Analytical Biochemistry 434 (2013) 99–104

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Development of a microarray detection method for galectin cancer proteins based on ligand binding

Hilde van Hattum [a], Nathaniel I. Martin [a], Rob Ruijtenbeek [b], Roland J. Pieters [a,*]

[a] Medicinal Chemistry and Chemical Biology, Utrecht University, 3585 CG Utrecht, The Netherlands
[b] PamGene International, 5211 HH's-Hertogenbosch, The Netherlands

ARTICLE INFO

Article history:
Received 22 August 2012
Received in revised form 21 October 2012
Accepted 4 November 2012
Available online 10 November 2012

Keywords:
Galectins
Carbohydrate binding proteins
Multivalent ligands

ABSTRACT

In this article, we describe the development of a novel detection method for the visualization of ligand-binding proteins. Current proteomic tools, such as the enzyme-linked immunosorbent assay (ELISA), are based on protein abundance rather than protein activity and can result in conflicting data. To address this issue, we developed an assay in which ligand binding is detected using a microarray approach with immobilized antibodies on a porous aluminum oxide matrix. The galectin family of proteins was used as a model system to evaluate the performance of this approach. Galectins selectively bind galactosides and are linked to cancer progression. Our assay employed antibodies directed against different galectins. The antibodies were immobilized on the microarray surface by use of protein A/G. In our example, galectin-1 and galectin-9 were then detected in cell lysates. Lysates were exposed to the anti-galectin surface, followed by washing and quantification with a general fluorescent galectin ligand. The optimal galectin ligand allowed detection of nanogram amounts of galectin using only 1 μg of antibody. Galectin-1 was visualized in HeLa and tumor cell lysates, indicating the potential of the method for a clinical setting.

The detection of specific biomolecules (DNA or proteins) in tissue samples provides valuable biological information for mechanistic elucidation and prognostic and diagnostic purposes. Current analytical tools, such as real-time polymerase chain reaction (rt–PCR)[1] and enzyme-linked immunosorbent assay (ELISA), have provided useful biological information [1–3]. However, these techniques suffer from some important disadvantages. First, rt–PCR measures the messenger RNA (mRNA) level in a sample instead of the protein level, but these levels are not directly correlated [4,5]. Second, ELISA measures the protein abundance rather than the amount of functionally active protein by making use of two different antibodies against the protein of interest. These antibodies capture and visualize the specific protein, respectively, thereby detecting the protein of interest irrespective of its functional status. Due to these disadvantages, proteomic techniques can also provide conflicting data. In addition, the current protein detection methods do not allow detection of different members of one protein family in a single experiment, again making it difficult to compare results.

Here we describe a protein detection assay that visualizes proteins by making use of their inherent ligand-binding capacity (Fig. 1). It is performed on a porous aluminum oxide material suitable for use in the construction of microarrays [6,7]. This method of activity-based profiling [8] ensures that the detected proteins are functionally active (i.e., bind their specific ligand), which is clearly an important consideration when quantifying proteins. The assay is set up in the following manner. The protein of interest is captured from a tissue sample using specific antibodies directed against this protein. After a washing step for the removal of the unbound proteins, the presence of the target protein is visualized by a fluorescently labeled ligand designed to bind the protein of interest. With a well-designed ligand, it is possible to visualize the presence not only of one target protein but also of all the members of a protein family. Such an assay not only would increase the efficiency of detection of several proteins in one single experiment but also would reduce sample volumes, sample consumption, time, and costs.

Here we describe a proof-of-concept investigation with the development of a microarray-based detection assay for the galectin protein family. The galectin protein family consists of 15 galactoside-binding proteins that are present in animals where they

* Corresponding author.
E-mail address: r.j.pieters@uu.nl (R.J. Pieters).

[1] *Abbreviations used:* rt–PCR, real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; NMR, nuclear magnetic resonance; HSQC, heteronuclear single quantum coherence; MS, mass spectrometry; MALDI–ToF, matrix-assisted laser desorption/ionization time-of-flight; BSA, bovine serum albumin; TSTU, *N,N,N',N'*-tetramethyl(succinimido)uronium tetrafluoroborate; PBS, phosphate-buffered saline; NHS, *N*-hydroxysuccinimide; DMSO, dimethyl sulfoxide; ASF, asialofetuin; DMF, dimethylformamide; RT, room temperature; CCD, charge-coupled device; NSCLC, non-small cell lung carcinoma; AU, arbitrary units; FITC, fluorescein isothiocyanate; TFA, trifluoroacetic acid.

0003-2697/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ab.2012.11.003

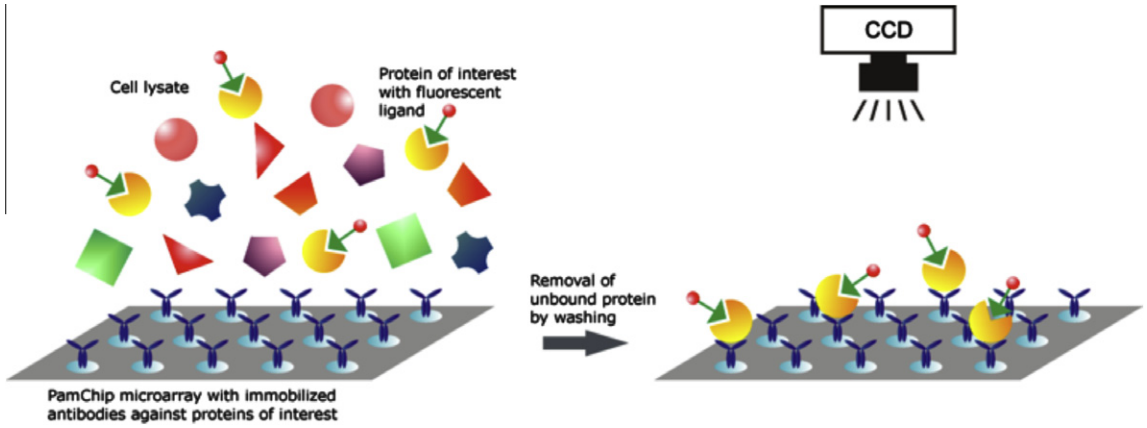

Fig.1. Schematic representation of the protein detection technology based on fluorescent ligand binding. Specific antibodies immobilized on the microarray chip capture target proteins from a cell lysate. All unbound proteins are removed by washing, and the presence of the protein of interest is visualized with a CCD camera. Of importance to note is that only functionally active proteins, capable of binding the fluorescent ligand, are detected in this approach.

function as cell–cell and cell–matrix interaction mediators [9,10]. Galectins are, however, also highly overexpressed in various processes related to cancer progression [11], including apoptosis [12–15], angiogenesis [16], and metastasis [17]. Therefore, galectins are excellent targets for tumor diagnostics and medical prognostic purposes [18]; hence, a galectin detection assay could serve very well in a clinical setting.

Materials and methods

Materials

All chemicals were obtained from commercial sources and used without further purification unless stated otherwise. Solvents were purchased at Biosolve (Valkenswaard, The Netherlands). Dowex Marathon C was purchased from Sigma–Aldrich Chemie (Zwijndrecht, The Netherlands). The nuclear magnetic resonance (NMR) analysis of all newly synthesized compounds (Fig. 2B) can be found in the supplementary material.

General methods

Thin layer chromatography (TLC) was performed on Merck precoated Silica 60 plates, and compounds were visualized by ultraviolet (UV) light and by staining with 10% H_2SO_4 in MeOH. Microwave reactions were performed in a Biotage Initiator in sealed vessels of 2–5 ml. Column chromatography was carried out on Merck Kieselgel 60 (40–63 mm). ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) spectra were recorded on a Varian G-300 spectrometer. ^{1}H–^{13}C correlated heteronuclear single quantum coherence (HSQC) NMR spectra were recorded on a Varian INOVA spectrophotometer (125 MHz). All recorded spectra were referenced to the solvent signal or tetramethylsilane (TMS). Electrospray ionization (ESI) mass spectrometry (MS) was carried out using a Shimadzu LCMS QP8000 system. Matrix-assisted laser desorption/ionization time-of-flight (MALDI–ToF) MS was recorded on a Shimadzu Axima–CFR with sinapinic acid as a matrix.

*General galectin ligand 1: Biotin–lactoside–BSA conjugate (**8**)*

Bovine serum albumin (BSA) was used as a scaffold to obtain a multivalent lactoside construct to serve as a general galectin ligand. Therefore, BSA was functionalized with compound **7** using the method described in Refs. [19,20]. In brief, compound **7** (37 mg, 73 μmol) and *N,N,N′,N′*-tetramethyl(succinimido)uronium tetrafluoroborate (TSTU) (35 mg, 117 μmol) were dissolved in 1,4-dioxane/H_2O (2 ml, 4:1). Triethylamine (10.2 μl, 37 μmol) was added, and the solution was stirred for 10 min. Then BSA (30 mg, 0.45 μmol) dissolved in sodium borate buffer (6 ml, 0.1 M, pH 8.5) was added, and the mixture was shaken overnight. The product was purified by dialysis against ddH_2O (2 × 2 h and overnight), lyophilized, and analyzed by MALDI–ToF MS. The obtained construct **8** contained on average 26 lactose moieties. Subsequently, the lactoside–BSA conjugate (2 mg in 500 μl of 1× phosphate-buffered saline [PBS]) was biotinylated with biotin–NHS (*N*-hydroxysuccinimide) (255 μg in 56 μl of dimethyl sulfoxide [DMSO]) for 90 min. The conjugate was purified by dialysis against 1× PBS (2 × 2 h and overnight) and analyzed by MALDI–ToF MS, which showed 2 or 3 biotin moieties per BSA. The biotin labels were installed to permit visualization of the ligand with a fluorescently labeled anti-biotin antibody (Fig. 2C).

*General galectin ligand 2: Biotin–ASF conjugate (**9**)*

Asialofetuin (ASF) is an enzymatically processed form of fetuin, which shows eight *N*-acetyl lactosamine moieties on the protein surface [21]. Therefore, it is also suitable as a general galectin ligand. ASF (2 mg in 500 μl of 1× PBS) was biotinylated with biotin–NHS (255 μg in 56 μl of DMSO) as described previously for BSA. The conjugate **9** was purified by dialysis against 1× PBS (2 × 2 h and overnight) and analyzed by MALDI–ToF, which showed 6 biotin moieties per ASF. The biotin labels were installed to permit visualization of the ligand with fluorescently labeled anti-biotin antibody (Fig. 2C).

S-Acetylthioacetate protein A/G and array functionalization

Protein A/G is a recombinant fusion protein containing immunoglobulin G (IgG) binding domains of both protein A and protein G, and it contains four Fc binding domains. Protein A/G was used to modify the porous aluminum oxide microarray chip, providing a general means for the attachment of antibodies on the chip surface [22,23]. Protein A/G (1.2 mg, 0.024 μmol, Pierce Biotechnology, Rockford, IL, USA) was first functionalized to contain a thiol moiety by dissolving it in PBS (390 μl, pH 7.4) and treating with *N*-succinimidyl *S*-acetylthioacetate (46 μg, 0.2 μmol) in dimethylformamide (DMF, 3.5 μl), followed by stirring for 45 min at room temperature (RT). The solution was then dialyzed against 1× PBS (2 × 2 h and overnight, pH 5.0) to obtain pure *S*-acetylthioacetate protein A/G. The protein (20 μl, 61 μg) was treated with