Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# A sensitive assay for the biosynthesis and secretion of MANF using NanoLuc activity

Junpei Norisada [a], Yoko Hirata [a,b], Fumimasa Amaya [c], Kazutoshi Kiuchi [a,b], Kentaro Oh-hashi [a,b,*]

[a] Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[b] United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan
[c] Department of Anesthesiology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kamikyo-ku, Kyoto 602-0841, Japan

ARTICLE INFO

*Article history:*
Received 23 April 2014
Available online xxxx

*Keywords:*
ER stress
MANF
NanoLuc

ABSTRACT

Mesencephalic astrocyte-derived neurotrophic factor (MANF) has been reported to prevent neuronal cell death caused by certain stimuli. Accordingly, the molecular features of MANF have been intensively investigated since the reporting of its cytoprotective actions. In addition to the characterization of the transcriptional regulation of MANF under pathophysiological conditions, it is important to understand its intracellular transport and secretion after translation. In this study, we developed a convenient and quantitative assay to evaluate the post-translational regulation of MANF using NanoLuc, a highly active and small luciferase. We inserted NanoLuc after the putative signal peptide sequence (SP) of MANF to construct NanoLuc-tagged MANF (SP-NL-MANF). Similar to wild-type (wt) MANF, SP-NL-MANF was secreted from transiently transfected HEK293 cells in a time-dependent manner. The overexpression of mutant Sar1 or wild-type GRP78, which has been reported to decrease wt MANF secretion, also attenuated the secretion of SP-NL-MANF. Using INS-1 cells stably expressing SP-NL-MANF, we found that the biosynthesis and secretion of SP-NL-MANF can be evaluated quantitatively using only a small number of cells. We further investigated the effects of several stimuli responsible for the expression of ER stress-induced genes on the secretion of SP-NL-MANF from INS-1 cells. Treatment with thapsigargin and high potassium significantly increased NanoLuc activity in the culture medium, but serum withdrawal dramatically down-regulated luciferase activity both inside and outside of the cells. Collectively, these results demonstrate that our method for measuring NanoLuc-tagged MANF as a secretory factor is highly sensitive and convenient not only for characterizing post-translational regulation but also for screening useful compounds that may be used to treat ER stress-related diseases such as neurodegenerative disease, ischemia and diabetes.



## 1. Introduction

The endoplasmic reticulum (ER) is responsible for folding and modifying newly synthesized transmembrane and secretory proteins [1,2]. Certain pathophysiological conditions disrupt ER functions and cause the accumulation of unfolded and/or misfolded proteins in the ER [3,4]. These situations, known as ER stress, activate various stress responses that are mediated by three major ER-resident stress sensors: PERK [5], IRE1 [6] and ATF6α [7,8]. A variety of genes have been identified as downstream targets of these three sensors, some of which, including ER-resident chaperones, control the quality of newly synthesized proteins in the ER and alleviate cellular damage [9]; others, such as GADD153, have been demonstrated to promote cell death in various types of cells [10]. Among these ER stress-induced factors, some have been reported to be actively secreted and to function both intracellularly and extracellularly [11–14].

Mesencephalic astrocyte-derived neurotrophic factor (MANF) was first identified as arginine rich, mutated in early stage of tumors (Armet), a protein with a high mutation rate in various tumors, the precise function of which is unknown [15]. Petrova et al. reported that MANF is secreted from a rat mesencephalic

*Abbreviations:* Armet, arginine-rich mutated in early stage of tumors; ER, endoplasmic reticulum; GRP78, 78-kDa glucose regulated protein; IRE1, inositol-requiring enzyme 1; MANF, mesencephalic astrocyte-derived neurotrophic factor; NanoLuc, a small nanoluciferase; RT-PCR, reverse transcription polymerase chain reaction; Tg, thapsigargin; Tm, tunicamycin; XBP-1, X-box binding protein 1.

* Corresponding author at: Department of Chemistry and Biomolecular Science, Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan. Fax: +81 58 293 2794.
*E-mail address:* oohashi@gifu-u.ac.jp (K. Oh-hashi).

http://dx.doi.org/10.1016/j.bbrc.2014.05.031
0006-291X/

type-1 astrocyte cell line and acts as a selective neurotrophic factor for dopaminergic neurons [16]. Accordingly, Armet is referred to as MANF in this study, even though the precise mechanisms by which it prevents both neuronal cell and non-neuronal death remain unknown [16–20]. MANF has been demonstrated to be a downstream target of ATF6α, ATF6β and sXBP1 [11,12]. Because of the promising cytoprotective action of MANF, in addition to the characterization of the expression of its gene, the mechanisms responsible for MANF post-translational regulation, including intracellular transport and secretion, have been interested in various types of diseases.

In this study, we developed a highly sensitive and convenient assay to investigate the biosynthesis and secretion of MANF using NanoLuc, a 19-kDa luciferase subunit from *Oplophorus gracilirostris* [21]. The sequence encoding NanoLuc was inserted into the MANF cDNA after the putative signal peptide sequence (SP) to demonstrate the usefulness of NanoLuc-tagged MANF (SP-NL-MANF) for analyzing the existence of MANF both inside and outside of cells using a cell line stably expressing SP-NL-MANF. Lastly, we estimated the luciferase activity inside and outside of cells in response to several stimuli affecting the expression of ER stress-induced genes.

## 2. Materials and methods

### 2.1. Materials

Thapsigargin (Tg) and tunicamycin (Tm) were obtained from Sigma–Aldrich. Antibodies against MANF were purchased from R&D Systems and Abcam.

### 2.2. Construction of plasmids

For the preparation of the MANF constructs, the wild-type (wt) MANF gene was cloned from cDNA derived from Neuro2a cells using RT-PCR and inserted into the pcDNA3.1 vector as described previously [12]. MANF fused to NanoLuc after the putative signal peptide sequence (22 aa) (SP-NL-MANF) was constructed and inserted into the pcDNA3.1 vector. Genes encoding GRP78 (wt GRP78) and GRP78 lacking four C-terminal amino acids (KDEL) (ΔC GRP78) were also amplified by PCR using mouse GRP78 cDNA, and the fragments were cloned into the pcDNA3.1 vector [12]. An HA-tagged Sar1 construct (H79G) was kindly provided by Dr. Wei Liu and Dr. Jennifer Lippincott-Schwartz [22].

### 2.3. Cell culture and treatment

HEK293, COS7 and Neuro2a cells were maintained in Dulbecco's modified Eagle's minimum essential medium containing 8% fetal bovine serum. INS-1 cells, a rat insulinoma cell-line [23], were cultured in RPMI1640 medium containing 8% heat-inactivated FBS. Transfection of the indicated constructs was performed using the Lipofectamine-Plus reagent (Life Technologies) according to the manufacturer's instructions. To establish INS-1 and COS7 cells stably expressing SP-NL-MANF, cells transfected with SN-NL-MANF were selected with the appropriate amount of G418. To detect both MANF protein and luciferase activities, cells were seeded into 6- or 12-well plates. For the analysis of luciferase activity, HEK293 cells or cells stably expressing SN-NL-MANF were seeded into 48- or 96-well plates, grown to semi-confluence and used for subsequent experiments. The treatments used in this study were follows: serum-free medium (SF), serum and glucose-free medium (SGF), high potassium chloride (KCl, 50 mM), Tg (0.2 μM) or Tm (1 μg/ml).

### 2.4. Reverse transcription polymerase chain reaction

To estimate the expression level of each gene by RT-PCR, total RNA was extracted from cells lysed with the TRIzol reagent (Life Technologies) and converted to cDNA by reverse transcription using random nine-mers as primers for superscript III RNase⁻ reverse transcriptase (RT) (Life Technologies), as previously

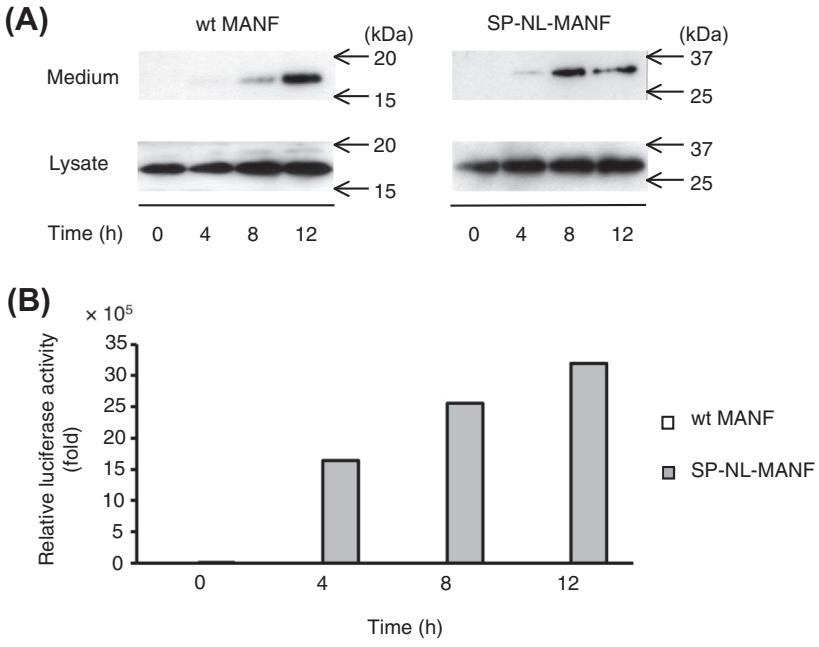


**Fig. 1.** Expression levels of wt MANF and SP-NL-MANF in transiently expressing HEK293 cells and their levels in conditioned media. Twenty-four hours after the transfection of each MANF gene into HEK293 cells in 12-well plates, the culture medium was replaced with fresh serum-free DMEM; the cells were then incubated for the indicated time. (A) The amount of each type of MANF in the cell lysate and culture medium were detected by a western blotting analysis, as described in the Section 2. (B) An equal amount of culture medium from HEK293 cells expressing each type of MANF was collected, and the luciferase activity in each sample was measured.

Please cite this article in press as: J. Norisada et al., A sensitive assay for the biosynthesis and secretion of MANF using NanoLuc activity, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.05.031