A Homogeneous Noncompetitive Immunoassay for the Detection of Small Haptens

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We describe a noncompetitive homogeneous immunoassay for small haptens based on the antigen-dependent reassociation of antibody variable domains and β-galactosidase (β-gal) complementation (open sandwich enzymatic complementation immunoassay). As a model system, the reassociation of two fusion proteins, an anti 4-hydroxy-3-nitrophénylacétyl (NP) antibody heavy-chain variable-region fragment fused to an N-terminal deletion mutant of β-gal (VHΔo) and the light-chain variable-region fragment fused to a C-terminal deletion mutant of β-gal (VLΔo), was monitored by the enzymatic complementation between the two. Upon simple mixing of the reagents with the sample, an antigen (NP)-dependent increase in enzymatic activity was observed. When 5-iodo-NP was measured, a 10 times higher sensitivity was observed, probably due to its higher affinity. Compared with our corresponding heterogeneous open sandwich enzyme-linked immunosorbent assay, ~1000-fold improvement in the sensitivity was attained, probably due to lower background VH–VL association. In addition, the assay required less time, handling, sample volume, and assay reagents.

The needs for the high-throughput analysis of small molecular substances such as drugs, pesticides, and hormones in the environment have never been higher. For such analyses, homogeneous assays rather than heterogeneous, are preferred due to their simplicity, ease of automation, and higher throughput. Immunological assays rather than biological or enzymatic ones are becoming more and more popular because of their versatility, sensitivity, and dynamic range. However, the sensitivity of homogeneous immunoassays especially for small haptens has been unsatisfactory to date mostly because of their general restriction to a competitive assay format. Noncompetitive sandwich immunoassays can achieve 100-fold more sensitivity than the competitive counterparts¹ and provide a linear range exceeding several orders of magnitude. However, for the detection of small molecules, including most drugs and hormones that are not large enough to have two distinct epitopes, a noncompetitive sandwich assay format is not feasible. Nevertheless, several attempts have been made to realize noncompetitive detection of small haptens using cross-linking¹, affinity separation² combined with enzymatic amplification,³ anti-idiotypic antibodies,⁴,⁵ or affinity-based capillary electrophoresis.⁶ However, besides their sensitivity, all these methods are heterogeneous systems, requiring additional steps including separation. As homogeneous immunoassays, classic methods of tryptophan fluorescence quenching,⁷,⁸ spin immunoassay,⁹ and assays for fluorochromes where binding influences its fluorescence (e.g., fluorescence polarization immunoassay¹⁰) have been popular, but all these have had limited applications with moderate sensitivity. In other words, no sensitive and generally applicable homogeneous noncompetitive immunoassay formats have been proposed for the detection of small haptens to date.

We previously described the “open sandwich” (OS) immunoassay, which employs antigen-dependent stabilization of VH–VL interaction. The OS immunoassay is based on a phenomenon that, in some antibodies, the association of separated VH and VL chains from the variable domain (Fv) of the antibody is strongly favored in the presence of antigen.¹¹ Compared with conventional two epitope sandwich immunoassays, antigen-driven association of VH and VL chains has proven to have several advantages such as the need for a single epitope, a wide dynamic range, short measurement times,¹² and potential for a homogeneous format.¹³,¹⁴

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that could allow easy automation. Importantly, the open sandwich format is compatible with an antigen of any size including the capacity to detect small molecules such as haptens. In fact, by the application of separated anti 4-hydroxy-3-nitrophenylacetyl (NP) antibody VH domain tethered with alkaline phosphatase and an immobilized VL from the same antibody, NP could be measured by an OS-ELISA at a sensitivity of 1 μM. Though the sensitivity attained was higher than the corresponding competitive assay, as this modest value shows, the property of this antibody B1-6(VH3L1) (Ka = 3.44 × 10^7 M) (see ref 15) was not ideal for the OS-ELISA because it has considerable background signal due to VH–VL interaction in the absence of antigen.

To create an OS homogeneous assay rather than such a heterogeneous format, the method to detect protein–protein interaction is of crucial importance. Several methods to monitor protein–protein interaction have been proposed including fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), surface plasmon resonance, and enzymatic complementation. We have successfully applied FRET and BRET to detect VH–VL interaction. These previous studies with HyHEL-10 antibody demonstrated that lower concentrations of VH/VL fragments yielded better assay sensitivity. This was probably because lower VH/VL concentration led to lower antigen-independent VH/VL association; thus, the effect of antigen-dependent association at lower concentration became more evident.

There is a homogeneous immunoassay that employs complementation within the β-galactosidase system under the name CEDIA. CEDIA is a powerful assay method and operates on many clinical chemistry analyzers in a fully automated format. It utilizes efficient complementation between α-peptide and Δα fragment (α complementation) and its inhibition by antibody-bound hapten-tagged α-peptide. One reason for such a rather complicated (double competitive) format is that the interaction between α and Δα is so strong that one cannot directly monitor protein (peptide)–protein (antibody) interaction by the complementation. Although a positive signal is generated, CEDIA remains sensitive.

Recently a novel β-gal complementation assay based on Δα and another deletion mutant, Δαβ, was described. In the assay, the interaction between the two mutants is weaker, and the interaction between the tethered protein domains in vivo can be determined by the recreated β-gal activity. In this study, we have chosen this β-galactosidase complementation and chemiluminescence.

EXPERIMENTAL SECTION

Plasmid Construction. For the expression of fusion proteins, pET Trx Fusion System 32 (Novagen, Madison, WI), a fusion expression system with Escherichia coli thioredoxin (Trx), was employed to enhance the solubility and disulfide formation of the expressed proteins in E. coli cytoplasm. The DNA fragments encoding lac2Δα and Δαβ were prepared from E. coli chromosomal DNAs from DH5α and HB101, respectively, by standard PCR using KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Primers for amplifying lac2Δα are 5’-CCCGGATCCGCAGGCGCGC-

Figure 1. (a) Structures of the coding region of the plasmids. pET/VHΔα, pET/VHΔαβ, pET/VLΔα, and pET/VLΔαβ were constructed to express the thioredoxin (Trx)-antibody variable-region-β-gal mutant fusion proteins in E. coli (Origami B (DE3, pLysS)) cytoplasm. Transcription is driven by T7 promoter with lac operator sequence (T7/lacO). All the plasmids code for a (His)6 tag between Trx and antibody domain and a (Gly 4 Ser) 4 flexible polypeptide linker (G4S4) 4 between the antibody and the β-gal domains. Restriction sites are shown above the construct. (b) Principle of the assay. Without antigen, the two fusion proteins remain monomeric; thus, the β-gal activity of each chimeric protein is low. The addition of antigen induces heterodimerization of the two chains, accompanied by the recreated β-gal activity tethered with VH and VL domains. (c) Structure of NP/NIP.

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Experimental
CATGACCATGATTACGTTACTGG-3' and 5'-CCCCCCTC-GAGTTATTCTTGACACGACAACTGG-3', and those for lacZα were 5'-CCCCGATTCCGGGCGCGGCTCGATCATTAGT-GACCATGACTGG-3' and 5'-CCCCCCTCAGATTACGTTAAGGTTGACTG-3' (the cleavage sites for NolI and Xhol sites were in boldface type). The amplified fragments were cloned into pPCR-Amp-Script (Strategene, La Jolla, CA) to give pPCR/Δα and pPCR/Δω. The nucleotide sequences for Δα and Δω fragments were confirmed to be correct using a SQ-5500 DNA sequencer (Hitachi, Tokyo, Japan) and a Thermosequenase sequencing kit (AP Biotech, Tokyo, Japan). Then the inserted fragments were digested with NolI and Xhol and cloned to pET32/VI13 between NolI and Xhol sites to give pET/V1(HEL)Δα and pET32/V1(HEL)Δω. The V1α(NP) and V1ω(NP) fragments, derived from a high-affinity mutant (Hw32) of antibody B1-8,15 were obtained by the digestion of pET32/V1α(NP)-Rluc and pET32/V1ω(NP)-EYFP with HindIII and XbaI, which are derivatives of pET32/V1α(HEL)-Rluc and pET32/V1ω(HEL)-EYFP, respectively.20 The obtained fragments were cloned to pET/V1(HEL)Δα and pET32/V1(HEL)Δω, digested with HindIII and XbaI, and designated pET32/V1αΔα, pET32/V1αΔω, pET32/V1ωΔα, and pET32/V1ωΔω, accordingly (Figure 1a).

Expression and Purification of the Proteins. E. coli Origami B (DE3) pLysS (Novagen), a strain lacking the thioredoxin reductase gene (trxB) and glutathione reductase gene (gor), was transformed with the expression plasmids and selected on 2YT agar plates containing 50 µg/mL ampicillin, 12.5 µg/mL tetracycline, and 15 µg/mL kanamycin. For all the cultivations thereafter, 2YT medium containing 50 µg/mL ampicillin, 12.5 µg/mL tetracycline, and 15 µg/mL kanamycin was used. A 400-ml aliquot of medium was inoculated with 5 ml of culture from a high-affinity mutant (H W33L) of antibody B1-8, 15 were mixed with 2 ml of NP-Sepharose in a rotator at 4 °C for 16 h that was made from EAH-Sepharose 4B (AP Biotech) and NP (Sigma-Aldrich, Tokyo, Japan) according to the manufacturer's protocol. The resin was packed to a column and washed 4 times with 4 ml of PBS; the bound materials were eluted with 4 ml of 2 mM NP in 1 M Tris-HCl, pH 8.0, by gravity flow. The eluates were extensively dialyzed against 2× PBS at 4 °C and stored at −20 °C in 50%glycerol (Wako, Osaka, Japan) and 0.01%BSA until use. The concentrations of the obtained proteins were determined by using the BCA assay kit (Pierce, Rockford, IL) with BSA as standard.

Examing β-Galactosidase Activity. Assay reagents were in 20 µL of 0.1 M sodium phosphate, pH 7.6, containing a pair of fusion proteins. The optimal concentration of the fusion proteins was experimentally determined to be 15 ng/mL. To these, 2.2 µL of sample solution containing either NP or NIP (Cambridge Research Biochemicals, Northwich, Cheshire, U.K.) in PBS, 0.1% Tween-20 was added. After a 5-min incubation in a white 96-well microplate (437796, Nalge Nunc, Rochester, NY) at 25 °C, each sample was added with 70 µL of Galacton-Plus substrate (Applied Biosystems, Foster City, CA) in 0.1 M sodium phosphate, 1 mM MgCl2, 0.1% Tween-20, pH 7.6. After a 40-min incubation at 25 °C, each sample was added with 100 µL of Siphophage-II enhancer (Applied Biosystems) in 0.1 M sodium phosphate, 1 mM MgCl2, 0.1%Tween-20, pH 12, and the chemiluminescence was measured for 6 s/well using a multwell luminometer AB-2100 (Atto, Tokyo, Japan).

RESULTS AND DISCUSSION

Expression and Purification of Fusion Proteins. As a model hapten–antibody system, detection of small hapten NP using an anti-NP antibody B1-8(Hw32) was attempted. To achieve this, four fusion protein expression vectors encoding B1-8(Hw32) variable domains tethered with β-gal mutants (pET/V1αΔα, pET/V1ωΔω, pET/V1αΔω, pET/V1ωΔα) were made (Figure 1a). Because of the difficulty in expressing β-galactosidase in E. coli periplasm, pET32 vector was used to express fusion proteins in TrxB–E. coli Origami B (DE3, pLysS) cytoplasm as thioredoxin fusion proteins to facilitate the soluble expression of disulfide-bonded molecules.13 According to SDS–PAGE, sufficient expression was always observed for V1α fusions, but that for V1ω fusions was variable (data not shown). Various culture conditions such as temperature and culture time were investigated, but only when the overnight preculture was stopped before overgrowth and immediately used for the subsequent inoculation was the expression for V1ω fusions markedly improved (Figure 2a). This was probably because of the cell toxicity of the V1ω fragment, where longer culture might have led to a significantly decreased proportion of full-length plasmids capable of expressing the proper fusion protein. To purify the fusion proteins, first purification by a metal affinity column was attempted. However, according to the preliminary evaluation of LacZ activity of the resultant proteins, we could not observe any reliable antigen-dependent change in the activity of any protein combinations (data not shown). So we adopted purification with the antigen affinity column, to get only the fusion proteins with a correctly folded variable domain. In principle, two pairs of fusion proteins V1αΔα/V1ωΔα and V1ωΔα/V1αΔω were expected to react both antigen binding and β-gal activities when added with antigen. The expressed proteins in cell lysate were combined in two and purified with a NP-Sepharose column to get active proteins retaining full affinity to NP. Before quantitation, the eluates were extensively dialyzed to remove bound NP. M moderate but sufficient amounts of fusion proteins (~15 µg/L culture for the both mixtures) of >90% purity were obtained as judged by protein quantitation and SDS–PAGE analysis (Figure 2a).

Optimization of Assay Conditions. Since the pairs of purified fusion proteins should retain binding affinity to NP and showed detectable recreated β-gal activity, their responsiveness to antigen was tested at several protein concentrations. When o-nitrophenyl β-galactopyranoside (ONPG) was used as a substrate, the signal was so weak that a prolonged reaction of at least 8 h was needed to detect sufficient antigen-dependent absorbance change (data not shown). The calculated recreated β-gal activity was at most 6.0 × 10−3 times as small as that of the wild-type enzyme, though
This value was in good agreement with a previous measurement. Since the apparent reaction rate was so slow that we changed the substrate to the chemiluminescent substrate Galacton plus, the reaction condition was also optimized. According to the manufacturer’s instructions, the pH of the enhancer solution was pH 7.6. However, when the pH was raised to pH 12.0, the number of photon counts increased by 100-fold, probably due to enhanced cleavage of a 1,2-dioxetane metastable intermediate. This in turn enabled reduction of the fusion protein concentration necessary for getting sufficient photons. According to the results (Figure 2b and c), the fusion protein pair that showed a better antigen response was \( V_H \Delta \alpha / V_L \Delta \alpha \), and the optimal protein concentration was \(-15 \text{ ng/mL}\). The value \((-0.05 \text{ nM})\) is far less than we previously used in FRET (0.16–0.85 \( \mu \text{M} \)) or BRET (0.02 \( \mu \text{M} \) for donor, 0.25 \( \mu \text{M} \) for acceptor) measurements. In addition, inclusion of 0.1% Tween-20 in the reaction buffers also reduced signal fluctuation, possibly by reducing nonspecific interaction of \( V_H \Delta \alpha / V_L \Delta \alpha \) and the plastic surface. The very low reagent concentrations lead to smaller antigen response. This was probably because of the predominated VH/VL dissociation even in the presence of antigen.

Antigen Concentration Dependency of \( \beta \)-Galactosidase Activity. After reaction optimization, the homogeneous immunoassay based on \( \beta \)-gal complementation was performed. Figure 3a shows the antigen concentration dependency in the recreated \( \beta \)-gal activity detected with chemiluminescence. As the antigen concentration increased, a clear increase in \( \beta \)-gal activity was observed, indicating the increased reassociation of the \( V_H \) and \( V_L \) domains of the fusion proteins due to coexisting antigen. It is notable that the lowest measurable concentration of NP (1 nM in the reaction mixture) was roughly 40 nM in the original sample (2.2 \( \mu \text{L/sample} \), which was almost 250 times less than that of previous OS-ELISA.5 5-Iodo-NP is known to bind to this antibody with the equilibrium dissociation constant of \(-10^{-8} \text{ M}\), which is a -10 times smaller value than that for NP \((-10^{-7} \text{ M}\)) .24 When NIP was used instead of NP in the samples, the minimum measurable concentration in the reaction mixture was roughly 0.1 nM, which was also one-tenth of that for NP measurement (Figure 3b). The difference in two results also illustrates the specificity of the assay, eliminating the possibility of nonspecific signal increase upon sample addition. These results clearly indicate that, in this system, sensitive quantitation of small hapten is indeed possible in a homogeneous noncompetitive format. The sensitivity obtained was on the order of \( K_d \) 100, which was superior to the value obtainable in normal competitive assays. Despite reduced antibody usage in a homogeneous system, the noncompetitive mode should have made the assay work better than competitive assays.

CONCLUSIONS

In this assay, through the enzymatic amplification and sensitive detection of chemiluminescence, we could substantially reduce the amount of \( V_H / V_L \) fusion proteins to get sufficient signal compared with previous FRET and BRET systems. It is now clear that a lower \( V_H / V_L \) concentration leads to lower background association and a correspondingly lower detection limit of antigen. By reducing the \( V_H / V_L \) fusion protein concentration, we could successfully detect their antigen-dependent association even when the antibody does not have the ideal property for OS-ELISA, which implies wide applicability of the assay to many hapten–antibody systems. One more advantage of the reduced reagent concentration is the lower reagent consumption. The concentration of the fusion proteins used (15 ng/mL) is almost 1/1000 of the \( V_H \)-PhoA protein concentration used (10 \( \mu \text{g/mL} \)) in previous OS-ELISA.

The homogeneous open sandwich immunoassay approach has been shown to work equally well with both low molecular weight and high molecular weight antigens. Though stabilization of whole


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antibody by haptens at the harsh condition of very low pH was reported, our system is superior because it is specifically designed to detect antigen-induced Fv stabilization at physiological pH, where minimal alteration of the binding activity as well as specificity is expected. The OS complementation requires only 1 h or less from mixing the solutions to measuring the $\beta$-gal activity. Although the assay appears to have the limitation that it requires a suitable antibody Fv that has weak VH-VL interaction without the antigen and is stabilized with the antigen, it is possible that many anti-hapten antibodies have such a property because almost all the anti-hapten antibody structures solved to date indicate mutual recognition of deeply buried hapten by both VH and VL fragments. Improvement of suboptimal VH and VL properties with a selection system using phage display for open sandwich-compatible Fvs is now in progress and has already been proposed for the selection of VH fragment recognizing related antigens.26

The present limitation of the system is its relatively small change (1.6–1.9-fold) in signal. This appears to be at least partly due to weak but significant $\Delta \alpha - \Delta \alpha$ interaction, since the extent of change is roughly in good agreement with that of a previously reported in vitro response (2-fold).28 We also observed a similar background signal when we used anti-hen egg lysozyme HyHEL-10 Fv, which has very low VH/VL affinity in the absence of antigen (H.U., in preparation). In future, optimization of the linker length or screening of more suitable $\beta$-gal mutant pairs may further improve the utility of this system.

Abbreviations: BSA, bovine serum albumin; BRET, bioluminescence resonance energy transfer; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; Fv, antibody variable region; HEL, hen egg lysozyme; IPTG, isopropyl $\beta$-D-thiogalactoside; NIP, 5-iodo-NP; 4-hydroxy-3-nitro-5-iodophenylacetyl; NP, 4-hydroxy-3-nitrophenylacetyl; ONPG, o-nitrophenyl D-galactopyranoside; OS-ELISA, open sandwich ELISA; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS-PAGE, polyacrylamide gel electrophoresis in sodium dodecyl sulfate; VH, antibody heavy-chain variable region; VL, antibody light-chain variable region; W33L, tryptophan 33 to leucine mutant

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