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7 Characterization of novel bicistronic Sindbis virus vectors, SinEGdsp and SinIRES-EG, in cultured neurons

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I. Abstract

Sindbis virus is a neurotropic virus whose modified RNA genome is used as a eukaryotic expression vector for neurons. Here, we developed bicistronic Sindbis vectors that can express multiple foreign genes, and characterized these Sindbis virus vectors with respect to the strength of their foreign gene expression and cytotoxicity in cultured neurons. The original SinRep5 vectors carrying lacZ or EGFP had higher expression efficiency in cultured neurons than in cultured glial cells. EGFP or lacZ expression was detected at 3 to 4 h and reached maximum levels 24 to 48 h after infection. Cell viability, however, began to decline 24 h after infection. Whole-cell voltage-clamp

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recordings from cortical neurons revealed that the voltage-dependent inward currents began to decrease at 12 h,while there were no significant alterations in other passive electrical membrane properties during infection. Gene expression efficiency was compared between the double subgenomic vector SinEGdsp and the modified vector SinIRES-EG carrying an internal ribosomal entry site. SinIRES-EG/lacZ had lower EGFP expression than did SinEGdsp/lacZ. Although rapid biological analysis is required before cytotoxicity appears, the novel double subgenomic Sindbis vector, SinEGdsp, provides a useful molecular tool for high level foreign gene expression.

II. Introduction

Gene transfer is a powerful tool for studying gene function in neurons and synapses. A number of studies have demonstrated that DNA virus vectors, such as herpes simplex type I, adenovirus, adeno-associated virus, and vaccinia virus, can be used to introduce a foreign gene into neural cells with various characteristics [1-4]. Herpes simplex type I vector appears to be selective for neurons, however, its usefulness is limited by its cytopathogenicity to the target cells [5]. Replication-defective adenoviruses, which show long-lasting expression of a foreign gene with minimal cytotoxicity, are useful vectors for a wide variety of post-mitotic cells [2,6,7]. Although neural cells are not natural adenovirus hosts, modified promoters enable neural expression of a recombinant gene both *in vivo* and *in vitro* [8,9]. Selective delivery of a recombinant gene to neurons, however, is particularly difficult because glial cells are often more susceptible to the adenovirus infection than neurons [2,10]. Retrovirus vectors, which can integrate the reverse transcribed virus genome as well as a foreign gene in post-mitotic cells, might be a useful alternative [11]. For example, HIV-derived lentivirus vectors were developed to introduce a recombinant gene into post-mitotic neurons *in vivo* [12].

Alphaviruses with RNA genomes, such as Sindbis viruses and Semliki Forest viruses, have been intensively investigated and their vector application has been greatly advanced [13,14]. Their replication-competent RNA can be easily *in vitro* transcribed from commercially available plasmid vector encoding the pseudoviral genome [15,16]. In nature, these viruses are transmitted by mosquitoes to vertebrate hosts, usually birds and mammals. Several Sindbis virus strains cause fatal infections in the central nervous system of newborn mice [17]. The Sindbis virus particle is enveloped and has an 11.7 kb-plus-strand RNA genome, which is capped at the 5' terminus and polyadenylated at the 3' terminus. After entry into the target cell, the genomic RNA functions as mRNA to produce the replicase enzyme that is essential for RNA-dependent transcription. The enzyme synthesizes full-length minus-strand intermediates for synthesis of viral genomic RNA as well as for transcription of subgenomic RNA using an internal promoter (Psg). The subgenomic RNA transcripts accumulate in the cytoplasm and the cellular ribosome machinery cap-dependently translated the viral proteins. In appropriate host cells, Sindbis virus infection can produce an estimated $5x10^5$ molecules per cell of the subgenomic mRNA, ~10^7-10^8 molecules per cell of the virion structural proteins, and more than 10^3 infectious particles per cell [18].

Various types of replication-competent Sindbis virus vectors were recently constructed and used for gene delivery to the central nervous system [19], sympathetic neurons [20], and dividing cells [14,21].

Biological advantages of the Sindbis virus vector are (i) short-term and high level induction of mRNA, (ii) protein expression without nuclear splicing, and (iii) technical ease in producing high titer recombinant virus by standard genetic engineering techniques. Restoration of cellular membrane conditions, neurotransmission, and the maintenance of cell viability might be important factors for gene delivery to neurons. Little is known about how Sindbis virus infection affects the physiological properties of individual neurons. We examined the cytotoxic effects of Sindbis virus-mediated gene expression and the electrophysiological properties of virus-infected cortical neurons. As accommodation of multiple foreign genes is often beneficial for such eukaryotic expression vectors, we developed two Sindbis vectors with a bicistronic structure (Fig.1). One is referred to as SinEGdsp because it contains two viral subgenomic promoters for mRNA synthesis. The other vector, referred to as SinIRES-EG, contains an internal ribosomal entry site (IRES). The IRES was first discovered as a *cis*-acting element in members of the picornavirus family, and subsequently found in non-viral mRNAs as well [22,23]. These *cis*-acting IRES elements mediate cap-independent translation initiation of bicistronic mRNA transcripts.

In this review article, we describe basic biological and physiological features of the Sindbis expression system. Cell specificity, time course of protein expression, cell toxicity and membrane properties are evaluated, and advantages and disadvantages of Sindbis vector-based gene delivery are discussed.

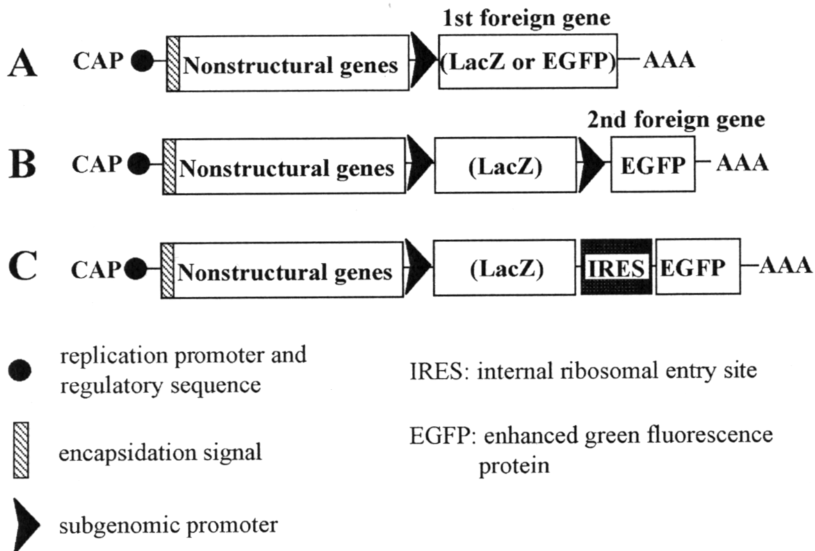

Figure 1. Diagram of wild-type SinRep5, SinEGdsp, and SinIRES-EG vector RNAs. (A) SinRep5 (SinRep/lacZ and SinRep/EGFP) structure. These vectors have a lacZ or EGFP gene as a foreign gene driven by a single subgenomic promoter. (B) SinEGdsp/lacZ structure. This vector has duplicate subgenomic promoters to transcribe mRNAs for the 1st foreign gene (lacZ) and the 2nd foreign gene (EGFP), independently. (C) SinIRES-EG/lacZ structure. This vector has a single subgenomic promoter and an internal ribosomal entry site (IRES) element to translate proteins of foreign genes (lacZ and EGFP) from a single mRNA.

III. Preparation of bicistronic Sindbis virus vectors

The viral packaging system requires two separate sets of RNA; the recombinant vector and helper RNAs. The recombinant vector RNA encodes the viral replicase (nsp1-4) genes and foreign gene(s). The helper RNA contains viral structural proteins that are essential for virus assembly and infectivity. One benefit of the system is provided by a unique packaging signal that is located at the 5' end in the recombinant vector RNA but is not present in the helper RNA. Therefore, only recombinant vector RNA is packaged into the virion as a virus genome, which allows for a single round of infection without virus replication [24]. Another benefit is provided by the helper RNA. In general, cell tropism for virus infection is determined by interaction(s) of the viral *env* protein and receptor molecule(s) of the cell surface. Highly efficient viral infectivity of neural cells is attained by a helper RNA encoding neurovirulent *env* protein [25].

Several of the Sindbis virus vector constructs were tested in this study (Fig.1). Wild-type SinRep5 contains the lacZ or EGFP gene as a foreign gene, which is transcribed under the control of the Psg promoter. Plasmid DNA; pSinRep5, and pSinRep/lacZ carrying the lacZ were commercially purchased from Invitrogen Co. Ltd (Carlsbad, CA). The pSinRep/EGFP was generated by ligation of the EGFP gene at multiple cloning sites (MCS) of pSinRep5 (Fig.1A). We used the monocistronic pSinRep5 constructs (SinRep/lacZ and SinRep/EGFP) as controls to evaluate the biological properties of modified bicistronic vectors.

We developed newly designed bicistronic vectors; SinEGdsp and SinIRES-EG (Fig.1B, 1C). A physical map of these wild-type vector plasmids and the partial nucleotide sequences are shown in Fig.2. In brief, the plasmid pSinEGdsp was generated from pSinRep/EGFP. An *Stu* I restriction site was incorporated upstream of the 5' flanking region of the subgenomic promoter region of pSinRep/EGFP using polymerase chain reaction (PCR) techniques. The *Stu* I-*Pac* I fragment (the 2nd Psg, entire EGFP gene, and polyA tail) was then inserted between the *Stu* I and *Pac* I restriction sites of the pSinRep5. Accordingly, this vector carries duplicate subgenomic promoters (Psg). A foreign gene is transcribed from the 1st Psg and the EGFP is transcribed as an internal marker from the 2nd Psg. The other vector, SinIRES-EG, had a single subgenomic promoter, but contained an IRES element for the second gene expression. In Fig.2, the *Sph* I-*Nco* I fragment contains the IRES of the encephalomyocarditis virus (ECMV). Both a foreign gene (cloned into MCS) and EGFP were translated from a single mRNA. We also introduced a lacZ gene to the above vectors and generated recombinant plasmids, SinEGdsp/lacZ and SinIRES-EG/lacZ, to compare the protein expression efficiency of both the bicistronic vectors. The recombinant vector plasmid was linearized by *Pac I* or *Not I* digestion in the 3' flanking region. The RNA transcripts were synthesized *in vitro* using an *in vitro* transcription kit (InvitroScript™ Cap SP6; Invitrogen) according to the manufacturer's instruction.

IV. Comparison of SinRep/lacZ infectivity among various cell types

Fig.3 shows experimental procedures of virus preparation and infection. For virion assembly, helper DH(26S) RNA transcript was co-transfected in baby hamster kidney (BHK) cells. Virus structural proteins including capsid and neurovirulent *env* proteins

were expressed from the helper RNA *in trans*, which allowed for packaging of the recombinant vector RNA into a virion.

Sindbis virus has a very wide host range, infecting many species from insect to higher vertebrates. There are strain variations that have different cell tropism and virulence. At first, we determined host cell range of this neurovirulent pseudovirus vector. Established cell lines such as BHK, P19 (a clone from mouse teratocarcinoma), C6 (rat glioma), NIH 3T3 (mouse fibroblast), HeLa (human cancer in uterus), and PC12 (rat pheochromocytoma) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with a suitable serum at optimal concentrations and used as viral hosts. The day before infection, the cells were plated at $5x10^4$ cells/cm^2. The primary neocortical culture was prepared from Sprague-Dawley rats (embryonic day 18) as described previously [26]. The dissociated culture was plated at a density of $1x10^5$ cells/cm^2. The culture medium was replaced every 3 d with fresh DMEM-N2 medium. Mixed glial cells were prepared from newborn rat brain and plated at a density of $2.5x10^4$ cells/cm^2. The mixed glial cells were further purified into astrocytes, oligodendrocytes, and microglia using a modification of the method originally described by Cole and de Vellis [27].

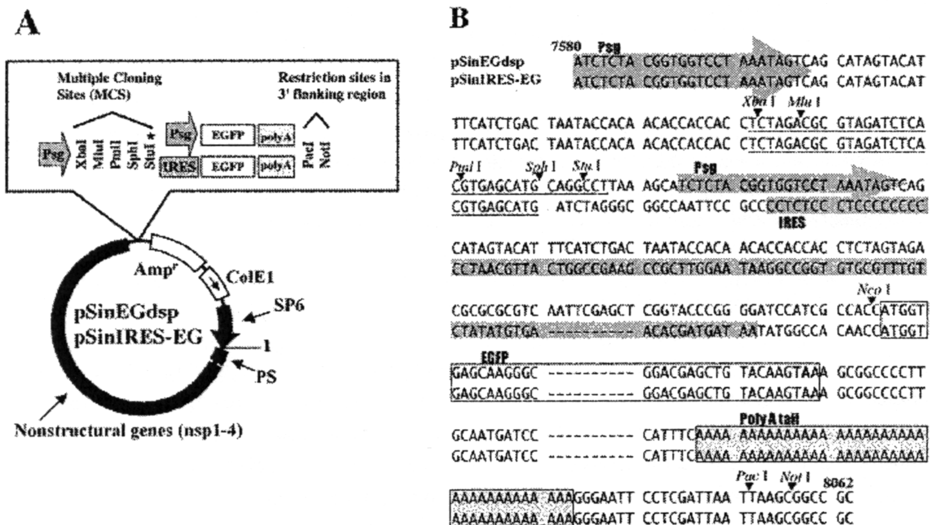

Figure 2. Structure of bicistronic Sindbis virus vectors; SinEGdsp and SinIRES-EGFP plasmid. These bicistronic vector plasmids originated from pSinRep5 (Invitrogen no.K750-01). (A) Map of restriction sites of pSinEGdsp and pSinIRES-EGFP vector. ★)pSinIRES-EGFP lacks this *Stu* I site. All recombinant plasmids can be linearized by *Not* I, or *Pac* I in the 3' restriction sites. Abbreviations: Sp6; Sp6 promoter region, PS; packaging signal unit, Nonstructural genes (nsp1-4); viral replicases, Psg; subgenomic promoter. (B) Partial nucleotide sequences of pSinEGdsp and pSinIRES-EG. Full length pSinEGdsp consists of 10805 nucleotides, in which the 2nd subgenomic promoter region and EGFP gene (863 nt.) are inserted between 7680 and 7688 of pSinRep5. Full length pSinIRES-EG consists of 11278 nucleotides, in which the IRES element of ECMV and the EGFP gene (1340 nt.) are inserted between 7676 and 7688 of pSinRep5. Nucleotide number starts at the packaging signal as referred to the pSinRep5.

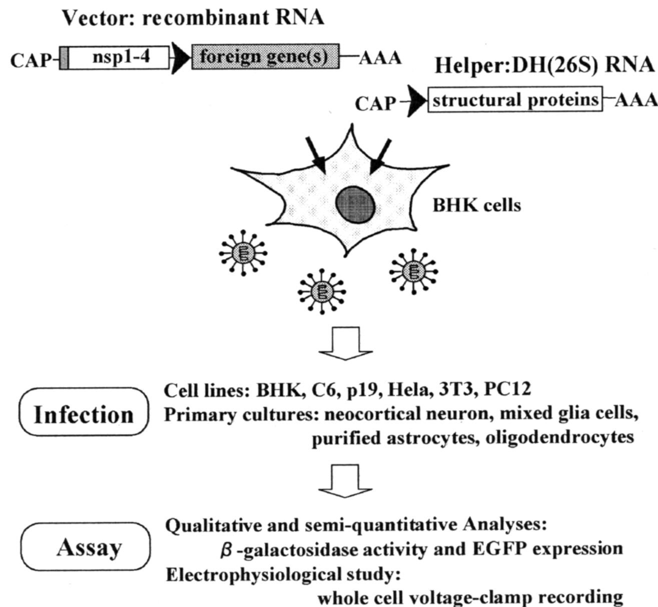

Figure 3. Experimental procedures for Sindbis virus construction and infection. Recombinant Sindbis virus vector was produced by co-transfection with a recombinant vector RNA and a helper RNA into BHK cells by electroporation. The optimal parameter/capacitance was 960 μF/0.23kV (Gene pulsar, BioRad, Hercules, CA) using a 1×10^7 cell suspension. After 24 h transfection, infectious virus was harvested in the culture supernatant and aliquots (0.5 ml) were stored at –80°C until use. Culture plates were tilted every 15 min during a 1-h incubation at 37°C. The cells were then gently washed and overlaid with fresh medium.

To estimate the strength of foreign gene expression in various types of cells, β-galactosidase enzyme activity was qualitatively and quantitatively determined. β-Galactosidase-positive cells were identified with the incubation of X-gal as a substrate. Quantitative assay for β-galactosidase enzyme activity was performed with cell lysate using 4-methylumbelliferyle-β-D-galactoside (MUG) as a fluorogenic substrate [28]. Qualitative and semi-quantitative analyses of EGFP expression were also performed. EGFP-positive cells were viewed by fluorescent microscopy, and the intensity of EGFP fluorescence was semi-quantitatively monitored using a fluoreplate reader, Fluorolite1000 (Dynatech Laboratories, Chantilly, CA). The whole-cell patch clamp method, which is a rapid and sensitive method for measuring membrane properties, was used for physiological evaluation.

Table 1 summarizes the wide host/tissue range of Sindbis virus infection in mammalian cells. In all of the examined cell lines, the virus had highly efficient lacZ expression, but different infectivity. In primary culture, neocortical neurons were more susceptible than cultured glial cells. The infectious unit of neuronal cultures was approximately 500-fold higher than that of glial cultures. In general, viral *env* glycoproteins are involved in the attachment-entry process for infection. Previous

Table 1. Comparison of SinRep/lacZ infectivity among primary culture and cell lines

primary culture	efficacy (%)	cell lines	efficacy (%)
BHK	100	BHK	100
Neocortical neurons	8.1	C6	30
Mixed glial cells	0.02	P19	113
Astrocytes	0.03	3T3	28
Oligodendrocytes	0.02	Hela	2
Microglia	Not detectable	PC12	0.1

The infection efficacy for each culture was calculated by counting X-gal stained cells infected 16-18 h post-infection. Infection efficacy was set to 100% for BHK cells.

studies have identified several receptor molecules for Sindbis viruses. A high affinity laminin receptor (67-63 kDa) was suggested to be a major receptor for Sindbis virus, which is highly conserved across the animal kingdom [29]. Recently, highly adapted Sindbis virus strain to BHK cells carries the *env* protein (E2) that interacts with heparan sulfate, a type of glycosaminoglycan (GAG) molecule [30,31]. The GAG molecules are found as sulfated polyanions in cytoplasmic membranes and distributed in a wide variety of vertebrate and invertebrate species in different concentrations [30,32]. Differences in SinRep/lacZ infectivity might reflect GAG expression levels as well as host/tissue distribution of a high affinity laminin receptor.

The time course of β-galactosidase enzyme activity in infected neurons and astrocytes is shown in Fig.4. Adenovirus vectors are widely used for post-mitotic cells, but foreign gene in the adenovirus vectors is often transferred in glia cells of brain tissue and slices [33-35]. Consistent with other reports, Sindbis vector achieves preferential gene transfer to neurons [19,36]. The culture was infected with SinRep/lacZ vectors at a multiplicity of infection (moi) of 1~2. In both cultures, lacZ expression was detected at 4 h, and reached maximum level 24 h after infection. Cell viability began to decline 24 h after infection in both types of cultures. Cell death was observed in astrocytes and neurons with a similar time course. In astrocytes, however, the lacZ expression efficiency was much lower than in neurons. This result indicated a poor relation between the amount of lacZ expression and the rate of cell death. In our study, lacZ expression was monitored as the viral infection index. After transcription of viral genomic RNA, the viral mRNA was presumably translated in a cap-dependent initiation manner. Recent studies revealed that neurons have unique translational machinery, especially in dendrites [37,38]. In brain tissue, the representative translation initiation factors are enriched in neurons rather than in glial cells [39]. This might explain why lacZ expression was greater in neurons than in glial cells.

Sindbis virus infection triggers glutamate release and excess glutamate leads to excitotoxic death of neurons [40]. When glutamate receptor antagonists [10 μM CNQX (6-cyano-7-nitroquinoaline-2,3-dione) for AMPA-type and kainate-type receptors and 25 μM AP-5 (D-2-amino-5-phoshonevalarete) for NMDA receptors] were added during the viral absorption period, cell toxicity was diminished in our preliminary study (data not shown). In addition, excess glutamate pre-existing in the virus stock might be problematic and influence cell viability. Interestingly, Sindbis virus-infected neurons *in vivo* or in slice culture survived longer than those in dissociated neuronal culture

[41,42]. In slice culture, the microenvironment produced by glial process around the synapse is likely more preserved than in dissociated culture. Such synaptic structure may help clearing the excess glutamate from the synaptic cleft. In contrast, in our experiment, neurons were over 90% pure in culture and this may make the culture more vulnerable to the excitotoxicity. Presumably, a neuron-glia mixed culture is more resistant against glutamate toxicity than a neuron-enriched culture.

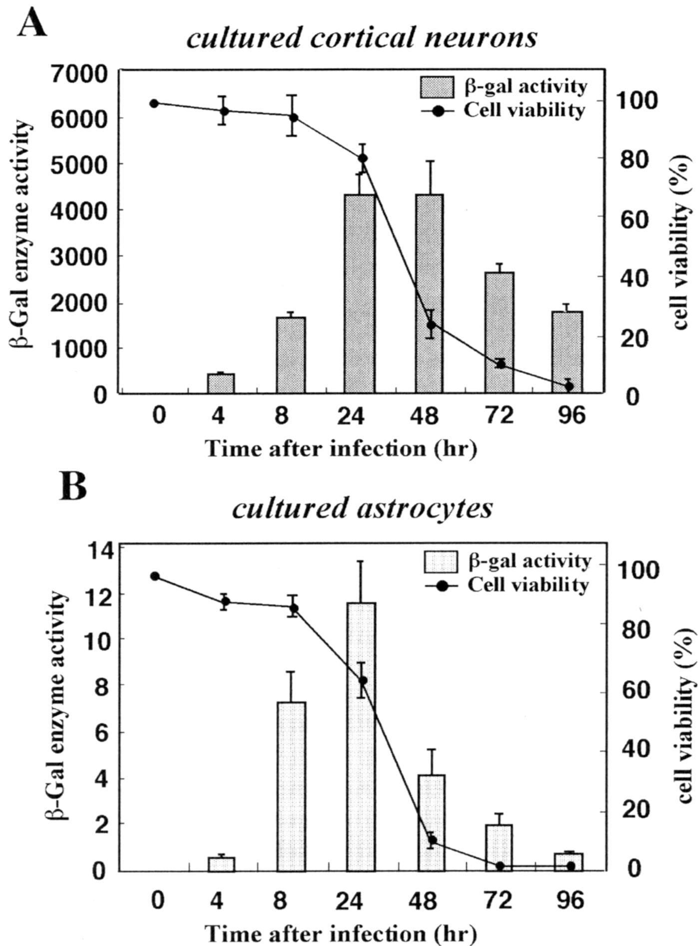

Figure 4. Time course of β-galactosidase activity and cell viability in cultured neurons (A) and glia cells (B). Cells were infected at an moi of 1~2 with Sindbis virus vector and further incubated for up to 96 h. At each time point, cells were lysed and assayed by β-galactosidase activity using 4-methylumbelliferyle-β-D-galactoside as a substrate. Gray bars (A) and dotted bars (B) show the fluorescent unit index of β-galactosidase activity (β-gal activity) per 1 μg of protein lysate. Cell viability was determined by trypan-blue exclusion. Percent (%) cell viability is indicated by a thick line. Data are presented as mean ± SD of three to four independent experiments.

V. Electrophysiological properties of neocortical neurons infected with SinRep/EGFP

To examine whether vector infection causes physiological changes in membrane properties, we analyzed passive and active electrophysiological properties using whole-cell recording (Fig.5). Cells grown for 4 d were inoculated with SinRep/EGFP virus at an moi of 0.2~0.6. EGFP expression was detected 3 h post-infection and the intensity of

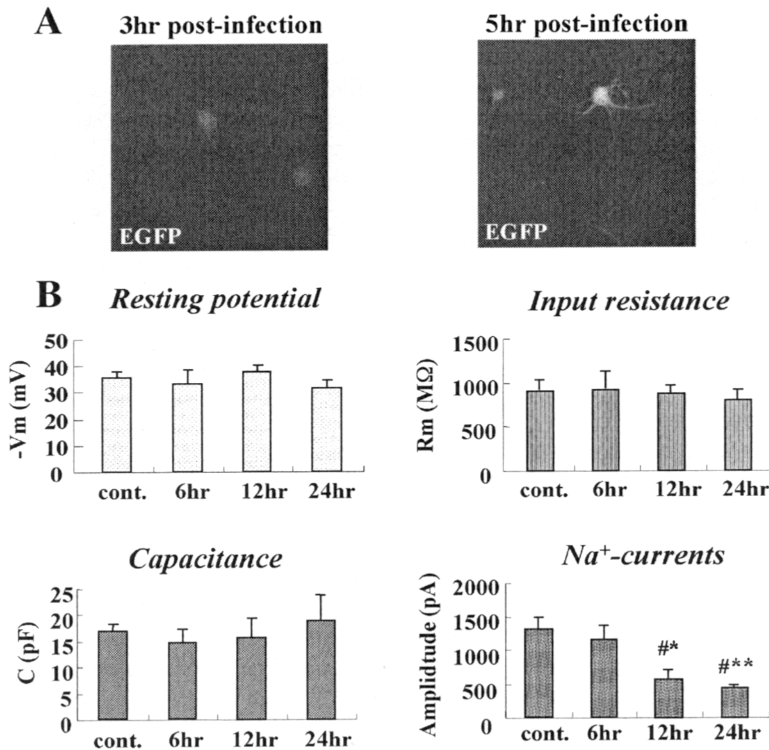

Figure 5. Electrophysiological properties of neocortical neurons infected with SinRep/EGFP. (A) EGFP expression 3 h (left) and 5 h (right) after infection. The primary neuronal culture (50 ~ 150 cells/mm^2) was infected with Sindbis vector at an moi of 0.2~0.6. (B) Electrical properties of the infected cells. Recording medium contained (in mM): 150 NaCl, 4KCl, 2 $CaCl_2$, 10 HEPES, 10 glucose, 1 $MgSO_4$, 10 sucrose (320 mOsm, pH7.4). Patch pipettes pulled to a tip resistance of 5-8 MΩ contained (in mM): 140 $KMeSO_3$, 10 KCl, 10 HEPES, 0.2 EGTA, 3 Mg-ATP, 0.3 Na-GTP (310 mOsm, pH7.4). Resting potential was measured within 30 s after whole-cell configuration and compensated junction potential. Input resistance and membrane capacitance were measured from current response to negative 5 mV pulse from –70mV. To calculate membrane capacitance, the elicited current transients were fitted with double exponentials curve using Clampfit (Axon Instruments)[70]. Voltage-gated currents were evoked by depolarizing voltage-steps to –10 mV from –100 mV. Significant differences between time points are indicated as follows: *, $p<0.05$ 12 h vs cont.; **, $p<0.01$ 24 h vs cont.; #, $p<0.05$ 12 h vs 6h. The Mann-Whitney U test was employed for statistical analysis.

the EGFP fluorescence increased at 5 h post-infection. Parameters important for membrane physiology, e.g., resting potential, input resistance, and membrane capacitance, were recorded at 0, 6, 12, and 24 h after infection. Because of shorter cultivation period, the resting potentials were slightly depolarized around -35 mV in control cells [43]. SinRep/EGFP infection did not cause any change in the resting potentials during experimental period. The input resistance of infected neurons was approximately 900 MΩ, indicating that the membrane conductance for ion current/flow was nearly constant before and after viral infection. In our study, the membrane capacitance was maintained at a steady level until 24 h after infection. Accordingly, there was no significant change in membrane potential, input resistance, or cell capacitance in the Sindbis virus-infected cells during the experimental period. In contrast, amplitudes in voltage-dependent inward currents, which represent voltage-dated sodium currents, were significantly decreased 12 to 24 h after infection. The physiological effects of foreign gene expression in culture should be recorded within 12 h after viral infection.

Recently, gene transfer of transmitter receptor channels was achieved using Sindbis virus vector for electrophysiological characterization [41,42,44-46]. The advantages of Sindbis vector application are evidenced in both slice culture and the brain *in vivo*. In electrophysiological studies, slice culture might be more beneficial for viral infection than dissociated culture. In slice culture, neurons infected with Sindbis virus had normal passive membrane properties for 1 to 3 d [41]. EGFP expression in slice culture, however, began to appear approximately 6 h after infection and its intensity reached a maximum around 48 h after infection [42]. The infected neurons appeared morphologically normal and viable for up to 5 d post-infection [36].

VI. Comparison of gene expression efficiency between bicistronic vectors, SinIRES-EGFP/lacZ and SinEGdsp/lacZ

For practical application of bicistronic Sindbis expression vectors, it is important that primary and secondary foreign genes do not interfere with each other's expression. Fig.6 shows the 1st foreign (lacZ) gene and the 2nd foreign (EGFP) gene expression from bicistronic vectors, SinEGdsp/lacZ and SinIRES-EG/lacZ. Neocortical neurons were infected with individual Sindbis virus vectors at an moi of 1~2 in culture. The β-galactosidase expression of both SinIRES-EG/lacZ (A) and SinEGdsp/lacZ (B) was indistinguishable. The EGFP expression level of SinIRES-EG/lacZ was different from that of SinEGdsp/lacZ. After infection by SinIRES-EG/lacZ, EGFP fluorescence was detectable in only a few cells and the EGFP intensity of the individual cells was relatively low during the 24 h post-infection period. As to subcellular distribution of EGFP, the fluorescence was detected mainly in cell bodies, and less in dendrites. In SinEGdsp/lacZ vector infection, however, EGFP fluorescence was detected even in dendrites of some neurons. Quantitative analysis revealed that the EGFP expression of SinIRES-EG/lacZ was significantly lower and reached a maximum level, which was approximately 20% of EGFP expression from SinEGdap/lacZ, after 24 h infection (data not shown). The time course of EGFP expression was almost parallel to that of β-galactosidase (Fig.7). Both bicistronic vectors (SinEGdsp/lacZ and SinIRES-EG/lacZ) had similar cell viability kinetics to monocistronic vectors (data not shown). IRES-dependent

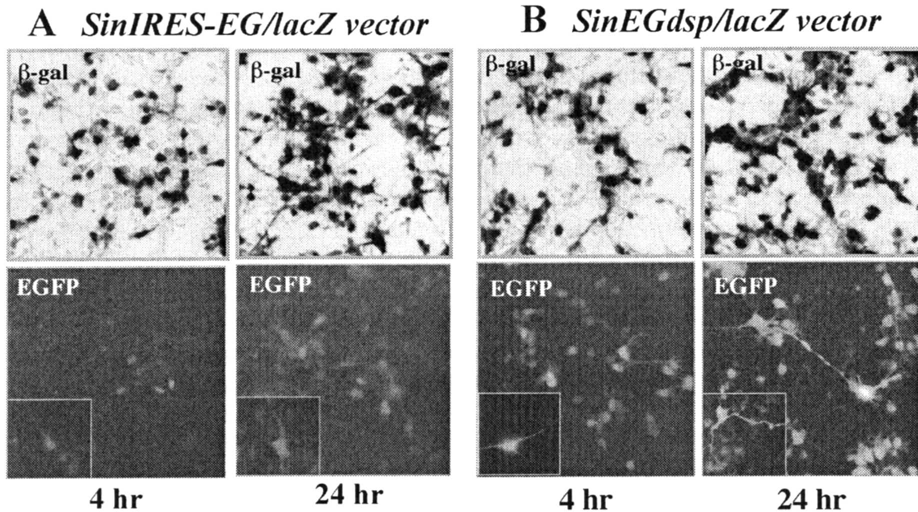

Figure 6. Bicistronic Sindbis vector-mediated gene expression in neuronal culture. Cells were infected with SinIRES-EG/lacZ (A) and SinEGdsp/lacZ (B) vectors. At 4 and 24 h after infection, cells positive for X-gal staining are presented in the upper panel and EGFP-positive cells are shown in the bottom panel. Inserts focus on dendritic EGFP fluorescence.

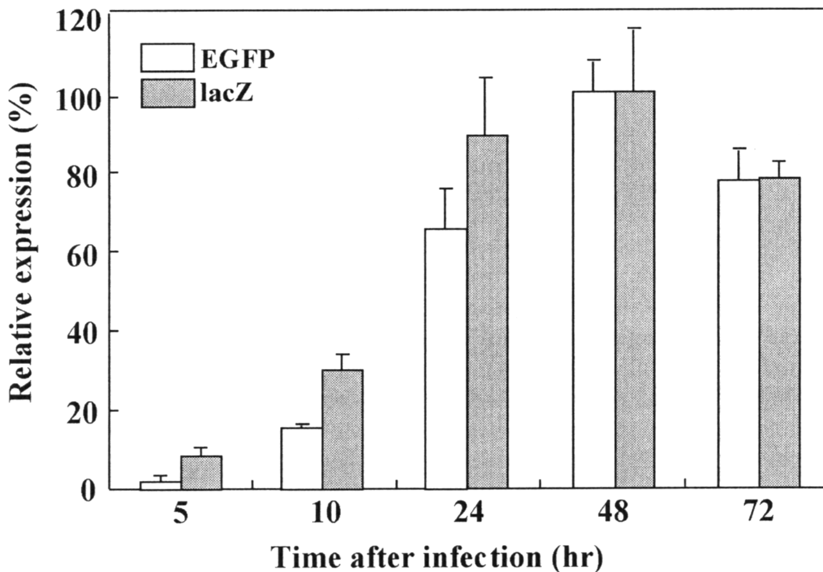

Figure 7. Parallel expression of EGFP and lacZ by SinEGdsp/lacZ vector. Both EGFP and lacZ expression reached maximum levels 48 h after infection. Relative expression (%) of EGFP and lacZ are indicated. Each relative expression % indicates EGFP or β-galactosidase units at the indicated time divided by their maximum expression units at 48 h post-infection.

gene expression was inferior in the Sindbis virus infection. In SinEGdsp/lacZ infection, almost all the neurons expressing lacZ were also positive for EGFP (90.5±4.4%) but with different intensity of fluorescence. These data indicated that the secondary gene expression (EGFP) was less likely to interfere in the level of the primary gene expression (lacZ) in SinEGdsp vector.

In this section, we demonstrated that novel bicistronic Sindbis vectors are useful for rapid and dual gene expression of the 1st foreign gene (lacZ) along with the 2nd foreign gene (EGFP) in cultured neurons. The IRES-dependent translation was less effectively driven by the Sindbis virus vector, SinIRES-EG. Recent studies revealed that IRES-mediated translation occurs throughout whole cell structures, but might be more efficient in dendrites [47]. The translation within the dendrites might provide a mechanism for a rapid local synthesis of proteins in response to certain synaptic stimulation [48-50]. In the present study, however, the IRES-dependent EGFP expression was detected mainly in the cell bodies, and less in dendrites. How other cellular stress(es) and synaptic activation lead to IRES-dependent gene expression using SinIRES-EG vector in neurons remains an interesting question.

VII. Application of Sindbis virus vectors

The feature described above points out the Sindbis virus expression system as a useful vector for introducing genes in both neurons *in vivo* and *in vitro*. For electrophysiological studies, Sindbis vector infection has been performed both *in vitro* and *in vivo* using slice culture and living animals, respectively. EGFP-tagged AMPA receptors were expressed in hippocampal CA1 neurons and had rapid delivery to spines and redistribution after synaptic NMDA receptor activation [41,44]. We reported the Sindbis vector-mediated expression of Ca^{2+}-permeable AMPA receptors at hippocampal CA1 synapses and induction of NMDA receptor-independent long-term potentiation [42]. In the latter experiment, the bicistronic Sindbis vector SinEGdsp was used to express the receptor molecule as well as the EGFP internal marker in cultured slices. SinEGdsp was also employed for expression of $GABA_A$ receptor subunits in cultured slices [45]. More recently, Sindbis vector was introduced into CA1 neurons of adult hippocampus by intra-cranial stereotaxic injection of virus solution [51]. The infected slices had healthy and stable electrophysiological responses as well as long-term potentiation, for at least 48 h post-infection. Sindbis virus-mediated gene transfer is an effective tool for studying gene function in synaptic plasticity in tissue.

The Sindbis vector also allows us to assess biochemical functions of foreign genes in neurons. In our laboratory, a Sindbis vector that carries EGFP protein fused with the AMPA receptor C-terminal peptides was constructed as a receptor decoy and applied to hippocampal culture [52]. Overexpression of the AMPA receptor decoy impaired normal subcellular distribution of the endogenous receptors, and functioned as a dominant negative molecule. Another group using the Sindbis vector demonstrated a novel RNA-based mechanism for upregulation of protein expression in a local subregion of the axon [53]. In brief, a fluorescent protein was detected as a marker for local translation within individual axons and growth cones, while transport of a membrane-anchored alkaline phosphatase was independently traced to the cell surface. Thus, application of the Sindbis virus vector is a powerful approach for biochemical analysis as well.

In nature, Sindbis virus infection sometimes causes rash, fever, and joint symptoms in humans. The safety of the Sindbis-based vector has not yet been determined. The possibility of replication-competent viruses produced by gene recombination is a risk of gene therapy. One report claims that recombinant viruses can be produced at a frequency of up to 3 x 10^{-3} infectious units/ml [54]. When a helper RNA packaging system is employed, recombination occurs less frequently. In this context, the highest caution must be used with Sindbis virus vectors and P2 level biohazard conditions are required for *in vivo* trials. To facilitate alphavirus application for gene delivery, a packaging cell line was established to produce virus particle stocks that are free from contaminating recombinant virus [55].

The practical use of Sindbis vector is limited by its cell toxicity in neurons. Mice exhibit age-dependent susceptibility to Sindbis virus infection and fatal encephalomyelitis is appeared in newborn mice [56]. During Sindbis virus infection, viral and cellular membrane fusion can initiate the apoptotic signal [57,58]. This is a good model for understanding not only the virus-specific activation of a programmed cell death/ apoptotic pathway, but also the cellular factors that possibly protect mature neurons from virus-induced apoptosis. Although the precise pathway of the apoptotic signal is not fully understood, the infection activates several caspases, a family of death-inducing cysteine proteases, resulting in cleavage of several cellular substrates [59-63]. Recently, it was reported that overexpression of several Bcl-2-related molecules (pro- and anti-apoptotic proteins) can protect mice from Sindbis virus-induced fatal encephalitis [64-67]. If manipulation of the apoptotic pathway can reduce the cytotoxicity of Sindbis virus vectors, the utility of the vector will be improved and virus vector-based gene therapy would be more acceptable.

To aim at protecting neurons in neurodegenerative diseases, interesting Sindbis vector-mediated gene delivery is studied. Spinal muscular atrophy (SMA) is a commom autosomal recessive disorder characterized by the loss of spinal cord motor neurons. Sindbis vector carrying an intact survival motor neuron protein (SMN) protects neurons from apoptosis and increases survival rates of virus-infected mice. [68]. Another group reported that overexpression of neprilysin in primary neurons from Sindbis vector shows clearance of extra- and intracellular amyloid β peptides, which are physiological metabolites and pathogenic agent of Alzheimer's disease. [69]. Sindbis virus-based gene therapy might be fruitful and desired for clinical application in brain diseases.

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