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Technical note

Detection of weak ligand interactions of leukocyte Ig-like receptor B1 by fluorescence correlation spectroscopy

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Abstract

Fluorescence correlation spectroscopy (FCS) can directly and quickly detect the translational diffusion of individual fluorescence-labeled molecules in solutions. Although FCS analyses for protein–protein interactions have been performed, the very weak interactions generally observed in cell–cell recognition of the immune system have not been examined in detail. Here, we report the FCS analysis for low-affinity and fast-kinetic binding (K_d greater than μM range) of the human inhibitory immune cell surface receptor, leukocyte immunoglobulin-like receptor B1 (LILRB1), to its ligands, MHC (major histocompatibility complex) class I molecules (MHCIs) by using the single-molecule FCS detection system which requires only a small amount of sample. Since the random labeling technique for LILRB1 disturbed the MHCI binding, we performed site-specific labeling of LILRB1 by introducing a cysteine residue at the C-terminus, which could be covalently attached with the fluorescence reagent, Alexa647. This technique can be applied to other type I membrane receptors. The low-affinity binding of LILRB1-Alexa647 to MHCIs (HLA-Cw4, and -G1) was detected by FCS, even though non-labeled MHCIs were only twice as big as the labeled LILRB1. Their dissociation constants (7.5 μM (HLA-Cw4) and 5.7 μM (HLA-G1)) could be determined and were consistent with surface plasmon resonance (SPR) data. These results indicate that the single-molecule FCS detection system is capable of analyzing the binding characteristics of immune cell surface receptors even in difficult cases such as (1) small amount of protein samples, (2) small difference in molecular weight and (3) weak affinity. Therefore, it is a powerful tool for characterization and high throughput inhibitor screening of a wide variety of cell–cell recognition receptors involved in immunologically relevant events.

Keywords: Fluorescence correlation spectroscopy; Leukocyte Ig-like receptor; MHC class I; Cell surface receptor; Weak interactions; Protein–protein interaction

Many biological events include weak protein–protein interactions, which have pivotal roles in the regulation of cellular function. For example, the cell–cell recognition of immune systems is controlled by weak protein–protein interactions of cell surface receptors (e.g. K_d in the μM range). Furthermore, these cell surface receptors are key molecules as drug targets and thus biophysical techniques to analyze such interactions are important for the screening of low molecular weight inhibitor compounds.

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The human inhibitory immune cell surface receptor, leukocyte Ig-like receptor B1 (LILRB1, also called as LIR1, Ig-like transcript (ILT) 2, CD85j), is expressed on the cell surface of a wide range of leukocytes including natural killer cells, T cells, B cells and myelomonocytic cells. It recognizes MHC (major histocompatibility complex) class I molecules (MHCIs) on target cells to mediate inhibitory signals and prevent the killing of normal cells expressing MHCIs. In other words, abnormal cells expressing little or no MHCIs can activate the cellular function of LILRB1-positive leukocytes due to lack of the LILRB1-mediated inhibitory signal. Our previous studies (Shiroishi et al., 2003; Kuroki et al., 2005; Shiroishi et al., 2006) using the surface plasmon resonance (SPR) technique showed that LILRB1–MHCI interactions show weak affinity with very fast dissociation rates, which are typical of cell–cell recognition receptors.

Fluorescence correlation spectroscopy (FCS) (Magde et al., 1974) can determine the translational diffusion coefficient of fluorescence-labeled molecules in solutions, which depends on molecular weight, structure, and the number of the molecules. Due to recent advances in laser and microscopic technologies (Borsch et al., 1998; Eggeling et al., 1998) FCS can exhibit single-molecule sensitivity. Thus FCS can be used for analysis of protein–nucleic acid, protein–drug and protein–protein interactions (Kinjo and Rigler, 1995; Kinjo et al., 1998; Meseth et al., 1999; Wolcke et al., 2003). However, to our knowledge, FCS analysis has not been applied to weak interactions of immune cell surface receptors, which have pivotal roles in relevant immune responses. Here we performed FCS analysis of the weak ligand binding of LILRB1, to investigate the capabilities of FCS under difficult conditions including: (1) very weak interactions, (2) when the difference in molecular weight between non-labeled ligand and labeled receptors is not very large, and (3) requirement of site-specific labeling techniques. Furthermore, we used the single-molecule FCS detection system requiring only a small amount of sample, which has great advantages for screening high throughput inhibitors.

Non-labeled recombinant ectodomains of MHCIs, HLA-Cw * 0401 (with peptide QYDDAVYKL), and HLA-G1 monomer (Cys42Ser mutant with peptide RIIPRHLQL) were produced according to our previous report (Shiroishi et al., 2003). Recombinant LILRB1 protein consisting of N-terminal D1–D2 domains in the extracellular region was expressed by *E. coli* as inclusion bodies, refolded and purified by gel filtration (Shiroishi et al., 2003). For FCS analysis, LILRB1 was randomly labeled by TAMRA fluorescence reagent (5-carboxytetramethyl rhodamine *N*-succinimidyl ester) following the standard protocol commercially provided (Molecular Probes, Eugene, OR). More than 90% of LILRB1 was chemically labeled with TAMRA. The TAMRA-labeled LILRB1 (5–10 nM) was incubated at 24 °C for 30 min with different concentrations of HLA-G1 (0–100 μM) and the single-molecule fluorescence detection FCS measurements were performed using the MF20 molecular interaction analytical system (Olympus, Tokyo, Japan) (Kobayashi et al., 2004). Only 25 μl of sample volume was required in one well of the 384-well plate for measurements. All experiments were performed with 10 s of data acquisition time per measurement, and measurements were repeated five times per sample. No interactions with HLA-G1 were detected even when the concentration was increased to 100 μM (Fig. 1A). This suggests that the random chemical modification disturbs the MHCI binding site of LILRB1.

In order to label LILRB1 protein site-specifically to maintain biological function, we introduced a Cys residue with one additional Gly residue at the C-terminal of LILRB1 (hereafter, designated as LILRB1-Cys). Because the C-terminal site of membrane-bound LILRB1 is directed to the cell membrane, its modification is unlikely to interfere with the MHCI binding. Preparation of LILRB1-Cys was the same as for non-labeled LILRB1 but with 1 M L-arginine in the refolding buffer. The Cys residue can be covalently attached with the fluorescence reagent Alexa Fluor 647 C_2-maleimide (Molecular Probes, Eugene, OR) near pH 7.0. The majority of the refolded LILRB1-Cys protein was purified with gel filtration as a monomer form of LILRB1-Cys (Fig. 1B). Purified LILRB1-Cys should be immediately labeled using Alexa647 C_2-maleimide to avoid the formation of the LILRB1 dimers mediating the intermolecular disulfide bonds of the C-terminal Cys residues.

In order to confirm whether Alexa647-labeled LILRB1 protein (hereafter, designated as Alexa647-LILRB1) has functional activity, its binding activity to anti-LILRB1 mAb, HP-F1, was examined. HP-F1 mAb can directly bind to functionally active LILRB1 on the cell surface by flow cytometric analysis and can also enhance the killing activity of LILRB1-expressing T cells (Colonna et al., 1997; Saverino et al., 2000). Alexa647-LILRB1 (5–10 nM) was incubated at 24 °C for 1 h with 0–330 nM of HP-F1 in 25 μl of PBS buffer (pH 7.5) containing 0.05% Tween 20. FCS measurements for Alexa647-LILRB1 were performed as for TAMRA-LILRB1 except that the He–Ne laser (633 nm) and a 580DF30 filter were used. HP-F1 (~ 150 kDa) is roughly seven times bigger than Alexa647-LILRB1 (22 kDa) and shows high affinity, typical of antibody–antigen