Biochemical and Biophysical Research Communications 449 (2014) 483–489

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

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ARTICLE INFO

Article history:
Received 23 April 2014
Available online 17 May 2014

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.

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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

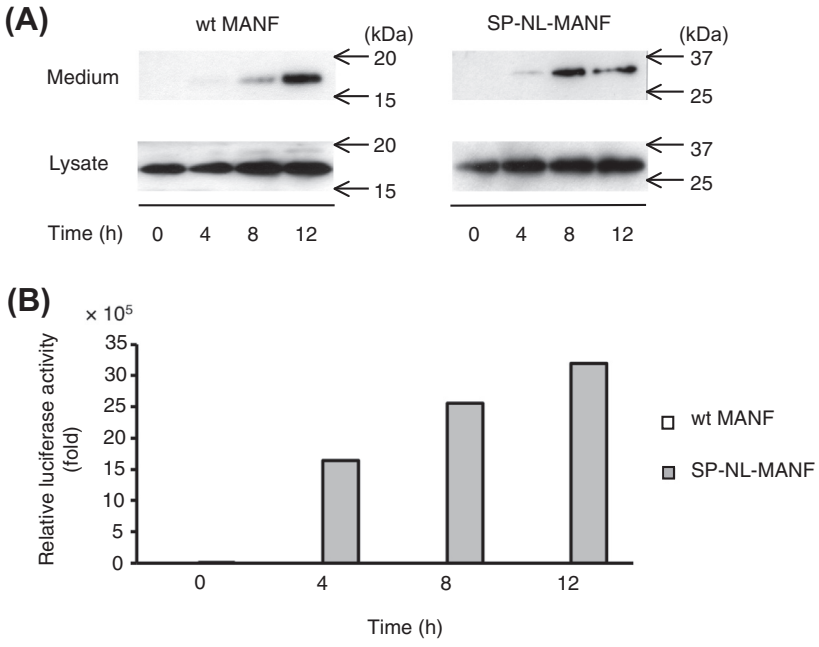

Fig. 1. Expression levels of wt MANF and SP-NL-MANF in transiently expressing HEK293 cells and their levels in conditioned media. Twenty-four hours after the transfection of each MANF gene into HEK293 cells in 12-well plates, the culture medium was replaced with fresh serum-free DMEM; the cells were 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.

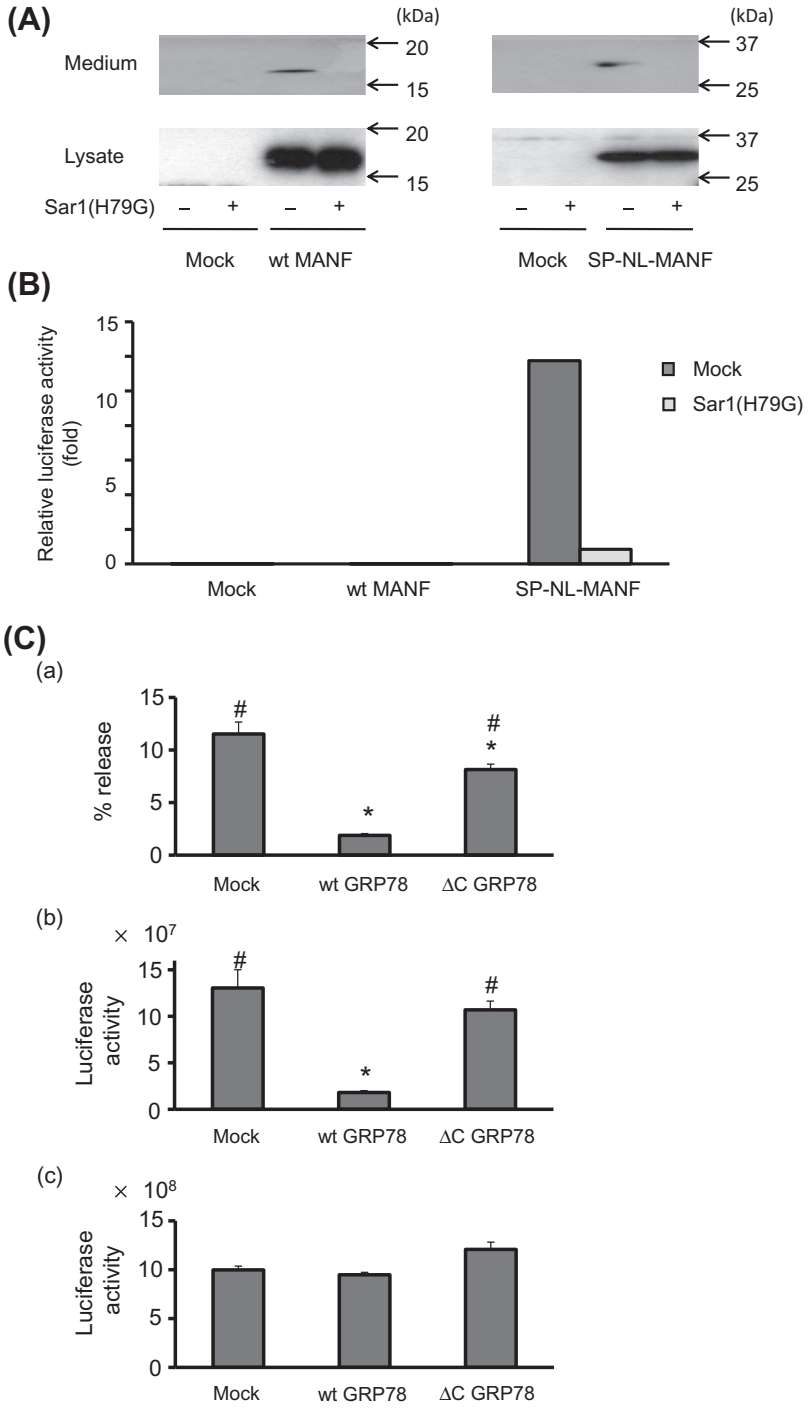

Fig. 2. Effects of mutant Sar1 and wt GRP78 co-expression on SP-NL-MANF secretion from HEK293 cells. (A) and (B) Twenty-four hours after the transfection of each MANF gene together with mutant Sar1[H79G] or the empty vector (mock) into HEK293 cells in 12-well plates, the culture medium was replaced with fresh serum-free DMEM; the cells were then incubated for an additional 12 h. (A) The amount of each 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 the culture medium from HEK293 cells expressing the indicated gene was collected, and the luciferase activity in each culture medium was measured. (C) Twenty-four hours after the transfection of SP-NL-MANF together with wt GRP78, ΔC GRP78 or the empty vector (Mock) into HEK293 cells in 48-well plates, the culture medium was replaced with fresh serum-free DMEM; the cells were then incubated for an additional 4 h. The culture medium (b) and cell lysate (c) from HEK293 cells expressing SP-NL-MANF were collected, and the luciferase activity in each sample was measured. The secreted ratio of SP-NL-MANF in each case (a) was calculated as described in the Section 2. The values represent the mean ± SD from 3 independent cultures. The data were analyzed by a one-way ANOVA followed by the Scheffe test to evaluate the effects of the co-expression of mock, GRP78 or ΔC GRP78 on the luciferase activity. The values marked with an asterisk or # are significantly different from the value of the mock-transfected or wt GRP78-transfected cells, respectively ($p < 0.05$).

described [12]. Specific cDNAs were mixed and amplified with a PCR reaction mixture (Taq PCR Kit, Takara). The RT-PCR primers used in this study were as follows: *MANF* sense primer, 5′-GTTTGT ATTTCTTATCTGGG-3′; *MANF* antisense primer, 5′-TCTTCTTCAGCTT CTCACAG-3′; *XBP1* sense primer, 5′-ACGCTTGGGAATGGACACG-3′; *XBP1* antisense primer, 5′-ACTTGTCCAGAATGCCCAAAAG-3′, *GAD D153* sense primer, 5′-GAATAACAGCCGGAACCTGA-3′; *GADD153* antisense primer 5′-GGACGCAGGGTCAAGAGTAG-3′, *GRP78* sense

primer, 5′- ACCAATGACCAAAACCGCCT-3′; *GRP78* antisense primer 5'- GAGTTTGCTGATAATTGGCTGAAC-3′, *GAPDH* sense primer, 5′-ACCACAGTCCATGCCATCAC-3′ and *GAPDH* antisense primer, 5′-TCCACCACCCTGTTGCTGTA-3′. The typical reaction cycling conditions were 30 s at 96 °C, 30 s at 58 °C and 30 s at 72 °C. The results represent 20–29 cycles of amplification. The cDNAs were separated by electrophoresis through 2.0% agarose gels and visualized using ethidium bromide.

2.5. Luciferase assay

After cells transiently or stably expressing each of the MANF constructs were treated with the indicated reagents, the culture medium and cell lysate prepared with 1 × Passive Lysis buffer (Promega) were collected. After a brief centrifugation, the luciferase activity in each culture medium and the lysate was measured using a NanoLuc assay system (Promega). By calculating the total luciferase activity in both the culture medium and cell lysate, the percentage of secreted SP-NL-MANF in each well was estimated. The experiments were repeated more than twice, and reproducibility was confirmed.

2.6. Western blot analysis

We detected the amount of MANF in the cell lysate and the culture medium as previously described, with a slight modification [12]. The cells were lysed with homogenate buffer [20 mM Tris–HCl (pH 8.0) containing 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% TritonX-100, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A]. After the protein concentration was determined, each cell lysate was dissolved with an equal amount of 2 × sodium dodecyl sulfate (SDS)-Laemmli sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS and 10% glycerol]. For the detection of each type of MANF in the culture medium, each culture medium collected after the indicated treatment was briefly centrifuged to remove detached cells and then mixed with one-forth volume of 4 × SDS sample buffer. Equal amounts of cell lysate and culture medium were separated on 15% SDS–polyacrylamide gels, immunoblotted onto polyvinylidene difluoride membrane (GE Healthcare) and identified by enhanced chemiluminescence (GE Healthcare) using antibodies against MANF (R&D Systems and Abcam).

2.7. Statistical analysis

The results are expressed as the means ± SD of the indicated number. The statistical analyses were carried out by a one-way ANOVA, followed by the Scheffe test. $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

Previously, we demonstrated the biosynthesis and secretion of mouse MANF by the transfection of various mutants of the MANF gene into HEK293 cells [12]. However, there are a few problems associated with evaluating the amount of MANF secreted into the culture medium. First, it is necessary to replace the serum-containing culture medium with a serum-free medium because the large amount of proteins in the former interfere with the separation of proteins by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Second, the cells should be incubated with serum-free medium for a relatively long time. Third, in some cases, the condensation of the culture medium is required to adequately detect

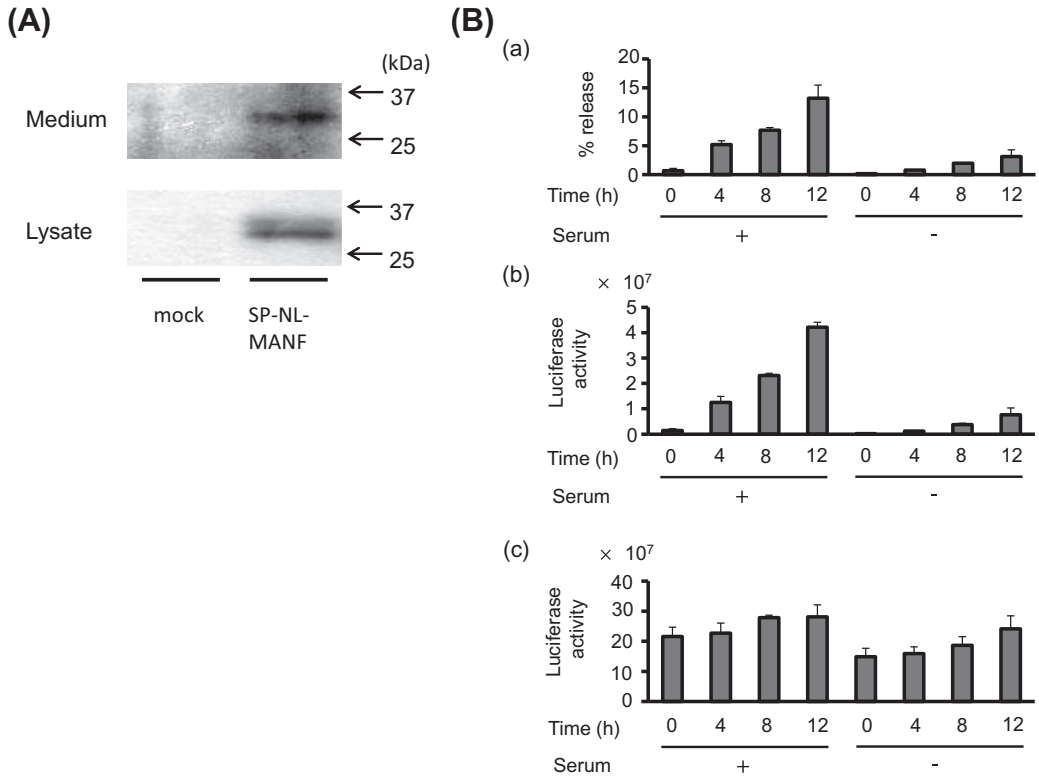

Fig. 3. Effect of serum addition to culture medium on the intra- and extra-cellular NanoLuc activities obtained from INS-1 cells stably expressing SP-NL-MANF. (A) The culture medium of INS-1 cells stably expressing SP-NL-MANF in 6-well plates was replaced with fresh serum-free medium, and the cells were incubated for an additional 12 h. The expression of SP-NL-MANF in cell lysate and culture medium was detected by a western blotting analysis, as described in the Section 2. (B) The culture medium of INS-1 cells stably expressing SP-NL-MANF in 48-well plates was replaced with fresh medium with or without serum, and the cells were incubated for the indicated time and each luciferase activity was evaluated as described in Fig. 2.

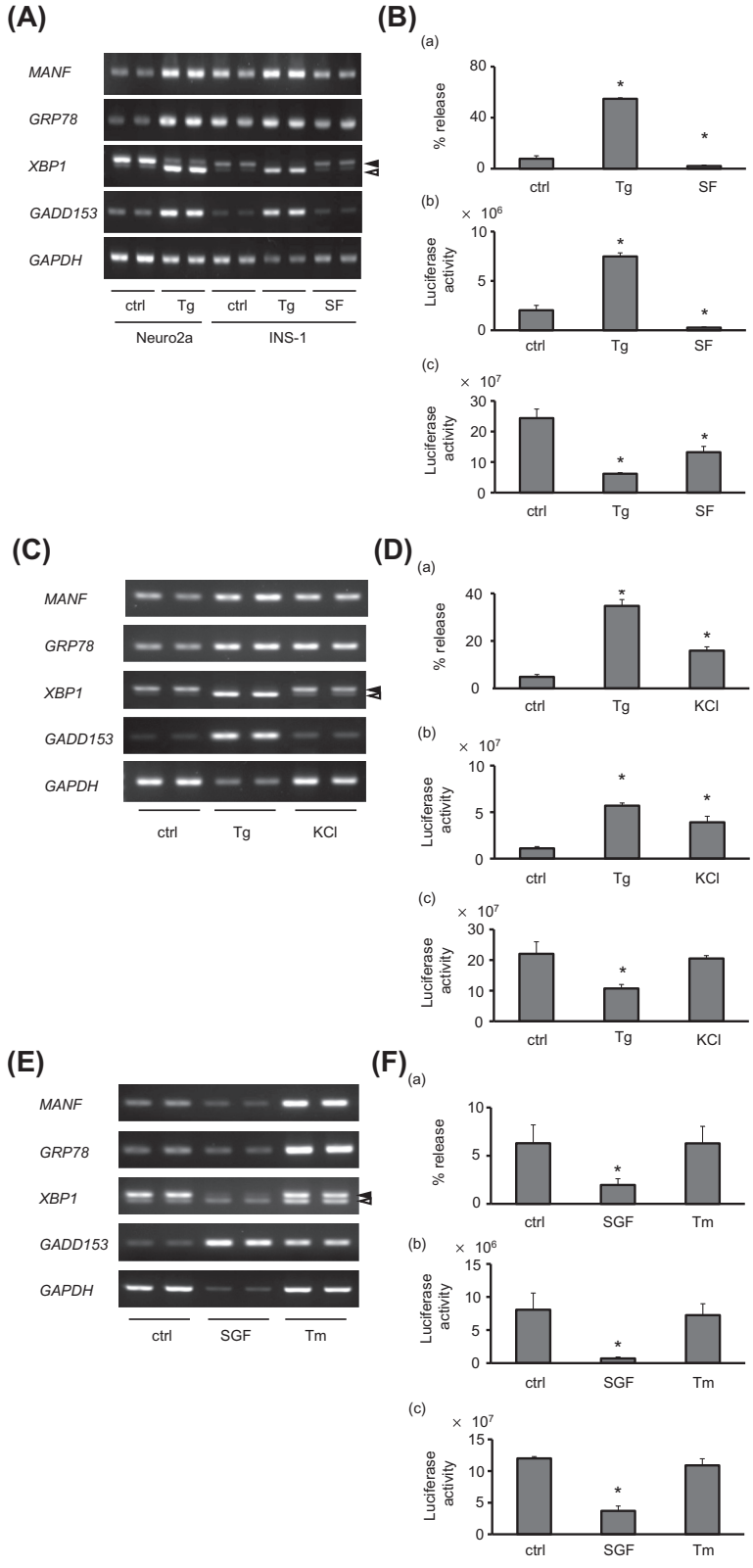

Fig. 4. Expression levels of ER-stress inducible genes by Tg, Tm or KCl stimulation or serum and glucose depletion in Neuro2a cells and INS-1 cells and the intra- and extracellular NanoLuc activities of INS-1 cells stably expressing SP-NL-MANF in response to each stimulus. Wild-type INS-1 cells in 6-well plates (A, C, E) or INS-1 cells stably expressing SP-NL-MANF in 48-well plates (B, D, F) were treated for 8 h (A, C, E) or 4 h (B, D, F), as follows: (A) Tg (0.2 μM), serum free (SF); (C) high potassium chloride (50 mM, KCl); (E) serum and glucose free (SGF), Tm (1 μg/ml). The expression levels of each gene in INS-1 cells were determined by RT-PCR as described in the Section 2. The luciferase activity in each culture medium (b), cell lysate (c) of INS-1 cells stably expressing SP-NL-MANF and the secreted ratio of SP-NL-MANF in each case (a) was evaluated as described in Fig. 2. The values represent the mean ± SD from 3 independent cultures. The data were analyzed by a one-way ANOVA followed by the Scheffe test to evaluate the effects of each treatment on the luciferase activity in each sample. The values marked with an asterisk are significantly different from the value of the untreated control cells ($p < 0.05$).

secreted MANF. To address these problems and to evaluate secreted MANF more sensitively and quantitatively, we focused our attention on NanoLuc, a novel 19-kDa luciferase [21]. NanoLuc consists of 171 amino acids and is smaller than both firefly luciferase (548 aa) and enhanced green fluorescent protein (239 aa), which are frequently used to analyze the transcriptional regulation of many genes and to detect the intracellular localization of many types of proteins. Therefore, if the behavior of a NanoLuc-tagged protein is similar to that of the wild-type protein, the measurement of NanoLuc activity derived from the modified protein will enable the evaluation of protein transport and secretion more efficiently and quantitatively. In this study, NanoLuc was applied for the analysis of MANF biosynthesis and secretion based on our previous study [12]. As shown in Fig. 1, both wt MANF and SP-NL-MANF were almost equally secreted into the culture medium (Fig. 1A); however, the luciferase activity increased in a time-dependent manner only in the culture medium of the SP-NL-MANF-transfected cells (Fig. 1B). To further estimate the usefulness of SP-NL-MANF, we investigated the effects of the co-transfection of mutant Sar1(H79G) and GRP78 together with SP-NL-MANF on the secretion of MANF, as based on the previous reports [12,19]. In addition to wt MANF, co-transfection of SP-NL-MANF together with mutant Sar1(H79G) was confirmed to decrease both the amount of NanoLuc-tagged MANF by a western blotting analysis (Fig. 2A) and the activity of NanoLuc in the culture medium (Fig. 2B). Furthermore, the co-transfection of wt GRP78 but not mutant GRP78 lacking four C-terminal amino acids, KDEL (ΔC GRP78) [24], together with SP-NL-MANF reduced the extracellular luciferase activity by approximately 10% of that of the control obtained from cells transfected with SP-NL-MANF only (Fig. 2C). As these results were well consistent with previous reports about MANF secretion [12,19], we considered that NanoLuc-tagged MANF is appropriate for evaluating the intracellular transport and secretion of MANF.

Next, we established two cell lines, INS-1 (Fig. 3) and COS7 (Supplementary Fig. 1) cells stably expressing SP-NL-MANF, and evaluated the secretion of MANF in 48- or 96-well plates. As shown in Fig. 3B, we confirmed that only a small number of cells was sufficient to estimate secreted MANF quantitatively through measurement of the NanoLuc activity inside and outside of the cells. We then investigated the secretion of SP-NL-MANF from INS-1 cells after treatment with five different stimuli. Because INS-1 cells have a developed ER due to the constitutive biosynthesis and secretion of insulin, these cells are burden with ER stress even under physiological conditions and would be vulnerable to intra- and extracellular stimuli disturbing ER homeostasis [25,26]. Indeed, the ratio of spliced XBP-1 (sXBP-1) to unspliced XBP-1 (uXBP-1) in INS-1 cells is relatively higher than that in a different type of cell, Neuro2a cells. This phenomenon indicates that IRE1 [6], one of three major ER resident stress sensors, is constitutively activated even without any stimuli, reflecting the above features of pancreatic β-cells. As shown in Fig. 4, the treatment of five agents that affect the expression of well-known ER stress-inducible genes in different manners influenced the biosynthesis and secretion of SP-NL-MANF in INS-1 cells. In consistent with a previous report [19], Tg treatment increased the NanoLuc activity in the culture medium, whereas it decreased the intracellular luciferase activity in an inverse proportion. Serum deprivation, which did not induce any ER stress-induced gene in INS-1 cells (Fig. 4A), significantly reduced the intra- and extracellular luciferase activities (Fig. 4B). Interestingly, the abundance of extracellular but not intracellular SP-NL-MANF was restored when bovine serum albumin (BSA) was added to the serum-free culture medium (Supplementary Fig. 2). These results indicate that the appropriate amount of proteins, including albumin, in the culture medium facilitates the secretion of MANF. The saposin-like charged structure of MANF, as predicted by Lindholm and Saarma [27], might associate with the lipid-associated factors in serum. The treatment with high potassium, which increased the expression of MANF and GRP78 mRNA in wt INS-1 cells to a lesser extent, up-regulated the extracellular level of SP-NL-MANF without significantly affecting the intracellular level (Fig. 4C and D). In contrast, both the Tm treatment and serum/glucose deprivation, which triggered ER stress, did not increase the secretion of SP-NL-MANF (Fig. 4E and F); instead, the latter reduced the intracellular level of SP-NL-MANF in INS-1 cells as did serum withdrawn alone. Collectively, these results suggest that MANF secretion is not simply proportional to the degree of ER stress responses in INS-1 cells.

In previous studies regarding the expression level and localization of MANF [12,19,28], the intra- and extracellular amount of MANF was estimated using western blotting analysis and microscopic imaging of GFP-tagged MANF. We consider that the present NanoLuc-based approach would facilitate the analysis of the intra- and extracellular behavior of MANF more accurately and quantitatively in a low background. Recently, ER resident proteins have been demonstrated to form highly dynamic complexes [29,30]. Therefore, both the characterization of relationships between GRP78 and MANF [12,19] and also the analysis of interactions with other ER-resident proteins using this NanoLuc-system might reveal a novel role of MANF in rescuing cells from ER stress. Additionally, very recently, Song et al. reported hepcidin-induced ferroportin internalization by fusing NanoLuc to the C-terminus of ferroportin [31]. Accordingly, SP-NL-MANF used in the present study might be a useful tool for identifying a receptor for MANF and characterizing their interactions. Furthermore, screening using the NanoLuc system would be helpful for identifying agents that increase MANF secretion and would be useful in applications for the prevention and treatment of several ER stress-related diseases, including neurodegenerative disease, ischemia and diabetes.

Acknowledgments

We are grateful to Dr. Wei Liu and Dr. Jennifer Lippincott-Schwartz and Promega Corporation for providing HA-tagged Sar1 construct (H79G) and NanoLuc gene, respectively. This work is in part supported by the Research Foundation for the Electrotechnology of Chubu (to K.O.), the Suzuken Memorial Foundation (to K.O.) and Grant-in-Aid for Challenging Exploratory Research (no. 26670692, to F.A.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.031.

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