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Comparative characterization of direct and indirect substrate probes for on-chip transamidating activity assay of transglutaminases

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ABSTRACT

The development of molecular probes is a prerequisite for activity-based protein profiling. This strategy helps in characterizing the catalytic activity and function of proteins, and how these proteins and protein complexes control biological processes of interest. These probes are composed of a reactive functional group and a reporter tag. The reactive group of these substrate probes has been considered to be important to their design, while the significance of the reporter tag is relatively underestimated. In this study we compare TAMRA-cadaverine and biotin-cadaverine, two substrate probes that have different reporter tags but an identical reactive functional group. We assess the on-chip transamidating activity of two transglutaminases; transglutaminase 2 and blood coagulation factor XIII. Activity assays were more easily executed when using the direct probe TAMRA-cadaverine. However the indirect probe, biotin-cadaverine, provided a wider dynamic range, higher signal-to-noise ratio, and lower limit of detection compared to TAMRA-cadaverine. Additionally, we successfully used the on-chip activity assay using the indirect probe to determine TG2 and FXIII activities in Hela cell lysates and human plasma samples, respectively. These results demonstrate that the reporter tag of the substrate probe is critical for protocol execution, sensitivity, and dynamic range of enzyme activity assays. Furthermore, this study provides a helpful guide for development of new probes, which is necessary for the identification of potential biomarkers and therapeutic targets for treating enzyme-related diseases.

1. Introduction

Activity-based protein profiling (ABPP) has emerged as a powerful strategy for assigning protein functions by identifying and characterizing the catalytic activity of proteins and their complexes that control biological processes of interest (Heal et al., 2010). ABPP is also used for the identification of enzyme substrates or inhibitors, the discovery of diagnostic markers, and the development of therapeutic reagents (auf dem Keller and Schilling, 2010; Gillet et al., 2008; Wiedl et al., 2011). Designing specific probes that either directly or indirectly detect the catalytic activity of enzymes is an essential prerequisite for ABPP (Sadaghiani et al., 2007; Speers and Cravatt, 2004).

Activity-based probes (ABPs) are chemical probes that directly detect the formation of a catalytically active site within an enzyme (Blum et al., 2009; Heal et al., 2010). Since the interaction of these probes with the protein is activity-based and does not rely upon product formation, they are useful for quantifying enzymes in an active state and for characterizing the binding affinity of enzyme substrates or inhibitors (Fonovic and Bogyo, 2008; Nomura and Casida, 2011). Two types of ABPs are used. The first are fluorescent probes that provide rapid and sensitive target detection. The second are biotinylated probes that are valuable for target purification and identification (Adam et al., 2002; Blum et al., 2009; Heal et al., 2010; Kong et al., 2012). Recently, substrate probes for ABPP have been also reported to detect and quantify enzyme activities using probes that themselves participate in the

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enzymatic reaction (Blum et al., 2009; Kwon et al., 2009, 2011a,b). Substrate probes are derived from the substrates of target enzymes. The substrate, or substrate mimetic, is synthesized and conjugated with a reporter tag, including fluorophores and biotin (Heal et al., 2010; Jung et al., 2009). The reporter tags are then used to quantify reacted substrates by either direct probing of the fluorophore or indirect probing of biotin with fluorophore-conjugated avidin (Kwon et al., 2009, 2011a,b; Olorundare et al., 2001). Increasing efforts are being made to improve the reactivity of probes by tuning their reactive group (Hilhorst et al., 2009). However, the effect the reporter tag has on the reactivity of the probe is not well understood.transglutaminase 2 (TG2) and blood coagulation factor XIII (FXIII) are isozymes of the transglutaminase family. They both catalyze protein cross-linking via transamidating the γ-carboxamide group of glutamine residues to the ε-group of lysine residues using a similar reaction mechanism (Sugimura et al., 2006), however they are differentially regulated (Kwon et al., 2011b). TG2 is implicated in the pathogenesis of a wide variety of diseases including celiac disease, cardiovascular disease, diabetes, cancers, and neurodegenerative disorders, such as Huntington's, Alzheimer's, and Parkinson's diseases (Iismaa et al., 2009; Park et al., 2010; Reif and Lerner, 2004). Additionally, TG2 is involved in various cellular responses including cytoskeletal reorganization, stabilization of the extracellular matrix, cell migration, and apoptosis (Nadalutti et al., 2011; Ohtake et al., 2007; Park et al., 2010). Plasma FXIII exists as a heterotetramer and is involved in hemostasis, angiogenesis, wound healing, and maintaining pregnancy (Muszbek et al., 2011a). Acquired FXIII deficiency has been reported in a number of diseases including inflammatory bowel disease, acute leukemia, liver disease, and ulcerative colitis (Karpati et al., 2000; Kwon et al., 2009; Muszbek et al., 2011b).

In this study, we compare the direct and indirect substrate probes, TAMRA-cadaverine and biotin-cadaverine, using on-chip transamidating activity and inhibition assays of two transglutaminases; TG2 and FXIII. On-chip activity profiling combined with a variety of detection methods is promising technology for the high-throughput analysis of enzyme activity and interaction with various biomolecules using only very small amount of sample over other activity assays (Jung et al., 2012). This comparative characterization of identical probes with different reporter tags provides a better understanding the effect of the reporter tag on the reactivity of the substrate probe. This data will be helpful for the development of new substrate probes.

2. Methods

2.1. Chemicals and reagents

3-Aminopropyltrimethoxysilane, ammonium hydroxide, hydrogen peroxide, 1,4-dithiothreitol (DTT), bovine serum albumin (BSA), Cy3-conjugated streptavidin, fibrinogen, thrombin, and isoniazid were obtained from Sigma-Aldrich (St. Louis, MO). Purified guinea pig TG2 was purchased from Oriental Yeast (Tokyo, Japan). Purified human plasma FXIII was obtained from Innovative Research (Novi, MI). 5-(Biotinamido)pentylamine (biotin-cadaverine) and tetramethylrhodamine-5-carboxamide cadaverine (TAMRA-cadaverine) were obtained from Pierce (Rockford, IL) and Zedira (Darmstadt, Germany), respectively. Monoclonal anti-mouse TG2 was purchased from NeoMarker (Fremont, CA).

2.2. Plasma samples

Human plasma samples were collected in sodium citrate tubes (Becton Dickinson, NJ) from normal group ($n = 41$) and from patients with hepatitis ($n = 21$), liver cirrhosis ($n = 41$), or HCC ($n = 25$). The plasma samples were obtained from Kangwon National University Hospital Biobank (a mamber of the National Biobank Korea, Korea) and stored at −80 °C until use. Experiments using human samples were performed with the approval of the local Institute's Ethics Committee for human subject research.

2.3. Cell culture

Hela cells were cultured in DMEM medium containing 10% fetal bovine serum, 1 unit/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 at 37 °C. Cells were treated with 20 μM all-*trans* retinoic acid (RA) for 6 days. Cell lysates were prepared at the indicated day by cell sonication in a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1 mM PMSF, 10 μg/mL aprotinin, and 10 μg/mL lupeptin) followed by centrifugation at 13,000 rpm for 10 min.

2.4. Western blot analysis

Cell lysates were separated by 10% SDS-polyacylamide gel electrophoresis and transferred to PVDF membranes. The membrane was incubated with a blocking solution containing 5% skim milk, 0.1% tween 20, 150 mM NaCl, and 20 mM Tris–HCl (pH 7.5), and the proteins were analyzed using anti-mouse TG2 antibody (1:1000, v/v) or anti-mouse β-actin antibody (1:2000, v/v) and horseradish peroxidase-conjugated anti-mouse IgG (1:1000, v/v).

2.5. Fabrication of well-type fibrinogen arrays

Well-type amine arrays were fabricated as previously reported (Jung et al., 2009). Briefly, glass slides (75 × 25 mm) were cleaned with a $H_2O_2/NH_4OH/H_2O$ (1:1:5, v/v) solution at 70 °C for 10 min. The slides were immersed in 1.5% 3-aminopropyltrimethoxiysilane solution in 95% (v/v) ethanol for 2 h and baked at 110 °C. Strips of Teflon tape with arrayed holes 1.5 mm in diameter were attached to the amine-modified glass slides to prepare the well-type arrays. Fibrinogen arrays were fabricated by incubating these amine arrays with 50 μg/mL fibrinogen for 1 h at 37 °C in 9.3 mM phosphate buffer (pH 7.4).

2.6. On-chip TG2 transamidating activity assay using two types of substrate probes

The on-chip TG2 transamidating activity assay was performed using fibrinogen arrays as previously reported (Kwon et al., 2009). Fibrinogen arrays were blocked with 3% BSA containing 0.1% Tween-20 in phosphate buffered saline (PBS; 8.1 mM Na_2HPO_4, 1.2 mM KH_2PO_4, pH 7.4, 2.7 mM KCl, 138 mM NaCl) for 30 min at 37 °C and sequentially washed with 0.1% Tween-20 in PBS and Milli-Q water. Reaction mixtures for TG2 transamidating activity were prepared by adding purified guinea pig TG2 or cell lysates into the reaction buffer containing 50 mM DTT, 2 mM $CaCl_2$, 0.01% Triton X-100, 1 mM biotin-cadaverine or TAMRA-cadaverine in 40 mM Tris–HCl, and 140 mM NaCl (pH 7.5). One microliter of the reaction mixtures containing TG2 was applied to the fibrinogen arrays, followed by incubation at 37 °C for 30 min. Biotin-cadaverine-incorporated fibrinogen was further probed by additional incubation of the arrays with 10 μg/mL Cy3-conjugated streptavidin at 37 °C for 30 min. The resulting arrays were scanned with a fluorescence scanner using a 543 nm laser (ScanArray Express GX, Perkin Elmer, Waltham, MA). Fluorescence intensities of array spots represent TG2 transamidating activity and were automatically measured with the embedded ScanArray Express GX program (Perkin Elmer).