Available online at www.sciencedirect.com
SCIENCE DIRECT®
Metabolic Engineering 7 (2005) 38–44
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Assay development and data analysis of receptor–ligand binding based on scintillation proximity assay

Shaoxian Sun*, Jonathan Almaden, Thomas J. Carlson, John Barker, Michael R. Gehring

La Jolla Laboratory, Structural Biology and Biochemistry, Pfizer Global Research & Development, 10628 Science Center Drive, San Diego, CA 92121, USA

Received 31 March 2004; accepted 13 May 2004
Available online 7 January 2005

Abstract

In this paper, we described the optimization of a generic binding assay to measure ligand–receptor interactions for peroxisome proliferator-activated receptors (PPARs). The assay is based on scintillation proximity assay, in which a protein is coated on scintillant-incorporated beads, and a radiolabeled ligand stimulates the beads to emit a signal by binding to the immobilized protein. An intrinsic binding affinity of unlabeled ligands is determined by competitive displacement of the radioligand. The protein coating and ligand binding are achieved in one step by simply mixing ligands, protein and beads in sequence. No additional steps of pre-coating and washing of beads are required. Protein is captured on beads effectively by electrostatic interactions, thus no affinity labeling of protein is required. In data analysis, ligands are grouped into two classes based on their binding affinities. For tight binding ligands, an equation is derived to accurately determine the binding affinity. Otherwise a general equation applies. This quantitative and high throughput assay provides a tool to screen a large library of molecules in search of potent ligands.

Keywords: Binding assay; Tight binding inhibitor; Scintillation proximity assay; PPARg

1. Introduction

Accurate and robust binding assays are highly desirable to screen the expanding and diverse set of chemical entities. Techniques in binding assays are generally based on absorbance, fluorescence (Gribbon and Sewing, 2003), such as fluorescence resonance energy transfer and fluorescence polarization, or radiometric assays. Reported here is a generic scintillation proximity assay (SPA) developed for peroxisome proliferator-activated receptors. Due to its simplicity and high-throughput format, the assay has broad applications in monitoring receptor–ligand interactions and enzyme reactions (Carpenter et al., 2002; Mallari et al., 2003; Udenfriend et al., 1987).

Briefly, the SPA assay involves coating a protein of interest on the SPA beads, and binding a radiolabeled ligand to the immobilized protein. The close proximity of the isotope to the scintillant incorporated in the beads allows the radiation energy to transfer to the scintillant and be detected in terms of counts or counts per minute (CPM). The energy from unbound radiomolecules is dissipated into the aqueous media and is too weak to be detected. No separation of the free from the bound radiomolecules is required in the SPA assay.

Peroxisome proliferator-activated receptors (PPARs) belong to the family of nuclear hormone receptors. They play crucial roles in the regulation of lipid and glucose metabolism, and are currently under extensive study as drug targets in the treatment of diabetes, dyslipidemia, and atherosclerosis (Francis et al., 2003). Similar to other family members of transcription factors, PPAR consists of a DNA-binding domain and a ligand-binding domain. PPAR ligands bind to the ligand-binding

*Corresponding author.
E-mail address: shaoxian.sun@pfizer.com (S. Sun).

1096-7176/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2004.05.004

domain, and induce activation by a structural conformation change in PPAR (Nolte et al., 1998). The activated PPARs recruit a cofactor protein required for transcriptional regulation of target genes.

Three PPAR isoforms have been discovered, namely as PPARalpha (a), gamma (g) and delta (d). PPARg is essential for adipocyte differentiation and has been indicated as a key player in insulin sensitization (Fajas et al., 2001). Thiazolidinediones (TZDs), a new class of drugs for type-2 diabetes, are known to be PPARg ligands (Martens et al., 2002). TZDs improve insulin sensitivity and lower glucose levels in vivo by activation of PPARg. However, weight gain and edema are side effects associated with TZD treatment. Much effort is focused on searching for novel PPAR ligands with high affinity and selectivity to possibly mitigate these side effects.

We developed a simple and quantitative SPA binding assay for PPARs. In the previous study of PPARg by SPA (Nichols et al., 1998), PPARg ligand-binding domain was labeled with biotin and pre-coated on streptavidine SPA beads. An additional wash step was required to eliminate the unbound PPARg. The determination of a ligand potency was limited to those ligands with a binding affinity $K_i \gg$ PPARg concentration in their data analysis. In this paper, we describe the coating of protein on SPA beads by electrostatic interaction, which does not require a specific affinity tag on the protein. No separate steps for coating and washing of SPA beads are needed. Furthermore we derived a tight binding equation to determine the K_i below the receptor concentration for those highly potent ligands. The assay is designed to measure the intrinsic binding affinity of ligands.

2. Methods

2.1. Materials

SPA beads were obtained from Amersham Pharmacia Biotech. Yttrium silicate (Ysi) His-tag SPA beads were supplied as a suspension in water at 20 mg/ml and used directly. Ysi polylysine SPA beads supplied in solid powder were suspended in PBS before use. Calcium and magnesium-free PBS was from Cellgro. Glycerol and β-mercaptoethanol were from Sigma.

Tritium labeled ligands including agonist darglitazone (Dar) and antagonist compound A, unlabeled darglitazone and compound B as a representative tight-binding ligand were made in house.

Human PPARg ligand-binding domain (hPPARg LBD, sequence 207–477) with a 6-histidine tag at its N-terminus was amplified from a human fat cell cDNA library and cloned into a Novagen pET24a(+) expression vector. The protein was over expressed in *E. coli* BL21 (DE3), and purified to nearly homogeneity by Ni NTA (Qiagen) affinity chromatography followed by a Poros 50HQ (Perseptive Biosystems) ion exchange column. The purified PPARg was stored in 20 mM Tris, pH 8.0, 100 mM NaCl, and 2 mM DTT at −80 °C. The protein concentration was determined by a Bio-Rad Bradford based protein assay using BSA as the standard (Bio-Rad).

2.2. General procedure of SPA assay

The radioligand was diluted into an assay buffer containing PBS, 14 mM β-mercaptoethanol and 10% glycerol. To a 96-well plate were added in sequence the diluted radioligand, PPARg and SPA beads. The Ysi SPA beads were stirred continuously using a magnetic stirrer in a bead-mixing reservoir (V&P Scientific, Inc) to obtain a homogeneous suspension during the dispensing step. The mixture was gently shaken for 2 h on a plate shaker. The SPA beads were allowed to settle for another hour. The assay plate was read on a Topcount scintillation counter (Perkin–Elmer). All the experiments were performed at room temperature.

2.3. Optimization of protein/beads ratio

The protein/beads ratio was optimized to achieve the maximal signal to background ratio. In one experiment, the amount of SPA beads was varied at fixed concentrations of protein and radioligand. The final concentrations in the assay were 10 nM ^{3}H-compound A, 5 nM PPARg, and 0.5–3 mg/ml Ysi His-tag SPA beads, or 20 nM ^{3}H-darglitazone, 5 nM PPARg and 0.5–3 mg/ml Ysi polylysine SPA beads. In another experiment, 2.5–10 nM PPARg was tested at a fixed amount of SPA beads.

2.4. Characterization of the radioligand binding to PPARg

The binding of antagonist ^{3}H-compound A to PPARg was measured in the presence of 0–200 nM ^{3}H-compound A, 5 nM PPARg, and 1 mg/ml Ysi His-tag SPA beads in an assay buffer containing PBS, 14 mM β-mercaptoethanol and 10% glycerol. Similarly, the binding of agonist ^{3}H-Dar was measured in the presence of 0–200 nM ^{3}H-Dar, 5 nM PPARg, and 1.0 mg/ml Ysi His-tag SPA beads or 1.5 mg/ml polylysine SPA beads. The non-specific binding of radioligand to SPA beads was determined in the absence of PPARg under the same condition. A binding curve was obtained by plotting the radioactive signal as a function of radioligand concentrations. The PPARg-specific binding was calculated by subtraction of the non-specific binding. The binding constant was determined by fitting the specific-binding data to a single site binding