Biosensors and Bioelectronics 91 (2017) 8–14

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

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

Detection of aberrant protein phosphorylation in cancer using direct gold-protein affinity interactions

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ARTICLE INFO

Keywords:
Protein phosphorylation
Gold-protein affinity
Electrochemistry
EGFR protein
Tyrosine kinase inhibitor

ABSTRACT

Protein phosphorylation is one of the most prominent post-translational mechanisms for protein regulation, which is frequently impaired in cancer. Through the covalent addition of phosphate groups to certain amino-acids, the interactions of former residues with nearby amino-acids are drastically altered, resulting in major changes of protein conformation that impacts its biological function. Herein, we report that these conformational changes can also disturb the protein's ability to interact with and adsorb onto bare gold surfaces. We exploited this feature to develop a simple electrochemical method for detecting the aberrant phosphorylation of EGFR protein in several lung cancer cell lines. This method, which required as low as 10 ng/μL (i.e., 50 ng) of purified EGFR protein, also enabled monitoring cell sensitivity to tyrosine kinase inhibitors (TKI) − a common drug used for restoring the function of aberrantly phosphorylated proteins in lung cancer. The reported strategy based on direct gold-protein affinity interactions avoids the conventional paradigm of requiring a phospho-specific antibody for detection and could be a potential alternative of widely used mass spectrometry.

1. Introduction

Scientific elucidation of cancer specific molecular aberrations has enabled the development of an increasing number of targeted therapies that are superior to conventional chemotherapy in specific settings (Stegmeier et al., 2010; Vanneman and Dranoff, 2012) and has been one of the major medical advances in lung cancer in the last few years (Dy and Adjei, 2002; Hirsch et al., 2003; Sun et al., 2007). For example, the ability of tyrosine kinase inhibitors (TKI) to restrict the activity of oncogenic phosphorylated kinase proteins have demonstrated long-term improvements in lung cancer survival and multiple TKI therapies are currently approved or in clinical trials (Camidge et al., 2014; Paez et al., 2004). However, despite the efficacy of TKIs a significant number of cancer patients acquire secondary mutations that impart varying degrees of drug insensitivity with consequent reactivation of the same or alternate phosphorylation pathway, leading to re-initiation of tumour growth (Camidge et al., 2014). The future long-term success of this therapeutic approach is dependent on accurate screening of, monitoring of and strategic therapeutic targeting of the phosphorylated status of these representative proteins.

Current platforms for proteomic analysis such as immunoassays (e.g., western-blot ELISA, immunohistochemistry and planar and bead arrays) using fluorescent or radiolabelled probes (Martin et al., 2003; Olive, 2004; Yan et al., 1998), mass spectrometry (Ficarro et al., 2002; Garcia et al., 2005; Yan et al., 1998), flow cytometry (Krutzik et al., 2004), and protein sequencing (Yan et al., 1998) can detect aberrant changes in the phosphorylated levels of proteins, enabling their use for monitoring the outcome of drug therapy on their target, and on related signalling networks of malignancies (Pierobon et al., 2015). There are however some limitations in the potential use of these methods for clinical purposes. Immunoassay-based assays typically rely on phospho-specific antibodies to target one or various phosphosites within the protein. Yet, phospho-specific antibodies are limited in the market, and determining the phosphorylation status of some proteins can thus be challenging. In addition, these antibodies are very expensive and can still lead to immunohistochemical artifacts and cross-reactivity between different epitopes (Mandell, 2003; Rush et al., 2005). Techniques such as mass spectrometry can determine the type and

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http://dx.doi.org/10.1016/j.bios.2016.12.012
Received 29 September 2016; Received in revised form 27 November 2016; Accepted 6 December 2016
Available online 07 December 2016
0956-5663/

site of phosphorylation on purified proteins without any phospho-specific antibody requirement, but these methods are affected by expensive instrumentation, long analysis procedures or the use of highly specific radioactive or fluorescent labels (Aebersold and Mann, 2003). Moreover, the determination of protein phosphorylation by mass spectrometry, is particularly complicated by the frequently low stoichiometry, the size and ionizability of peptides bearing the modifications, and their fragmentation behaviour in the mass spectrometer (Aebersold and Goodlett, 2001; Mann and Jensen, 2003; Mann et al., 2002). Over the last decade, biosensors have showed they are uniquely positioned to overcome these limitations and few formats have already enabled rapid monitoring of kinases activity without the need for phospho-specific antibodies (Bhalla et al., 2014, 2015; Liu et al., 2014; Martić et al. 2012; Oldach and Zhang, 2014; Shin et al., 2014; Yin et al., 2015a, 2015b). In this type of biosensors, usually a peptide is immobilized onto the sensor surface and either optical or electrochemical methods are employed for detecting the enzymatic addition of phosphates to the peptide. Although enabling fast and accurate detection, a major limitation of this format is that the sensor surface has to be biofunctionalized with the substrate peptide specific for each type of kinase tested. This process requires previous knowledge of the target peptide composition for a given kinase and tedious biofunctionalization chemistries to attach them to the sensing surface. In addition, these approaches often rely on chemical labelling (e.g., ATP labelling with ferrocene (Martić et al. 2012)). Other optical biosensing formats, such as those using genetically encodable fluorescent reporters have enabled live-cell visualization of protein phosphorylation without requiring attachment of peptides to a sensing surface (Oldach and Zhang, 2014). However, the biosensor design and development represents an extremely lengthy work and usually require transfection and expression of biosensor proteins within the cell, which is not compatible with the clinical settings. Therefore, the simple and fast analysis of the phosphorylation status of a purified protein, which could be implemented in clinical settings, remains a challenging endeavour.

In order to address this challenge, we focused on the conformational changes arisen in proteins due to phosphorylation. Phosphorylation acts as a molecular switch enabling proteins to adopt at least two extreme on/off conformations (Huse and Kuriyan, 2002). Although structurally very similar, these conformations differ on the spatial arrangement of several amino-acid residues located inside and outside the protein kinase domain. For example, the epidermal growth receptor (EGFR) undergoes dimerization and subsequent auto-phosphorylation upon binding its cognate ligand. This autophosphorylation, that mainly occurs on EGFR cytoplasmic tyrosine residues, induces a conformational change affecting most of the cytoplasmic domain (Sako et al., 2000). We therefore reasoned that phosphorylation-induced conformational changes, such as those reported for EGFR, could alter the intrinsic affinity of native proteins towards gold surfaces due to amino-acids with higher gold-affinity (e.g., cysteine) being exposed/hidden on the protein's surface, or due to hydrophobic/hydrophilic fragments misplaced from their original positions. The measure of proteins affinity towards bare gold surfaces might thus enable a simple approach to determine protein's phosphorylation levels; especially because protein gold-adsorbed levels are generally easy to quantify with optical or electrochemical methods. Following this rationale, we have developed a simple phospho-specific antibody-free approach that can detect protein's phosphorylation via direct protein-bare gold affinity interactions followed by electrochemical quantification. The practical application of the assay was showcased through the analysis of EGFR proteins derived from selected TKI-sensitive and insensitive lung cancer cell lines, which show various degrees of phosphorylation. We also successfully applied this assay in monitoring sensitivity to tyrosine kinase inhibitors in these cell lines.

2. Experimental section

Details on materials and methods are included in the Supplementary Information.

2.1. Cell culture and immunopurification of target EGFR protein

Lung cancer cell lines NCI-H1666, NCI-H1975, and HCC827 were grown in RPMI 1640 containing 10% fetal bovine serum, 1% antibiotics, and 1% glutamax. The EGFR protein was generically isolated from the selected cell-lines regardless of its phosphorylation status via magnetic purification using protein A/G magnetic beads and a phospho-independent anti-EGFR antibody according to manufacturer's instructions. For validation experiments the phosphorylated-EGFR isoform (P-EGFR) was selectively isolated via a secondary IP to the previously EGFR eluted protein using the same conditions as above, but with a pan phospho-specific (P-Tyr-100) antibody. The concentration of any eluted protein was quantified by bicinchoninic acid (BCA) protein assay and the purity of EGFR extracted from the three cell lines was confirmed by electrophoresis followed by gel staining with coomassie blue.

2.2. EGFR characterisation by western-blot

Western blot was performed to confirm the presence of EGFR in each cell line and to determine its phosphorylation status in the immunopurified fraction by respectively using the phospho-independent anti-EGFR and pan phospho-specific (P-Tyr-100) primary antibodies. For TKI drugs-sensitivity experiments western-blot used a phospho-specific (phosphoY1068) anti-EGFR primary antibody.

2.3. Electrochemical quantification of adsorbed EGFR levels on the gold electrode

Differential Pulse Voltammetric (DPV) experiments were carried out using CH1040C (CH Instruments) with a three electrode system consisting of a gold working electrode (2 mm in diameter), Pt counter electrode, and Ag/AgCl reference electrode in PBS solution containing 2.5 mM $[K_3Fe(CN)_6]$ and 2.5 mM $[K_4Fe(CN)_6]$ electrolyte solution.

3. Results and discussion

3.1. Assay principle

Our novel assay for protein phosphorylation detection is schematically depicted in Scheme 1. The target protein is isolated from lung cancer cells via immunoprecipitation (IP) using magnetic beads previously functionalized with a generic phospho-independent antibody, i.e., an antibody specific to the targeted protein irrespective of their phosphorylation status. Contrary to phospho-specific antibodies, these generic antibodies are widely available in the market for almost any protein type, and they are more cost-effective. Next, the IP-extracted protein is dropped onto the bare gold electrode surface and allowed to adsorb for a specific period of time. Phosphorylation levels are finally inferred from the total protein adsorption levels, which are quantified electrochemically using Differential Pulse Voltammetry (DPV) in the presence of $[Fe(CN)_6]^{3-/4-}$ redox system. In this method, the ability of the redox molecules for interacting with the gold surface is reduced with increasing concentration of protein adsorbed onto the gold surface, resulting in less electrical current recorded. We have previously used this redox system for quantitation of the gold-adsorption levels of DNA (Koo et al., 2014; Sina et al., 2014), RNA (Koo et al., 2016a, 2016b) and protein molecules (Yadav et al., 2016) and found that the adsorption of these molecules onto gold substrates is a highly reproducible process when performed under optimized and well-controlled conditions. Moreover, it is also possible to perform robust