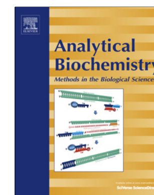
Analytical Biochemistry 442 (2013) 62–67

Contents lists available at ScienceDirect

Analytical Biochemistry

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



Analysis of protein tyrosine phosphatase interactions with microarrayed phosphopeptide substrates using imaging mass spectrometry

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ARTICLE INFO

Article history:
Received 27 June 2013
Received in revised form 15 July 2013
Accepted 19 July 2013
Available online 29 July 2013

Keywords:
MALDI
Imaging mass spectrometry
Peptide microarray
YopH
Phosphatase assay
SPRi

ABSTRACT

Microarrays of peptide and recombinant protein libraries are routinely used for high-throughput studies of protein–protein interactions and enzymatic activities. Imaging mass spectrometry (IMS) is currently applied as a method to localize analytes on thin tissue sections and other surfaces. Here, we have applied IMS as a label-free means to analyze protein–peptide interactions in a microarray-based phosphatase assay. This IMS strategy visualizes the entire microarray in one composite image by collecting a predefined raster of matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectrometry spectra over the surface of the chip. Examining the bacterial tyrosine phosphatase YopH, we used IMS as a label-free means to visualize enzyme binding and activity with a microarrayed phosphopeptide library printed on chips coated with either gold or indium–tin oxide. Furthermore, we demonstrate that microarray-based IMS can be coupled with surface plasmon resonance imaging to add kinetic analyses to measured binding interactions. The method described here is within the capabilities of many modern MALDI–TOF instruments and has general utility for the label-free analysis of microarray assays.

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Peptide microarrays are increasingly used in large-scale studies of protein–protein interactions. Short peptide sequences derived from protein motifs are often sufficient to recreate native interactions, and such peptides can be synthesized and printed on a large scale for use in binding or enzymatic assays [1]. Miniaturization of bioassays by high-density printing of microarrays minimizes reagent consumption while maximizing throughput. A significant barrier to implementation of peptide microarrays, however, is the lack of analytical methods to visualize these events. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS)[1] is a soft ionization technique for analysis of proteins, peptides, lipids, nucleic acids, and other high-molecular-weight molecules. For MALDI–MS, analytes are cocrystallized with a chemical matrix and ejected into the gas phase as ions by laser ablation. MALDI–imaging mass spectrometry (MALDI–IMS) takes advantage of this targeted sampling to acquire data directly from the surface of flat samples, generally thin tissue sections, that have been coated in matrix (reviewed in Ref. [2]). This strategy facilitates the identification of analytes with molecular specificity while preserving the original spatial distribution in situ.

Phosphatase assays generally employ small chromogenic substrates, such as pNPP (*p*-nitrophenyl phosphate), or fluorescently labeled phospho-specific antibodies to visualize phosphorylation status of peptide substrates after exposure to enzyme (reviewed in Ref. [3]). False signals in these probe-based assays are influenced by amino acid sequence context, and for the case of antibodies probes must be specific for a single phosphorylated residue (p-Tyr, p-Ser, p-Thr, etc.). MALDI–IMS can potentially overcome many limitations of probe-based assays by using label-free detection of dephosphorylation based on mass shift. Here, we have applied MALDI–IMS as a novel readout for a peptide microarray-based phosphatase assay. Our experiments measured interactions between the protein tyrosine phosphatase YopH produced by the bacterium *Yersinia pestis* and a small library of phosphorylated peptides derived from a targeted motif [4,5] in epidermal growth factor receptor (EGFR). Local sequence is thought to play an important role in phosphatase substrate affinity, and the arrayed peptides were designed with substitutions and truncations predicted to affect YopH binding and activity.

Although IMS was previously applied to analyze tissue microarrays and in situ synthesized peptide microarrays [6–8], our study demonstrates that IMS can directly visualize the results of binding and enzymatic assays by providing a detailed characterization of microarrayed substrates at the molecular level. In addition, this

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[1] *Abbreviations used:* MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; IMS, imaging mass spectrometry; EGFR, epidermal growth factor receptor; 11-MUA, 11-mercaptoundecanoic acid; CHCA, α-cyano-4-hydroxycinnamic acid; SA, sinapinic acid; DTT, dithiothreitol; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; sulfo-NHS, *N*-sulfohydroxysuccinimidyl ester; wt-YopH, wild-type YopH; m-YopH, mutant (C403A/D356A) YopH; ITO, indium–tin oxide; SAM, self-assembled monolayer; PBS, phosphate-buffered saline; SPRi, surface plasmon resonance imaging; TOF, time-of-flight; IgG, immunoglobulin G.

0003-2697/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ab.2013.07.031

method simultaneously evaluates microarray printing quality by localizing and identifying peptides, products, and contaminants.

Materials and methods

Reagents

11-Mercaptoundecanoic acid (11-MUA), α-cyano-4-hydroxy-cinnamic acid (CHCA), sinapinic acid (SA), formic acid (Fluka brand), and dithiothreitol (DTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1-Ethyl-3-[3-dimethylamino-propyl]carbodiimide hydrochloride (EDC) and *N*-sulfohydroxy-succinimidyl ester (sulfo-NHS) were purchased from Thermo Scientific (Rockford, IL, USA).

Peptides and proteins

The following biotinylated peptides were synthesized, purified to ⩾95% by high-performance liquid chromatography (HPLC), and confirmed by MS by Peptide 2.0 (Chantilly, VA, USA): biotin-AHX-VDADA(pY)LI-amide, biotin-AHX-VAAAA(pY)LI-amide, biotin-AHX-VDAAE(pY)LI-amide, biotin-AHX-VAAAE(pY)LI-amide, biotin-AHX-VAADE(pY)LI-amide, biotin-AHX-VDADE(pY)LI-amide, biotin-AHX-VDADEYLI-amide, biotin-AHX-ADE(pY)L-amide, and biotin-AHX-VVDADE(pY)LIPQQG-amide (Table 1). Peptides were reconstituted for printing (500 μM) in 10 mM citrate buffer (pH 6.6). Recombinant *Y. pestis* proteins consisting of wild-type YopH (wt-YopH) and catalytically inactivated (mutant C403A/D356A) YopH (m-YopH) were kind gifts of Dave Waugh (National Cancer Institute–Frederick National Laboratory), prepared as described previously [5]. Mouse anti-pTyr antibody was purchased from Cell Signaling (Danvers, MA, USA, pTyr-100, cat. no. 9411), and NeutrAvidin was purchased from Thermo Scientific.

Microarray printing

Microarrays used in these experiments were printed on 25 × 75 × 1.1-mm glass slides coated (30–60 ohms) with indium–tin oxide (ITO, Delta Technologies, Loveland, CO, USA) or on polycarbonate slides coated with gold (GE Healthcare, Piscataway, NJ, USA). The print surfaces of the slides were covered with self-assembled monolayers (SAMs) of 11-MUA by immersion in a 1-mM solution in ethanol for 2 h (22 °C) followed by extensive rinsing with 100% ethanol. Reactive esters were created on the SAM carboxyl moiety by exposure to a solution containing 200 mM EDC and 50 mM sulfo-NHS in water for 10 min (22 °C). Excess EDC/sulfo-NHS solution was rinsed away with water, and a solution of 100 μg/ml NeutrAvidin in phosphate-buffered saline (PBS, pH 7.4) was applied to the print surface. Excess NeutrAvidin was rinsed away with PBS followed by water. Unreacted esters were blocked for 5 min with 1 M Tris (pH 9.0, 22 °C), rinsed with water, and dried. The slides were used immediately for microarray printing. Biotinylated peptides were printed on the surface in a 9 × 3 microarray format using an inkjet microarray printer (ArrayJet, Edinburgh, Scotland), delivering 1.5 nl of 500 μM peptide solution to produce spots with 1.0-mm center-to-center spacing.

YopH phosphatase and binding assays

For the phosphatase activity assay, 1 μM wt-YopH (amino acid residues 164–468) was prepared in 10 mM citrate buffer (pH 6.6) containing 100 mM NaCl and 1 mM DTT. The wt-YopH (200 μl) was applied to the print area of an ITO-coated slide and incubated for 10 min (22 °C). The protein solution was removed, and the print area was washed with 10 mM citrate buffer (pH 6.6) containing 100 mM NaCl followed by filtered deionized water. The slide was dried thoroughly under a nitrogen gas stream before matrix application. For the binding assay, 2.5 μM m-YopH was prepared in 10 mM citrate buffer (pH 6.6) containing 100 mM NaCl and 1 mM DTT. m-YopH (200 μl) was applied to the print area of an ITO-coated slide and incubated for 30 min (22 °C). The protein solution was removed, and the print area was washed with 10 mM citrate buffer (pH 6.6) containing 100 mM NaCl followed by filtered deionized water. The slide was dried under a nitrogen gas stream before matrix application.

Surface plasmon resonance imaging

Surface plasmon resonance imaging (SPRi) experiments were carried out with a GE Flexchip using a 500-μl/min flow rate, temperature of 25 °C, and HBS–EP (10 mM Hepes [pH 7.4], 150 mM NaCl, 3 mM EDTA, and 0.005% Tween 20) as a running buffer. SPRi experiments consisted of a 30-min block step with Flexchip blocking buffer (GE Healthcare), a 15-min equilibration with running buffer, a 15-min injection with anti-phosphotyrosine monoclonal antibody diluted 1:1000 in running buffer supplemented with 1 mm DTT, and a 2-min dissociation initiated by injection of running buffer. The cover slip of the SPRi microarray chip was carefully removed with tweezers, and the microarray surfaces were washed in filtered deionized water and dried under a nitrogen gas stream before matrix application.

Matrix application for MALDI–MS analysis

The MALDI matrices consisted of 5 mg/ml CHCA or 10 mg/ml SA dissolved in 50% acetonitrile aqueous solution with 0.1% formic acid. An automated sprayer (TM-Sprayer, HTX Technologies, Carrboro, NC, USA) was used for matrix application, using 0.25 ml/min 50% acetonitrile aqueous solution as a mobile phase, nitrogen pressure of 10 psi (140 °C) as a nebulizing gas, and the following settings: 1200 mm/min stage velocity, 3 mm track spacing, 1.5 mm offset between consecutive passes, with six total passes (three in horizontal direction and three in vertical direction).

Imaging mass spectrometry

IMS experiments were carried out on an AB Sciex 5800 MALDI–TOF–TOF (tandem time-of-flight) with an Nd:YAG laser operating at 349 nm with a fixed beam diameter of 75 μm (Applied Biosystems, Foster City, CA, USA). Slides were placed in a microscope slide adaptor for the steel AB Sciex MALDI target (LaserBio Labs, cat. no. IMG-120) and affixed to the surface with copper tape that bridged the conductive surfaces of the MALDI plate and slide (Electron Microscopy Sciences, Hatfield, PA, USA, cat. no. 77801). For image acquisition, 4800 Imaging software (Applied Biosystems) was used to raster the microarray surfaces collecting and averaging 50 spectra from points spaced 75 μM from center to center in *x* and *y* directions. Peptide analyses were performed in reflector positive mode scanning the *m*/*z* 500 to 2500 range with a focus at *m*/*z* 1250. For detection of m-YopH and NeutrAvidin, linear mid mass positive mode was used to scan the *m*/*z* 7000 to 70,000 range with a focus *m*/*z* of 33,000. Monoclonal antibody (immunoglobulin G, IgG) was detected in linear high mass positive mode scanning the *m*/*z* 100,000 to 200,000 range with a focus at *m*/*z* 150,000. See Supplementary Table 1 of the supplementary material for additional details about MALDI settings.

Imaging data analysis

Optical images of the array area were exported from the Flexchip for coregistration with MS images. MS images were

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