFr and each of the mutations are presented in Figure 2(b). Changes in localization due to the altered sequence were calculated from nuclear to cytoplasmic ratios using CellProfiler.^{16,17} All of the mutated OGFr-EGFP fusion proteins displayed a significant decrease in the NC ratio (Figure 2c). The NC ratio of the EGFP control construct ranged between 1 and 1.5 in our work and others,¹⁸ while OGFr-EGFP had a NC ratio of approximately 2, supporting its predominant nuclear localization. All of the mutations resulted in decreased nuclear localization, and in some cases, nuclear exclusion. The OGFr-EGFP construct lacking NLSs, termed subNLS, demonstrates nuclear exclusion and serves as a positive control. Thus, the sequence

Figure 2 Mutational analysis of the putative NES. (a) A schematic highlighting the leucines of the NES mutated to alanine in isolation or in combination. (b) Representative images of each of localization of each of the mutations described in A. Scale bar represents 10 μ m. (c) Histogram of the quantitative analyses of nuclear to cytoplasmic (NC) ratios for each of the mutations described in (a) and visualized in (b). Error bars represent SEM. Statistically different from wildtype OGFr at $p < 0.001$ (***). (d) BrdU index demonstrating the loss of function with subNES and subNLS mutations. Error bars represent SEM. Significantly different from EV at $p < 0.01$ (**), and from OGFr at $p < 0.01$ (++). (A color version of this figure is available in the online journal.)

changes to the putative NES caused a significant change in receptor localization. To further assess function related to the requirement for a NES, cell proliferation was evaluated by BrdU incorporation assays. It has previously been demonstrated that overexpression of wildtype OGFr-EGFP significantly decreases incorporation of BrdU, indicating decreased proliferation resulting from interactions of endogenous OGF with excess receptor.⁷ Mutation of the NLSs preventing OGFr trafficking into the nucleus resulted in cell growth that was normal, indicating that OGFr must translocate into the nucleus to invoke its inhibitory growth activity.⁷ Similar to the results recorded with NLS mutations, cultures transfected with subNES also demonstrated a loss of function in BrdU assays (Figure 2d), indicating that the putative NES is essential for OGFr function. These observations further support the decrease in NC ratios, and the identification of nuclear export pathways for OGFr.

To determine whether the putative NES functions as an NES in isolation, a construct was made with the NES tagged directly to EGFP. NES-EGFP localized to regions similar to those for EGFP alone potentially suggesting that the NES is a weak export signal and does not compete well with passive diffusion (Figure 3). In cultures treated with LMB, the NES-EGFP displayed a significant increase in NC ratio indicating that NES is capable of functioning as an export signal in isolation (Figure 3).

These data suggest that there are other functional regions in OGFr that contribute to the localization activity. Three studies were conducted to investigate how the mutated NES altered OGFr trafficking. Alterations in the sequence associated with the NES and the stability of the receptor were investigated by examination of degradation. Three putative ubiquitination sites identified in the literature^{19–21} reveal that one site is at residue 219 within the NES.^{19,20} It was hypothesized that altering the residues around residue 219 could affect OGFr degradation. COS-7 cells transfected with subNES were cultured in the presence of MG132, a potent proteosomal degradation inhibitor previously demonstrated to inhibit degradation in COS-7 cells.^{22,23} Exposure to MG132 had no significant affect on the NC ratios of subNES (Figure 4a), suggesting that proteosomal degradation does not significantly alter localization of mutant OGFr-EGFP (Figure 4b).

The second approach was to determine if endogenous OGFr was dimerizing with exogenous OGFr and altering the localization. To test this, NC ratios in the presence of LMB were measured and revealed that LMB significantly increased the NC ratio of OGFr-EGFP. Thus, OGFr-EGFP is exported in a CRM1-dependent manner because LMB treatment had no significant influence on EV or subNLS. Moreover, treatment with LMB had no significant affect on the NC ratio of subNES (Figure 5), suggesting that dimerization does not account for the altered localization of the NES mutants.

OGFr could be exported from the nucleus in a CRM1-dependent manner, as well as be exported by additional CRM1 independent pathways. CRM1-dependent export signals are easily identified based on their known consensus sequence, whereas CRM1 independent export signals are more difficult to identify by sequence alone. The

Figure 3 NES functions in isolation. (a) NC ratios of NES-EGFP in the presence and absence of LMB. ***Indicates statistically significant in comparison to NES-EGFP, $p \leq .001$. (b) Representative images of each of the treatment groups evaluated in (a). Large images are optimized for viewing; insets represent images that were processed using CellProfiler. (A color version of this figure is available in the online journal.)

majority of OGFr remains uncharacterized, so the possibility of an additional export signal remains.

Finally, the regional localization of the putative OGFr NES was evaluated. The NES is located near the N terminus of the protein, whereas the three NLSs are located near the middle of the protein. OGFr has seven imperfect tandem repeats in the C-terminus of the protein² that are uncharacterized. To evaluate the effect of the tandem repeats on receptor localization, all repeat sequences except the first one were removed by site-directed mutagenesis to form deltaTR. A schematic diagram of deltaTR (removal of 6 of the 7 repeats) is presented in Figure 6(a). When this construct was transfected into cells, deltaTR localized almost exclusively to the nucleus (Figure 6c). The NC ratios were calculated and compared to wildtype as well as other mutants. DeltaTR demonstrated a significantly higher NC

Figure 4 MG132 does not affect localization of subNES. Cells were treated with DMSO or MG132 and the NC ratios were evaluated. (a) Histograms represent the NC ratios for each treatment group. Values represent means $\pm$ SEM; significantly different at $p < 0.001$ (***). (b) Representative images of each treatment group in A. Scale bar represents 10 μ m. Large images are optimized for viewing, while insets represent images that were processed using CellProfiler. (A color version of this figure is available in the online journal.)

ratio than any of the other mutations (Figure 6b), indicating that this region contributes to the localization of the receptor. This region could serve as a regulatory region or as a CRM1 independent export signal. Interestingly, deltaTR also demonstrated a loss of function (Figure 6d), similar to both subNES and subNLS, indicating that this region is essential for the inhibitory function of the receptor.

Figure 5 LMB does not affect localization of subNES. COS-7 cells were treated with 70% methanol or LMB. (a) Histograms of NC ratios for each treatment group. Values represent means $\pm$ SEM; significantly different at $p < 0.001$ (***). (b) Representative images of each treatment group from graph in (a). Scale bar represents 10 μ m. Large images are optimized for viewing, while insets represent images processed using CellProfiler. (A color version of this figure is available in the online journal.)

Discussion

It has previously been demonstrated that the nuclear import of OGFr is critical for its inhibitory function.⁷ However, little is known about how OGFr is exported from the nucleus, and whether altered export affects the function of the receptor. The present study demonstrated that endogenous as well as exogenously expressed OGFr accumulates in the nucleus in response to LMB, indicating that OGFr is exported in a CRM1-dependent manner. One

Figure 6 The effect of tandem repeats on localization and function of OGFr. (a) Schematic representing the removal of all but one of the tandem repeats. (b) Histograms of NC ratios for each treatment groups. Data represent means $\pm$ SEM; significantly different from OGFr at $p < 0.01$ (**) or $p < 0.001$ (***). (c) Representative images of cells in each treatment group analyzed in (b). Scale bar represents 10 μ m. Large images are optimized for viewing; insets represent images that were processed using CellProfiler. (d) BrdU labeling of cells (wildtype or transfected with deltaTR); EV is empty vector control. Cells expressing wildtype OGFr had significantly less cell replication in comparison to EV and deltaTR at $p < 0.05$. Values represent mean cell counts $\pm$ SEM. (A color version of this figure is available in the online journal.)

NES was identified at residues 217-225. Leucine to alanine mutations of this NES resulted in decreased nuclear accumulation quantitated by calculating the average NC ratio per group.

The OGFr NES (217-225) was validated using construct NES-EGFP and assessment of nuclear localization. The putative OGFr NES demonstrated a significant accumulation in the nucleus in response to LMB, indicating that this sequence is a functional NES. However, mutation of the

proposed NES resulted in decreased NC ratios and even appeared to be excluded from the nucleus. Several explanations for this unexpected result were explored. Degradation was a possibility to explain NES mutant localization. Three residues in OGFr are ubiquitinated,^{19,20} and residue 219 is responsive to MG132,¹⁹ indicating that OGFr adheres to classical poly-ubiquitination that marks proteins for degradation by the proteasome. Since this residue is within the NES, changing the residues surrounding this

ubiquitination site could change how the protein is recognized for degradation. It has been demonstrated that the same proteasomal degradation machinery that is present in the cytoplasm is also present in the nucleus and responsible for degradation of specific proteins.^{24,25} Thus, by sequestering OGF_r in the nucleus, it is plausible that nuclear degradation was increased, thereby altering the NC ratios. These results were not observed. OGF_r-EGFP displayed a significant decrease in the NC ratio, indicating that the nuclear levels of receptor were constant, whereas cytoplasmic levels increased, indicating that OGF_r-EGFP is degraded in the cytoplasm, not in the nucleus by the proteasome.

These experiments also demonstrated that the tandem repeat region found in the C-terminus of OGF_r contributes to the localization of the receptor. The structure of OGF_r is currently unknown but websites such as FoldIndex²⁶ predict that the majority of the receptor is unstructured. Thus it is unlikely that the removal of the tandem repeats significantly alters OGF_r structure. However, the tandem repeats could contain a CRM1-independent NES that would facilitate nuclear export through an undetermined exportin. Alternatively, the tandem repeats could be a region needed for a protein-protein interaction that regulates the localization of the receptor. These studies will be the focus of independent research.

CRM1-dependent export is the most common export mechanism for proteins, and the identification of a leucine rich NES supports the hypothesis that OGF_r can be exported from the nucleus in a CRM1-dependent manner. In summary, we demonstrate that OGF_r has at least one functional NES that consist of the leucines at residues 217, 220, 223 and 225, and is capable of being exported from the nucleus in a CRM1-dependent manner. However, we have also demonstrated that there may be other mechanisms at play, and further studies are needed to further characterize the contributions of the tandem repeats to the localization of the receptor.

Authors' contributions: NK performed the research, analyzed data, and drafted the manuscript as part of her doctoral research. ISZ and PJM conceived of the ideas, designed the overall study, interpreted data and composed the final paper.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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