Molecules and Cells

Supplementary Materials

Purification of MBP-TRIM25

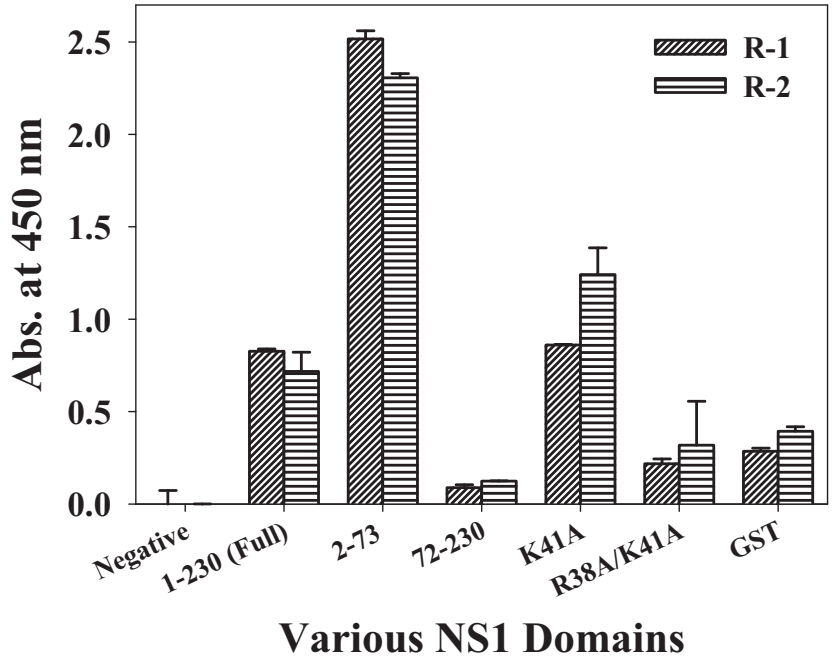
MBP-tagged TRIM25 protein was expressed in *E.coli* Rosetta 2 (DE3)^{TM} cells (Millipore). Transformed cells were grown in LB broth containing 0.2% glucose at 37 °C. Then, 0.2 mM IPTG was added and the cells were incubated under shaking conditions for 18 h at 25 °C, after which the cells were harvested by centrifugation. The pellet was resuspended in T1 buffer (20 mM Tris/Cl(pH 7.4), 1 mM EDTA, 1 mM sodium azide, and 1 mM DTT) and sonicated. After centrifugation, the supernatant was loaded onto a DEAE ion-exchange column (GE healthcare) and the flow throw was collected. Next, the pooled sample was applied to amylose resin (NEB). The column was washed with T2 buffer (T1 buffer + 200 mM NaCl) and eluted with T3 buffer (T2 buffer + 10 mM maltose). The purity of the proteins was analyzed by 10% SDS-PAGE. The final yield of MBP-TRIM25 was determined by the Bradford assay with BSA as the standard and the samples were stored at −80 °C.

Purification of His-RIG-I

His-tagged RIG-I was expressed in *E. coli* BL21(DE3) cells. The transformed cells were grown in LB broth at 37 °C and then further incubated at 4 °C for 3 h. Subsequently, 0.25 mM IPTG was added and the cells were incubated under shaking conditions for 18 h at 25 °C, after which the cells were harvested by centrifugation. The pellet was resuspended in R1 buffer (50 mM HEPES; pH 7.4, 10% glycerol, 500 mM NaCl, 0.2% NP-40, 10 mM imidazole, 1 mM PMSF, 0.5 mg/ml lysozyme, and protease inhibitor cocktail) and sonicated. After centrifugation, the supernatant was applied to a Ni-NTA chelating agarose (Incospham). The column was washed with R2 buffer (50 mM HEPES; pH 7.4, 10% glycerol, 500 mM NaCl, 0.2% NP-40, and 50 mM imidazole) and the His-tagged RIG-I was eluted by increasing the imidazole concentration. The purest fractions were applied to a heparin-Sepharose column (GE healthcare), and the resin was washed with R3 buffer (50 mM Tris/Cl(pH 7.4), 10% glycerol, 0.2% NP-40, and 20 mM NaCl) and eluted with increasing concentrations of NaCl (up to 500 mM). The final yield of His-RIG-I was determined by the Bradford assay (Bio-Rad) with BSA as the standard.

Determination of the aptamer binding region of NS1

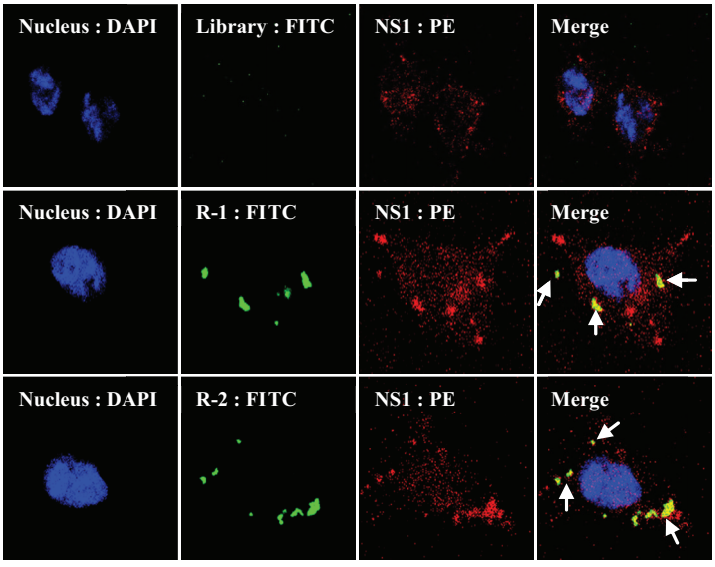
To identify the aptamer binding region on NS1, ELISA was performed as described in Materials and Methods, with slight modifications. GST-tagged RBD (2–73 amino acids), ED (72–230 amino acids), and mutants (K41A and K41A/R38A) of the NS1 protein were purified as described previously (Woo et al., 2013). 3′-Biotinylated RNA aptamers were synthesized by terminal transferase using Biotin-16-dUTP (Roche) (Rosemeyer et al., 1995). A streptavidin-coated plate was incubated with 100 μl/well of the selected 3′-biotinylated aptamer (100 nM in PBS) for 1 h at room temperature. The wells were blocked with 5% BSA in PBST for 1 h and further incubated with 100 μl/well of truncated and mutant NS1 proteins (full length, RBD, ED, R38A mutant, and R38A/K41A double mutant at 100 nM in PBS) for 1 h at room temperature. The bound aptamers were detected using a GST antibody-conjugated HRP (1:1000 in PBS, Pierce). Then the color developing reaction was performed as described in Materials and Methods.



Supplementary Fig. S1. Comparison of binding ability of aptamers to truncated and mutant NS1 by ELISA. The immobilized 3′-biotinylated aptamer was incubated with GST-tagged truncated and mutant NS1. After addition of GST antibody-HRP, the amount of the NS1-aptamer complex was measured by ELISA. Lane 1, aptamer only without NS1; Lane 2, amino acids 1–230 (full-length NS1); Lane 3, amino acids 2–73 (RBD); Lane 4, amino acids 72–230 (ED); Lane 5, K41A mutant; Lane 6, R38A/K41A double mutant; Lane 7, GST only for a negative control.

Confocal imaging

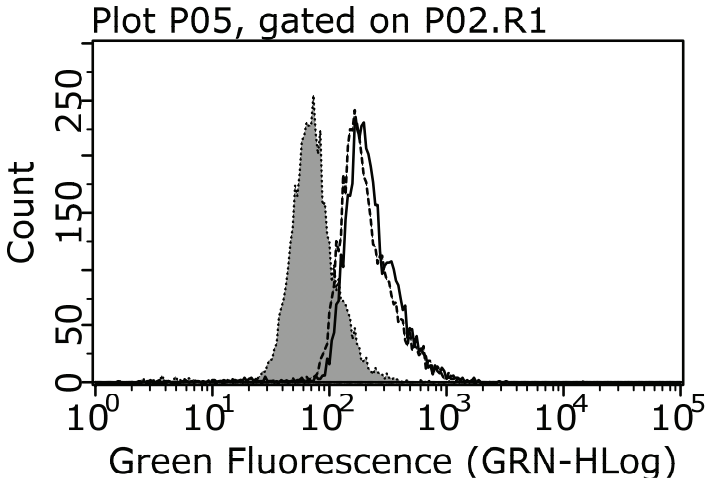
The 3′ end of the RNA aptamer was labeled with fluorescein-12-dUTP using terminal transferase (Roche) for confocal imaging. Then, 4×10^4 HEK293T cells were seeded in eight-chambered slides. After 24 h incubation, the cells were transfected with 0.2 μg of pcDNA6-AIV NS1 and 2 μg of FITC-labeled RNA aptamer using lipofectamin™ 2000 (Invitrogen) as described previously (Woo et al., 2013). After 6 h incubation, the media were substituted with DMEM containing 1% polyI:C. After 48 h incubation, the transfected cells were fixed with 4% paraformaldehyde for 10 min, permeabilized using 0.1% (v/v) Triton X-100 for 5 min, blocked with 1% BSA in PBS for 30 min, and incubated with diluted PE-labeled His-tag antibody (1:100 in PBS, Abcam) for 30 min at 4 °C. Then, 3 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) was added to the sample for 10 min, and it was washed several times with PBS. Confocal imaging was obtained using a Carl Zeiss LSM 700 laser scanning microscope and analyzed using ZEN imaging software. The FITC-labeled RNA library was used as a negative control.



Supplementary Fig. S2. Binding assay of NS1 with the selected aptamers in HEK293T cells. Confocal imaging of HEK293T cells treated with NS1 and aptamers. Cells were transfected with NS1 and an aptamer: the RNA library as a negative control (row 1), the selected FITC-labeled R-1 aptamer (row 2), and the selected FITC-labeled R-2 aptamer (row 3). Cells were visualized by the distribution of the nucleus (blue color stained with DAPI), the selected aptamer (green color by FITC), and NS1 (red color by PE conjugated anti-6X His-tag antibody). Overlays of the fluorescence images are shown. Arrows indicate the localization of the FITC-labeled aptamer bound to NS1.

Flow cytometric analysis

To confirm the selectivity of RNA aptamers, 1×10^6 HEK293T cells were transfected with 24 μg of pcDNA6-AIV NS1 using lipofectamin™ 2000 and incubated for 6 h. The media were substituted with DMEM containing 1% polyI:C. After 48 h incubation, transfected cells were harvested with trypsin-EDTA, fixed in 4% paraformaldehyde solution, permeabilized with 0.1% (v/v) Triton X-100 for 15 min, blocked with 1% BSA in PBS for 15 min, and reacted with 2 μg FITC-labeled aptamer in PBS for 30 min at 4 °C. Then, the samples were further incubated with diluted PE-labeled His-tag antibody (1:100 in PBS, Abcam) for 30 min at 4 °C and washed several times with PBS. Cells were suspended in 500 μl PBS containing 10% fetal calf serum and 1% NaN_3. The fluorescence was determined with a Guava easyCyte flow cytometer (Millipore) by counting 10,000 events. The FITC-labeled RNA library was used as a negative control.



Supplementary Fig. S3. Flow cytometric analysis of aptamer binding to NS1. The FITC-labeled RNA library (gray integral), R-1 aptamer (full line), and R-2 aptamer (dotted line) were measured for binding to NS1 in cells by flow cytometry. The mean fluorescence signal represents binding affinity: RNA library (76.30), R-1 (230.84), and R-2 (208.08).

REFERENCES

Rosemeyer, V., Laubrock, A., and Seibl, R. (1995). Nonradioactive 3'-end-labeling of RNA molecules of different lengths by terminal deoxynucleotidyltransferase. Anal Biochem *224*, 446-449.

Woo, H.M., Kim, K.S., Lee, J.M., Shim, H.S., Cho, S.J., Lee, W.K., Ko, H.W., Keum, Y.S., Kim, S.Y., Pathinayake, P., et al. (2013). Single-stranded DNA aptamer that specifically binds to the influenza virus NS1 protein suppresses interferon antagonism. Antiviral Res *100*, 337-345.