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A mammalian cell-based reverse two-hybrid system for functional analysis of 3C viral protease of human enterovirus 71

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Abstract

Although several cell-based reporter assays have been developed for screening of viral protease inhibitors, most of these assays have a significant limitation in that numerous false positives can be generated for the compounds that are interfering with reporter gene detection due to the cellular viability. To improve, we developed a mammalian cell-based assay based on the reverse two-hybrid system to monitor the proteolytic activity of human enterovirus 71 (EV71) 3C protease and to validate the cytotoxicity of compounds at the same time. In this system, the GAL4 DNA binding domain (M3) and transactivation domain (VP16) were fused, in-frame, with 3C or $3C^{mut}$. The $3C^{mut}$ was an inactivated protease with mutations at the predicted catalytic triad. The reporter plasmid contains a secreted alkaline phosphatase (SEAP) gene under the control of GAL4 activating sequences. We demonstrated that M3-3C-VP16 failed to turn on the expression of SEAP due to the separation of M3 and the VP16 domains by self-cleavage of 3C. In contrast, SEAP expression was induced by the M3-$3C^{mut}$-VP16 fusion protein or the M3-3C-VP16 in cells treated with AG7088, a potent inhibitor of human rhinoviruses (HRVs) 3C protease. Potentially, this protease detection system should greatly facilitate anti-EV71 drug discovery through a high-throughput screening.

Keywords: Human enterovirus 71; 3C protease; SEAP; AG7088; Drug screening

Human enteroviruses (EVs)[2] are members of the family Picornaviridae, which contain several serotypes: poliovirus (3 serotypes), coxsackievirus A (23 serotypes), coxsackievirus B (6 serotypes), echovirus (28 serotypes), and enterovirus (68–71, 73–78, 89–91 serotypes) [1–5]. These viruses are associated with a variety of different clinical syndromes,

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[1] Present address. TaiGen Biotechnology, Taipei, Taiwan 11470, Republic of China.
[2] *Abbreviations used:* EV, enterovirus; HFMD, hand, foot, and mouth disease; EV71, human enterovirus 71; IRES, internal ribosomal entry site; HRV, human rhinovirus; SEAP, secreted alkaline phosphatase; RT–PCR, reverse transcriptase PCR; MT, metallothionein; EGFP, enhanced green fluorescent protein; ORF, open reading frame; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; TNT, transcription and translation; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone.

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including upper respiratory tract illness, aseptic meningitis encephalitis, myocarditis, and hand, foot, and mouth disease (HFMD) [6]. Recently, human enterovirus 71 (EV71) has been implicated in several small and large outbreaks in the Asia–Pacific region that caused severe complications or death in children under 5 years of age [7–9]. In the 1998 EV71 outbreak in Taiwan, there were more than 120,000 infected children, 78 of whom died [8,10,11]. It is clear that effective antiviral drugs are needed to conquer this disease.

EV71 consists of a simple virus capsid and a single strand of positive-sense 7.5 kb RNA genome [12]. On infection, the 5′ nontranslational region serves as an internal ribosomal entry site (IRES) and controls the expression of a large polyprotein consisting of approximately 2161 amino acid residues. This polyprotein is then processed proteolytically by host and viral proteases into at least 11 mature proteins that are encoded in the following sequence: NH_2-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D-COOH [4]. In many of defined enteroviruses, including poliovirus and human rhinovirus (HRV), the 3C protease plays a major role during the virus replication life cycle [13]. In addition to its pivotal role in viral replication, 3C protease of poliovirus and EV71 were shown to kill cells, by apoptosis with a mechanism involving caspase activation, that were correlated with the pathogenesis of certain diseases in the central nervous system [14,15]. Sequence analysis for enterovirus 3C protease revealed no obvious homology with mammalian proteases. Therefore, 3C protease has been considered as a potential target for anti-EV71 drug discovery. Several different anti-HRV compounds targeting 3C protease have been designed based on the known sequence of the preferred peptide substrate, Leu-Phe-Gln [16]. These mimetic peptides were modified using the solved three-dimensional structure of HRV's 3C protease. Although this structure-aided rational design of protease inhibitors has generated a few potent lead compounds, no antiviral drugs for picornavirus are yet available for clinical administration [17,18]. It would be valuable to devise an alternative methodology to discover more protease inhibitors.

Dasmahapatra and coworkers developed a yeast two-hybrid system to study the coxsackievirus 3C protease inside cells [19]. However, the yeast system might not be ideal for drug screening because the yeast cell wall and mammalian cell membrane are very different with respect to compound permeability. In addition, the mammalian system would enable us to screen for 3C protease inhibitors inside cells that are more reflective of the natural environment of 3C protease. Although several cell-based protease activity assays have been developed for screening of protease inhibitors in the cellular environment [20–23], most of these assays have a significant limitation in that numerous false positives can be generated for the compounds that are interfering with the cellular viability. Based on the principle of the reverse two-hybrid system [24], we developed a new mammalian cell-based assay to monitor the proteolytic activity of 3C. It is noteworthy that the cellular toxicity of tested compound could be measured expediently at the same time. Positive hits discovered based on this system will be scored based on the increase in secreted alkaline phosphatase (SEAP) secreted into culture medium. In contrast, compounds with cellular toxicity or with activity that interferes indirectly with the secretion mechanism of SEAP would, in contrast, result in a decrease in the level of SEAP secretion. Therefore, false positive hits could be reduced substantially with this system. To test the functionality of the cell-based reporter assay for drug screening, we used an irreversible inhibitor of HRV 3C, AG7088, to examine the inhibition of autocatalytic activity of EV71 3C protease in mammalian cells. The results showed that AG7088 exhibited a dose-dependent induction of SEAP expression due to the inhibition of EV71 3C protease. Collectively, this assay system would allow for a simple and sensitive screening method for discovery of potential protease inhibitors that are permeable to cell membrane.

Materials and methods

Viruses and cell cultures

EV71 isolates in the 1998 outbreak were obtained from the clinical virology laboratory in Chang Gung Memorial Hospital (Linkou, Taiwan). For RNA isolation, EV71 infected cells were detached by shaking or scraping and collected in 15-ml centrifuge tubes. After spinning down the cells, the supernatant was decanted and 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to lyse the cells and dissociate the nucleoprotein complex. Following the phenol-chloroform extraction and RNA precipitation steps, RNA pellet was dried and dissolved in RNase-free water. The isolated viral RNA was used to generate reverse transcriptase PCR (RT–PCR) product encoding the 3C protease and flanking sequences.

Plasmid construction

Construction of pBAK8-MTEGFP-M3VP16

The cloning vector pBacPAK8-MTEGFP was modified from pBacPAK8 (Clontech, Mountain View, CA, USA). MT (metallothionein) promoter and EGFP (enhanced green fluorescent protein), including poly(A) signal fusion cassette [25], were inserted into pBacPAK8 with the *Eco*RV site. The M3VP16 DNA fragment was amplified by PCR using pM3-VP16 (Clontech) as the template with primers containing *Sal*I/*Eco*RV restriction sites and then ligated with the *Xho*I/*Sma*I vector backbone of pBAK8-MTEGFP to construct pBAK8-MTEGFP-M3VP16.

Construction of pBAK8-MTEGFP-M3-3C-VP16 and pBAK8-MTEGFP-M3-3C^{mut}-VP16

Enterovirus TW/2231/98 protease gene, whose coding region spans from the C-terminal 15 amino acids of 3A to the N-terminal 15 amino acids of 3D (Δ3A-3B-3C-Δ3D),

was amplified by RT–PCR with two primers containing the *Eco*RI/*Eco*RI site. The sequences of the primers used for RT–PCR are 5′-3C-F, 5′-CGGAATTCTCTCTCAC TGGTGTATGTTATT-3′, and 3C-R, 5′-CGGAATTCCC AACCTGCCAGTTTCTTTGAT-3′. The *Eco*RI cloning sites are underlined. The fragment was inserted into the middle of M3VP16 in pBAK8-MTEGFP-M3VP16 with the *Eco*RI site to create an intact open reading frame (ORF) of M3-Δ3A-3B-3C-Δ3D-VP16. For a control, we created the M3-Δ3A-3B-3C^{mut}-Δ3D-VP16 fusion construct by site-directed mutagenesis using PCR at the putative catalytic triad of the 3C gene; both His40 and Cys147 were substituted to Gly (Fig. 1). The sequences of primers for point mutations at the presumed catalytic triad are 3C^{mut} (H40G)-F, 5′-GCTCCCCAGAGGCTCCCAACCAG-3′, 3C^{mut} (H40G)-R, 5′-CTGGTTGGGAGCCTCTGGGGA GC-3′, 3C^{mut} (C147G)-F, 5′-AGGCAGGACAGGGTGGT GGTGTTG-3′, and 3C^{mut} (C147G)-R, 5′-CAACACCACC ACCCTGTCCTGCCT-3′. The mutated fragment was inserted with *Eco*RI to construct pBAK8-MTEGFP-M3-Δ3A-3B-3C^{mut}-Δ3D-VP16.

Construction of pGAL4-SEAP and pBAK8-MTEGFP-GAL4-SEAP

The GAL4 binding site was derived by PCR from pG5CAT (Clontech) using primers containing the *Kpn*I/*Nru*I cutting site. The product was cloned into pSEAP2-control (Clontech) with the *Kpn*I/*Nru*I cutting site to construct pGAL4-SEAP. In this plasmid, the SV40 promoter was replaced with the GAL4 binding site to control the

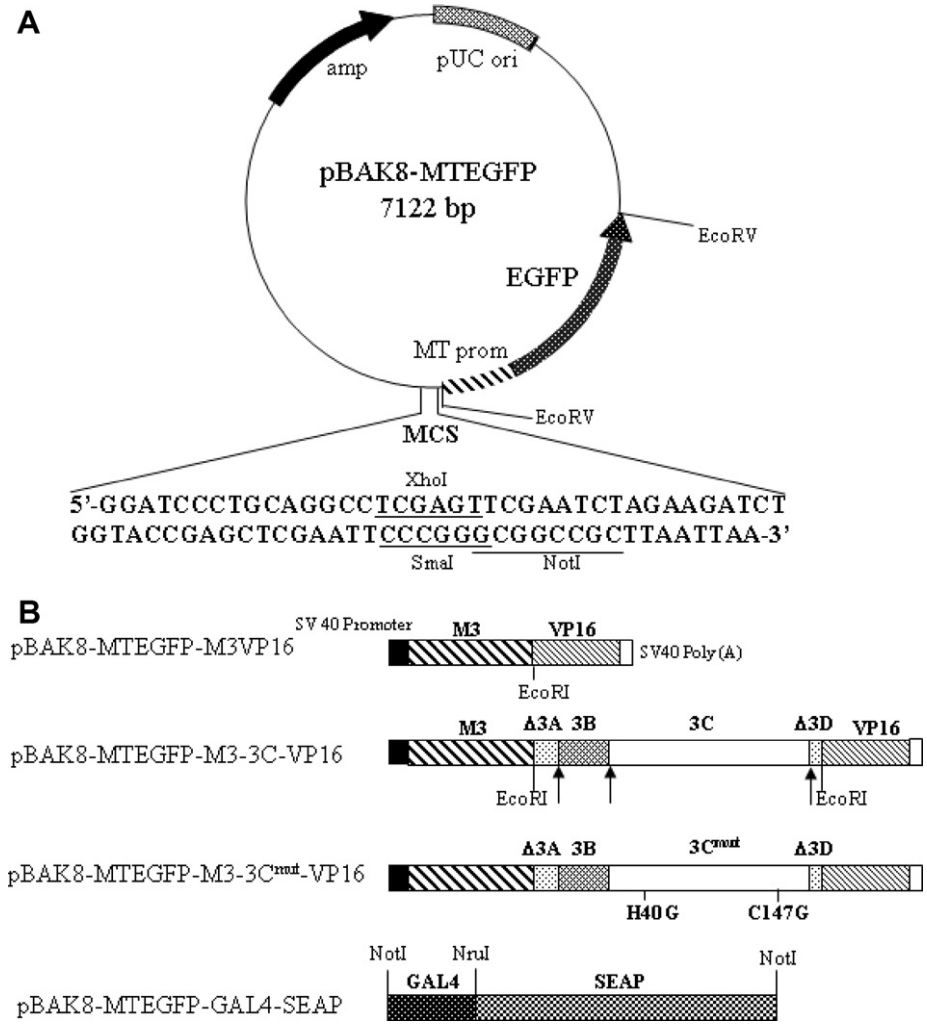


Fig. 1. Diagrams of chimeric transactivators and reporter construct. (A) Structure of the plasmid pBAK8-MTEGFP, in which EGFP gene was under the control of MT promoter (MT prom). The plasmid was used as vector backbone for insertion of various transactivators and reporter. The sequences of multiple cloning sites (MCS) are also shown, and the selected restriction sites in this study are underlined. (B) The names of the plasmids encoding the respective expression units are indicated at the left. The M3VP16, containing the DNA binding domain (M3) and the activation domain (VP16), in pBAK8-MTEGFP-M3VP16 plasmid was driven by the SV40 promoter. The coding region spanning from 3A C-terminal 15 amino acids to 3D N-terminal 15 amino acids was inserted into the middle of M3VP16 at the *Eco*RI site, resulting in pBAK8-MTEGFP-M3-3C-VP16 plasmid. The pBAK8-MTEGFP-M3-3C^{mut}-VP16 was created by site-directed mutagenesis to contain double mutations substituting His40 and Cys147 into Gly residues, destroying the protease activity of 3C. Possible 3C-cleavable sites within the M3-3C-VP16 fusion protein are indicated by arrows. In the reporter construct, pBAK8-MTEGFP-GAL4-SEAP, the transcription of SEAP was under the control of five consensus GAL4 binding sites.

expression of the reporter gene, SEAP. Finally, the GAL4-SEAP expression cassette was amplified by PCR using pGAL4-SEAP2 as the template with primers containing the *Not*I/*Not*I cutting site and then ligated with the *Not*I vector backbone of pBAK8-MTEGFP to construct pBAK8-MTEGFP-GAL4-SEAP.

Transfection of COS-7 cells and SEAP activity assay

COS-7 cells (monkey kidney cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. For transient transfection, cells were seeded in a 24-well plate at 6×10^4 cells per well in 1 ml of DMEM/10% fetal bovine serum (FBS) and incubated at 37 °C overnight until cells were 70% confluent. Then cells were transfected with various plasmid constructs using the Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen). As an internal control, 0.1 μg pCMV-GAL was cotransfected with 0.5 μg of each expression vector. The SEAP activity of the culture media and the β-galactosidase activity of cell lysates were measured at 48 h posttransfection by using a Phospha-Light assay kit (Tropix, Foster City, CA, USA) and a Galacto-Star assay kit (Tropix) according to the manufacturer's instructions. The chemiluminescence was detected with a TopCount Microplate Scintillation and Luminescence Counter (Packard, Meriden, CT, USA).

Western blotting

COS-7 cells were seeded in a 24-well plate at 6×10^4 cells per well in 1 ml of DMEM/10% FBS and incubated at 37 °C overnight. Subsequently, cells were transfected with various plasmid constructs using the Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen). Western blot analysis was performed according to standard procedures [26]. The membrane was stained with mouse polyclonal anti-3C antibody or mouse monoclonal anti-actin antibody (Chemicon International, Temecula, CA, USA).

Protease digestion of in vitro transcription and translation products

The template for substrate M3-Δ3A-3B-3C-Δ3D-VP16 and M3-Δ3A-3B-3C^{mut}-Δ3D-VP16 protein was obtained by PCR with the forward primer, 5′-GCTTACTGGCT TATCGAAATTAATACGACTCACTATAGGGAAGCT ACTGTCTTCTATCGAAC-3′, containing the promoter sequence for the T7 RNA polymerase (underlined), and the reverse primer, 5′-ACTGCTCTAGACTACCCACCG TACTCGTCAA-3′, containing a stopped codon. PCR products were transcribed and translated in vitro with rabbit reticulocyte lysates (Promega, Madison, WI, USA). For ^{35}S-labeled substrates, reaction was carried out at 30 °C for 2 h in the presence of ^{35}S-labeled methionine (Amersham, Piscataway, NJ, USA) at 100 μCi/ml. To assay the cleavage activity of 3C protease, aliquots of purified 3C protease was added to the translated hybrid protein for 30 min at 30 °C. Reactions were stopped by the addition of sample buffer (50 mM Tris–HCl [pH 6.8], 100 mM dithiothreitol [DTT], 2% sodium dodecyl sulfate [SDS], and 0.1% glycerol) and then boiled for 3 min. The cleavage of radiolabeled products was then analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) on 4 to 20% Tris–glycine gel (Invitrogen).

Recombinant histidine-tagged 3C protease and AG7088

The 3C protease of EV71 (strain TW/2231/98) was subcloned into pET23a plasmid (Novagen, Madison, WI, USA). The plasmid was transformed into BL21 *Escherichia coli* host. The 3C protease was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The histidine-tagged 3C protease was then purified using nickel-chelating column. Protein was dialyzed against storage buffer (25 mM Tris [pH 7.0], 50 mM NaCl, 0.1% Triton X-100, 50% glycerol, 1 mM ethylenediaminetetraacetic acid [EDTA], and 1 mM DTT), and protein concentration was determined by Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as the standard. The purified protease was stored at −70 °C in small aliquots. Compound AG7088 was synthesized in Jim-Min Fang's laboratory [27].

Results and discussion

Characterization of 3C protease activity fused inside the M3-3C-VP16 and M3-3C^{mut}-VP16 constructs

To demonstrate whether the created fusion transcription factor containing 3C protease would cleave itself as designed to separate the DNA binding (M3) and transactivation (VP16) domains, PCR fragments were generated to contain the full-length fusion protein under the control of the T7 promoter. The fusion proteins were produced and labeled with ^{35}S radioisotope in rabbit reticulocyte lysate using the in vitro transcription and translation system (TNT) for 1 and 3 h, respectively. The labeled protein products were separated on a 4 to 20% SDS–PAGE gel. The gels were dried and analyzed by autoradiography. As shown in Fig. 2, there was a distinct band whose size (∼ 52 kDa) is similar to the sizes of the predicted full-length products, M3-Δ3A-3B-3C-Δ3D-VP16, M3-3C-VP16 (lanes 1 and 2), and M3-3C^{mut}-VP16 (lanes 3 and 4). Two clear bands were observed in the M3-3C-VP16 sample but not in the M3-3C^{mut}-VP16. This band is likely to be the M3-3C degradation products, M3-Δ3A-3B-3C-Δ3D and M3-Δ3A-3B-3C (predicted MWs ∼ 43.2 and 41.4 kDa, respectively). To confirm the identities of cleaved products that were derived from 3C-mediated proteolytic processing, ^{35}S-labeled M3-3C-VP16 and M3-3C^{mut}-VP16 proteins were treated with purified 3C protease and incubated for 30 min. The uncleaved product (M3-Δ3A-3B-3C-Δ3D-

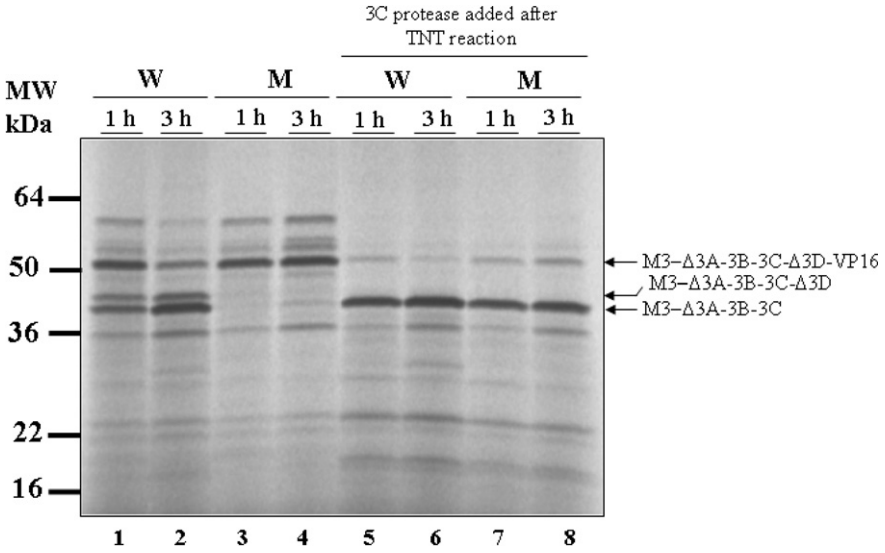


Fig. 2. Cell-free TNT analysis of 3C protease cleavage. PCR products derived from pBAK8-MTEGFP-M3-3C-VP16 (W) and pBAK8-MTEGFP-M3-3C^{mut}-VP16 (M) were reacted with reaction mixtures. The reaction mixtures were incubated for 1 and 3 h, respectively, at 30 °C in rabbit reticulocyte lysate containing ^{35}S-labeled methionine and cysteine. At the indicated time points, 5 U/μl recombinant 3C protease was further added into reaction mixtures for 30 min at 30 °C. ^{35}S-labeled TNT products were resolved by 4 to 20% SDS–PAGE and autoradiograph. The arrowheads indicate the expected sizes of M3-Δ3A-3B-3C-Δ3D-VP16 (52.0 kDa), M3-Δ3A-3B-3C-Δ3D (43.2 kDa), and M3-Δ3A-3B-3C (41.4 kDa). W and M indicate the expression vector containing wild-type 3C protease and mutant 3C protease, respectively.

VP16) disappeared and the cleavage product (M3-Δ3A-3B-3C) was accumulated in M3-3C^{mut}-VP16, consistent with the self-cleavage of M3-3C-VP16 (lanes 2, 6, and 8).

When general serine protease inhibitors, *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and *N*-tosyl-L-lysine chloromethyl ketone (TLCK), were included in the TNT experiment (Fig. 3), the degradation event was mitigated at 1.0 mM (lanes 5, 6, 11, and 12). The effects of TPCK and TLCK on protein degradation were not observed at 0.5 mM (lanes 3, 4, 9, and 10). At 5 mM TPCK and TLCK, the TNT experiment failed to produce any products (lane 7, 8, 13, and 14). This may be due to the toxic effect of TPCK and TLCK on the transcriptional and/or translational apparatus in the TNT system. These results showed that the molecular constructs were established successfully to meet our needs and provided a convenient cell-free system for monitoring drug action.

A cell-based reporter assay for monitoring EV71's 3C protease activity

To evaluate whether the blockage of the self-cleavable M3-3C-VP16 fusion protein was able to drive the SEAP reporter gene expression under the control of GAL4

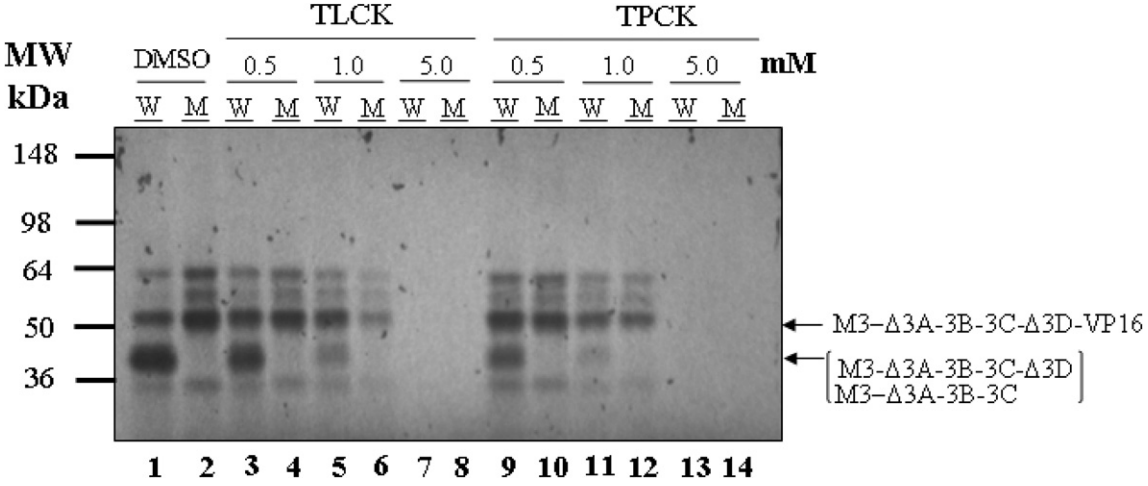
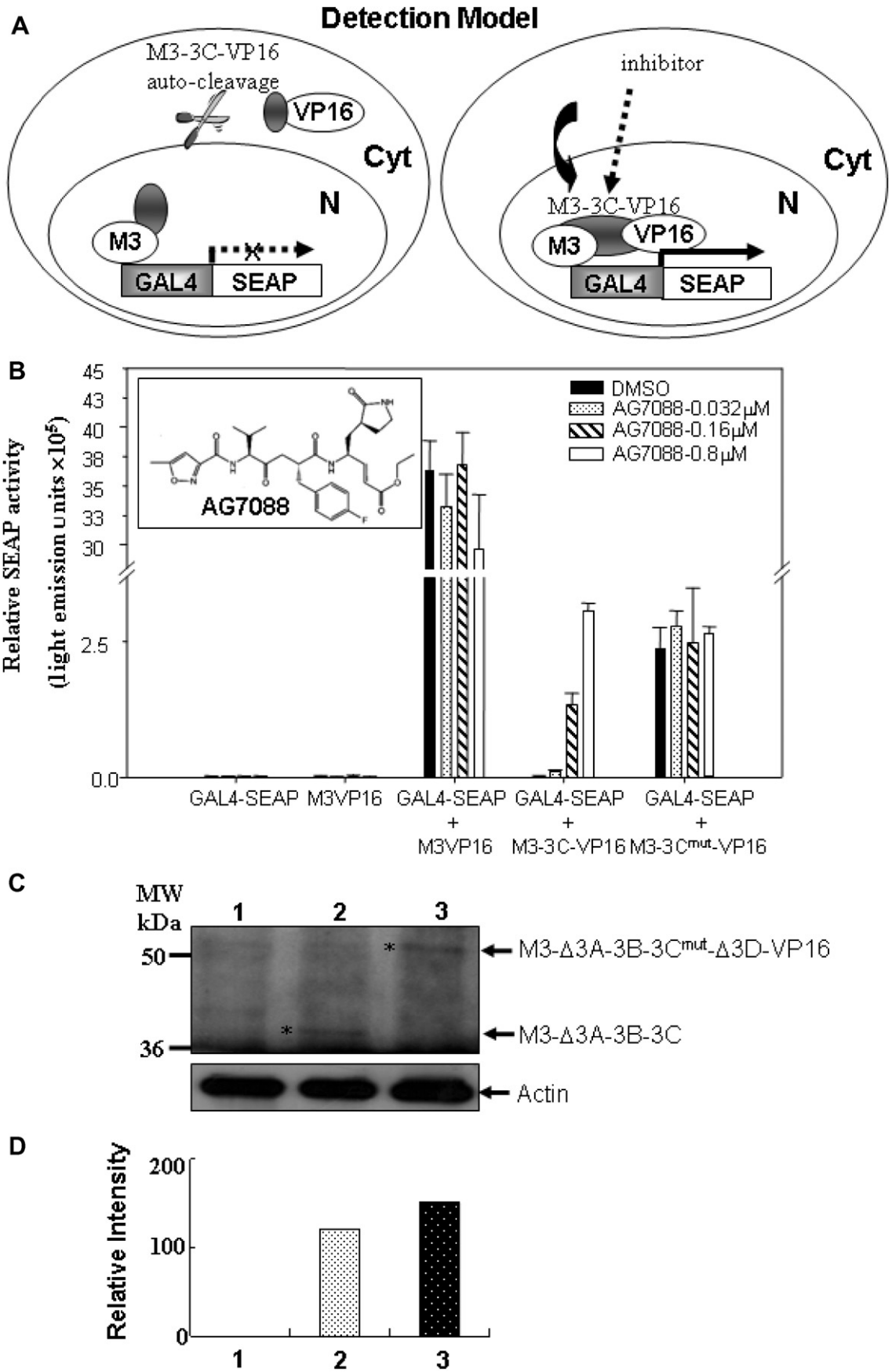


Fig. 3. Effects of protease inhibitors on the activity of the 3C protease. PCR products derived from reaction pBAK8-MTEGFP-M3-3C-VP16 (W) and pBAK8-MTEGFP-M3-3C^{mut}-VP16 (M) were reacted with reaction mixtures containing protease inhibitors or appropriate solvent controls using a cell-free TNT system (Promega) in the presence of ^{35}S-labeled methionine and cysteine. Following incubation for 3 h at 30 °C, reaction products were analyzed by SDS–PAGE and autoradiograph. Lanes 1 and 2 show a solvent control (DMSO). Lanes 3 to 14 show the reaction products treated with the indicated amounts of TLCK and TPCK. The arrowheads indicate the expected sizes of M3-Δ3A-3B-3C-Δ3D-VP16 (52.0 kDa), M3-Δ3A-3B-3C-Δ3D, and M3-Δ3A-3B-3C (41.4–43.2 kDa). W and M indicate the expression vector containing wild-type 3C protease and mutant 3C protease, respectively.

response element (UAS_G), an irreversible inhibitor of HRV 3C, AG7088, was used to treat cells at various concentrations. COS-7 cells were cotransfected with the following combinations of plasmids: (i) pBAK8-MTEGFP-GAL4-SEAP only, (ii) pBAK8-MTEGFP-M3VP16 only, (iii) pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3VP16, (iv) pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3-3C-VP16, and (v) pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3-$3C^{mut}$-VP16 (in which the M3-$3C^{mut}$-VP16 served as a control). The detection model is indicated in Fig. 4A. When cells were transfected with pBAK8-MTEGFP-GAL4-SEAP only, SEAP activity in the culture supernatant was only 2-fold higher than that of the nontransfected cells. This indicated that the GAL4 response element rendered low genetic background in controlling a reporter gene expression. When pBAK8-MTEGFP-GAL4-SEAP plasmid was cotransfected with pBAK8-MTEGFP-M3VP16 plasmid (which codes for the intact transcription factor without 3C fused in between the DNA binding and transactivation domains), SEAP activity in the cell culture supernatant was approximately 3 orders of magnitude higher than that in cells with only pBAK8-MTEGFP-GAL4-SEAP transfection. Importantly, when cells were cotransfected with pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3-3C-VP16 plasmids, SEAP activity in the media was only 2.5 times higher than that in the control (cells transfected with pBAK8-MTEGFP-GAL4-SEAP only). This indicated that the M3-3C-VP16 protein did not turn on much SEAP gene expression, most likely due to the fast self-cleavage rate of 3C protease given that there are three potential sites in the M3-3C-VP16 fusion protein for 3C to exert self-cleavage activity. When cells were cotransfected with pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3-$3C^{mut}$-VP16, the SEAP activity was approximately 10 times higher than that in the previous experiment. Therefore, the results appeared to have an acceptable window (the signal/noise ratio was $\sim$ 10-fold) to allow the screening of 3C protease inhibitors using this cell-based screening system. As for a SEAP activity analysis in the above cotransfected cells, there was a dose-dependent increase in secretion of SEAP from cells treated with AG7088, confirming that the inhibitory activity on self-cleavage of M3-3C-VP16 would effectively maintain the transactivation activity (Fig. 4B). As shown in Fig. 4C, it is evident that the actual cleavage of the fusion protein occurred in mammalian cells, as revealed by Western blot analysis. Expression of the fusion protein containing the active 3C protease resulted in the degradation of M3-Δ3A-3B-3C-Δ3D-VP16. The dominant band is likely to be the M3-Δ3A-3B-3C degradation product (Fig. 4C, lane 2). As expected, no cleaved fragments could be detected on expression of the fusion protein containing the mutated 3C protease (Fig. 4C, lane 3), consistent with the results from the cell-free TNT experiments (Fig. 2). In addition, densitometric quantification of the cleaved- and uncleaved-specific bands confirmed that the accumulation of the precursor was equal to the amount of degradation products from the autocatalytic cleavage by 3C protease (Fig. 4D). Taken together, these results indicate that no significant endogenous proteolysis in the cleavage of inactive M3-$3C^{mut}$-VP16 construct inside cells and the lower activity of GAL4-SEAP induced by M3-$3C^{mut}$-VP16 transactivation, as compared with M3-VP16, may be due to the lowered transcriptional level or the lower efficiency of nuclear translocation of the enlarged fusion protein.

EV71 has caused large epidemics, and the infection may be fatal in young children. Currently, there is no vaccine or drug to prevent or cure this viral infection. Viral 3C protease appears to be an attractive target for development of therapeutics. In addition to its important role in viral maturation, 3C protease activity of EV71 recently was shown to induce apoptosis in a human cell line [15]. A few cell-based assays have been developed for screening inhibitors of viral proteases. A yeast two-hybrid system to study the coxsackievirus 3C protease inside cells has been developed [19]. In the current study, we have developed and validated the cell-based screening system for screening EV71 3C pro-

Fig. 4. A cell-based reporter assay for EV71 3C protease activity. (A) Model of transcriptional activation by GAL-inducible reporter gene. M3-3C-VP16 fusion proteins contain the GAL4 DNA binding domain (M3), the transactivation domain (VP16), and the cleavage sites of 3C protease. When the inhibitor was introduced, the blockage of protease activity resulted in the nuclear translocation of M3-3C-VP16 and the subsequent activation of reporter gene. A hybrid protein, M3-$3C^{mut}$-VP16, containing the mutant 3C protease served as a control of the activation of GAL4 transcription. N, nucleus; Cyt, cytoplasm. (B) Activity of AG7088 against 3C protease. COS-7 cells were cotransfected with the following combinations of plasmids: (i) pBAK8-MTEGFP-GAL4-SEAP only, (ii) pBAK8-MTEGFP-M3VP16 only, (iii) pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3VP16, (iv) pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3-3C-VP16, and (v) pBAK8-MTEGFP-GAL4-SEAP and pBAK8-MTEGFP-M3-$3C^{mut}$-VP16. Shown is dose-dependent induction of GAL4-driven SEAP transcription by AG7088. The cells were incubated in the absence or in the presence of 0.032, 0.16, and 0.80 μM AG7088 for 12 h, and culture media were subjected to SEAP assay. In this experiment, pCMV-GAL was cotransfected to monitor the difference in transfection efficiency and β-galactosidase activity was used to normalize the SEAP of each transfection. The result is shown as a comparison with the induction of SEAP activity based on the interaction of the DNA binding domain (GAL4) and the transactivation domain (VP16). Each point represents the average of three independent experiments, and error bars reflecting standard deviations are shown. (C) Western blot analysis of self-cleavage by 3C protease inside cells. Lysates of COS-7 cells transiently transfected with pBAK8-MTEGFP-M3-3C-VP16 or pBAK8-MTEGFP-M3-$3C^{mut}$-VP16 were harvested at 48 h posttransfection. The cleaved and uncleaved products were detected by immunoblotting with an anti-3C antibody. The actin antibody was used as an internal control for protein amount. Lane 1 shows the untransfected cells. Lanes 2 and 3 show the pBAK8-MTEGFP-M3-3C-VP16-transfected and pBAK8-MTEGFP-M3-$3C^{mut}$-VP16-transfected cells, respectively. The arrowheads indicate the M3-Δ3A-3B-3C (41.4 kDa), M3-Δ3A-3B-$3C^{mut}$-Δ3D-VP16 (52.0 kDa), and actin. (D) Densitometric quantification of the untransfected cells (lane 1), M3-Δ3A-3B-3C (lane 2) and M3-Δ3A-3B-$3C^{mut}$-Δ3D-VP16 (lane 3) bands in the Western blot.


A
Detection Model
M3-3C-VP16
auto-cleavage
VP16
Cyt
N
M3
GAL4
SEAP
inhibitor
M3-3C-VP16
VP16
B
Relative SEAP activity
(light emission units ×10⁵)
AG7088
DMSO
AG7088-0.032μM
AG7088-0.16μM
AG7088-0.8μM
GAL4-SEAP
M3VP16
GAL4-SEAP + M3VP16
GAL4-SEAP + M3-3C-VP16
GAL4-SEAP + M3-3Cmut-VP16
C
MW kDa
M3-Δ3A-3B-3Cmut-Δ3D-VP16
M3-Δ3A-3B-3C
Actin
D
Relative Intensity

tease inhibitors. A major advantage of the cell-based assay is the elimination of false positives caused by toxic compounds because toxic compounds do not give rise to higher levels of reporter gene expression.

Several reagents have been used to treat enterovirus infections and showed variable effectiveness against the neurotropic EV71 in vitro, including enviroxime [28], pleconaril.[29], and lactoferrin [22,30]. In the case of pleconaril, with the capsid binding capability of capsid protein VP1, it was an available inhibitor for treatment of life-threatening enteroviral infections such as meningitis, encephalitis, and myocarditis [29]. However, pleconaril has been found to have limited activity against EV71 [31]. AG7088 is a potent irreversible inhibitor of HRV 3C protease with broad-spectrum antipicornavirus activity, including 48 HRV serotypes, 4 human enterovirus strains, and 46 untyped field isolates of HRV [17,32–34]. Binford and coworkers extended these findings when they evaluated the antiviral activity of AG7088 against 23 clinical isolates of HRV and 4 additional human enterovirus strains (CVB2, CVB5, EV6, and EV9). Based on the nucleotide sequence comparison and structural analysis among various isolates, the results demonstrated that 14 conserved amino acid residues played a critical role in 3C protease–AG7088 binding interactions [35]. A novel finding of our study was that AG7088 also showed potent inhibitory activity on EV71 3C protease (Fig. 4B). In conclusion, our assay system is a suitable tool to facilitate the discovery of antiviral drugs against EV71 infection.

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