Development of the Direct and Timesaving *in vitro* Assay Methods for Anti-HIV Compounds through Fluorescently Labeled gp41 Domains

Choon Woo Lim, Bong-Suk Jin, Eun Gyeong Yang, and Yeon Gyu Yu*

Division of Life Sciences, Korea Institute of Science and Technology, PO Box 131, Seoul 130-650, Korea
Received August 23, 2003

Key Words : Ectodomain of gp41, Protein-protein interaction, *In vitro* assay, Fluorescently labeled proteins

Fluorescence techniques have been widely employed in chemistry and biology for over a century.[1] Various fluorescence methods[2] have been developed for elucidating specific interactions in biological systems as well as for application on screening system for drug development. Nowadays, many current researches are interested in monitoring protein-protein interactions, which has an important key in understanding complex biological phenomena. As a model system of protein-protein interaction we have characterized the fusion protein (gp41) of human immunodeficiency virus (HIV). The ectodomain of gp41, which is a transmembrane subunit of the envelope protein of HIV, composed of a coiled coil and membrane proximal helical domains.[4] Since the strong interaction between the two domains of gp41 has important roles in mediating fusion between membranes of the virus and target cell,[5] the *in vitro* assay systems targeting the activity of gp41 have been extensively studied and developed for the research of drug discovery.[6] Previously, we have reported the *in vitro* assay system,[3] which is mainly based on the conventional ELISA for screening inhibitory compounds against gp41. Here we report that a novel *in vitro* assay system has been developed using the fluorescence labeled protein. The coiled coil or the C-terminal membrane proximal helical domains (C-αH) of gp41 were inserted into the C-terminus of thioredoxin (Trx) or glutathione S-transferase (GST), respectively, to generate the chimera proteins, Trx-N and GST-C, as described previously.[3] Then, the GST-C was conjugated with the thiol-reactive Rhodamine dye (Rhodamine Red C_2 maleimide)[7] as shown in Figure 1. Since the labeling site of the fluorescence dye is located at the GST domain, it could not interfere the inherent interaction between the two helical domains. Conjugation methods and measurements of labeling efficiency were followed by the recommended procedures of the supplied company.[7]

The interaction between Trx-N and Rhodamine dye labeled GST-C has been measured using a fluorescence plate reader with λ_{ex} = 560 nm and λ_{em} = 580 nm.[8] The experimental procedures are partially based on ELISA methods. The Trx-N is immobilized on the surface of 96-well plates by incubation at room temperature for 1 h. After blocking un-reacted surface with 5% skim milk and washing 6 times with PBST buffers (100 mM Sodium Phosphate, 150 mM NaCl and 0.5% Tween 20 at pH 7.0), the rhodamine-labeled GST-C (Rh-S-GST-C) added to the plate for 1 h. After removing unbound protein by washing with PBST buffer 5 times, the bound Rhodamine-labeled GST-C was denatured and separated from the surface into the solution phase by the incubation with 8 M urea. The fluorescence intensity of the eluted Rhodamin-labeled GST-C was monitored and analyzed by the fluorescence plate reader. As this assay requires an incubation time of only one hour after blocking steps, it is considerably faster than the conventional ELISA assay that requires several additional time-consuming washing and incubation steps. In addition, the fluorescence intensity is directly proportional to the amount of Rhodamine labeled GST-C due to the absence of signal amplification step during the detection or color development process.

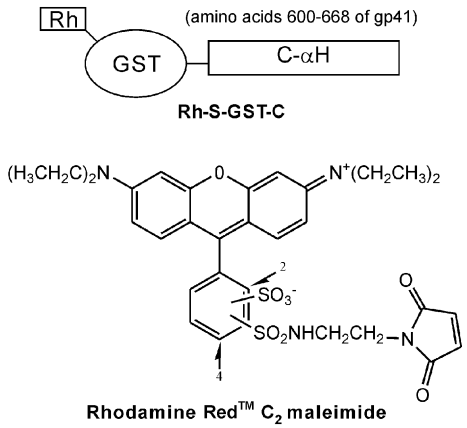

Figure 1. Graphic representations of Rhodamine-labeled GST-C.

The dose dependent binding of the Rh-S-GST-C on the surface immobilized Trx-N (filled circle) and the basal level of fluorescence intensity of the Rh-S-GST-C bound to Trx (open circle) indicated that the binding between Trx-N and Rh-S-GST-C is mediated by the interaction between the coiled coil and C-αH region gp41, and Rh-S-GST-C has no nonspecific binding to the blocking agent or thioredoxin protein under the assay condition.

From the standard curve equation[9] of Rh-S-GST-C, the amounts of the bound Rh-S-GST-C on the surface could be determined and therefore, the apparent dissociation constants[10] (K_d) between GST-C and Trx-N proteins have been evaluated to be 8.7×10^{-7} M^{-1}, assuming the binding mode is simple one to one. This value is consistent with the results of the previous reports.[3] To confirm the specificity of the

*To whom correspondence should be addressed. Phone: +82-2-958-5936, Fax: +82-2-958-5909, e-mail: ygy@kist.re.kr

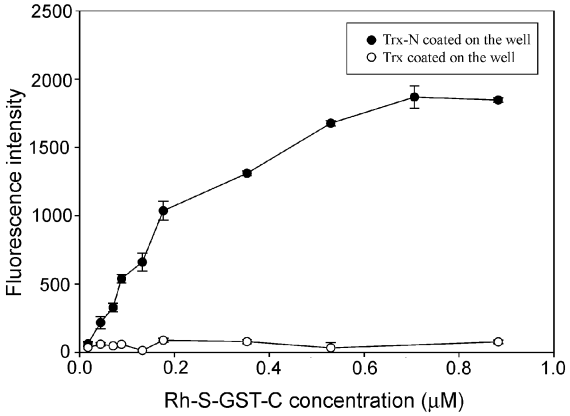

Figure 2. Dose dependent binding of the Rh-S-GST-C on the surface-immobilized Trx-N. Trx-N (filled circle) or Trx (open circle) were coated on the surface by the incubation of the protein solution (6 μg/mL) overnight at 4 °C respectively. After blocking the surface with 5% skim milk solution, different concentration of the Rh-S-GST-C were applied on the plate, and the amount of bound Rh-S-GST-C were measured after the treatment of 8 M urea. The excitation and emission wavelengths are 560 nm and 580 nm.

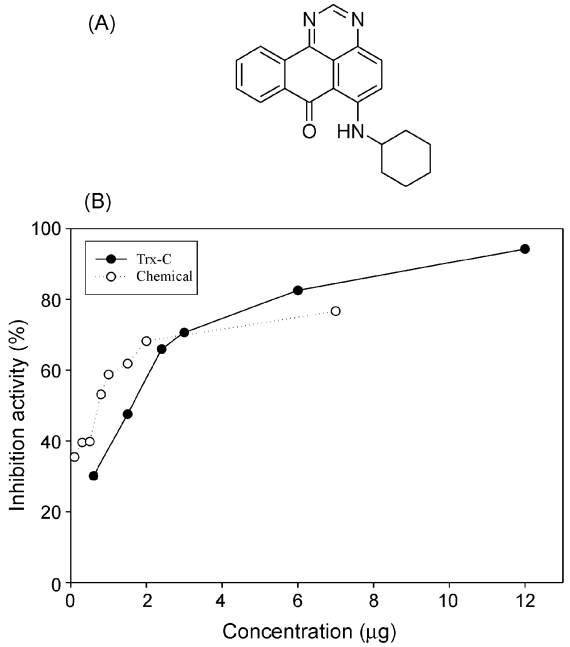

Figure 3. (A) Structure of KY100. (B) Inhibition of the interactions between Rh-S-GST-C (6 μg/mL) and the surface-coated Trx-N by Trx-C (filled circle) or KY100 (open circle), respectively. Different concentration of Trx-C and KY100 were mixed with Rh-S-GST-C and applied to Trx-N coated plate. The percentage of inhibition activity was calculated from the relative reduced amount of the bound Rh-S-GST-C due to Trx-C or KY100.

interactions, the competitive inhibition by another C-αH containing protein at the C-terminus of thioredoxin (Trx-C) or a chemical compound (KY100: Fig. 3A) that had been previously identified to prevent the interaction between the coiled coil and C-αH region of gp41[11] (unpublished data) has been tested. The Trx-C and KY100 were shown to effectively inhibit the interaction between Rh-S-GST-C and Trx-N as shown in Figure 3B. The concentrations for 50% inhibition were measured as 2 and 1 μg/mL for Trx-N and KY100, respectively, implying that this assay system could be used to screen anti-HIV inhibitory compounds with high sensitivity.

In conclusion, we have demonstrated that the developed assay system is a direct, timesaving, and sensitive method for screening inhibitory compounds against the activity of gp41. The usage of directly fluorescence labeled protein has advantages of low level of non-specific binding and high sensitivity, which is comparable with ELISA assay. This type of assay could be applied for other type of protein-protein interactions.

Acknowledgements. This work was supported by grants from the 21C Frontier Research Program, Functional Proteomics Research, Ministry of Science and Technology, Korea.

References

1. *Applied Fluorescence in Chemistry, Biology and Medicine*; Rettig, W.; Strehmel, B.; Schrader, S.; Seifert, H., Eds.; Springer: New York, 1999.
2. Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*; Kluwer/ Plenum Publishers: New York, U. S. A., 1999.
3. (a) Ryu, J.-R.; Lee, J.; Choo, S.; Yoon, S. H.; Woo, E.-R.; Yu, Y. G. *Mol. Cells* **1998**, *8*, 717-723. (b) Ryu, J.-R.; Jin, B.-S.; Suh, M.-J.; Yoo, Y.-S.; Yoon, S. H.; Woo, E.-R.; Yu, Y. G. *Biochem. Biophys. Res. Commun*. **1999**, *265*, 625-629.
4. (a) Vaishnav, Y. N.; Wong-Staal, F. *Annu. Rev. Biochem*. **1991**, *60*, 577-630. (b) Chan, D. C.; Fass, D.; Berger, J. M.; Kim, P. S. *Cell* **1997**, *89*, 263-274. (c) Weiserhorn, W.; Dessen, A.; Harrison, S. C.; Skehel, J. J.; Wiley, D. C. *Nature* **1997**, *387*, 426-430.
5. (a) Dubay, J. W.; Roderts, S. J.; Brody, B.; Hunter, E. *J. Virol*. **1992**, *66*, 4748-4756. (b) Chen, S. S.-L.; Lee, S.-F.; Hao, H.-J.; Chuang, C.-K. *J. Virol*. **1998**, *72*, 4765-4744.
6. (a) Labrosse, B.; Pleskoff, O.; Sol, N.; Jhons, C.; Henin, Y.; Alizon, M. *J. Virol*. **1997**, *71*, 8230-8236. (b) Labrosse, B.; Treboute, C.; Alizon, M. *J. Virol*. **2000**, *74*, 2142-2150. (c) Debnath, A. K.; Radigan, L.; Jiang, S. *J. Med. Chem*. **1999**, *42*, 3203-3209.
7. The thiol-reactive dye is a mixture of structural isomers, which was purchased from the Molecular probes company and conjugation and labeling degree determination were followed by the recommended procedure of the molecular probes. These are also available on the web of the company. The degree of labeling is about 0.7, that is, dyes are conjugated to seven of ten protein molecules.
8. The fluorescent signals were obtained from the multi-label HTS counter, Victor^2 of the Perkin Elmer life science in the 96-well black plate (Fluotrac 600), which were purchased from the Greiner Bio-One Company.
9. We measured the changes of fluorescent signals, according to the various concentrations of the probe proteins (Rh-S-GST-C) and quantified the bound proteins (Rh-S-GST-C) from the equations of the standard curves.
10. The volume of incubation buffer was adjusted to 100 μL in each well. Trx-N (Surface-bound) + Rh-S-GST-C (Solution) $\leftrightarrows$ Complex (Surface-bound)
K_d = [Trx-N][Rh-S-GST-C]/[Complex], $10^{-4}K_d$ = (a-y)(x-y)/y
If $K_d' = 10^{-4}K_d$ and x' = 1/(x-y)
K_d' = (a-y)/x'y, y = a/(K_d'x' + 1)
If y' = 1/y, y' = (K_d'/a)x' + 1/a
11. (a) The chimera protein Trx-C was expressed in BL21(DE3) strain of *E. coli*. The C terminal membrane proximal helical domains (C-αH) of gp41 were inserted into the C-terminus of thioredoxin (Trx) to generate the chimera protein, Trx-C.