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Absolute quantitation of endogenous proteins with precision and accuracy using a capillary Western system

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ABSTRACT

Precise and accurate quantification of protein expression levels in a complex biological setting is challenging. Here, we describe a method for absolute quantitation of endogenous proteins in cell lysates using an automated capillary immunoassay system, the size-based Simple Western system (recently developed by ProteinSimple). The method was able to accurately measure the absolute amounts of target proteins at picogram or sub-picogram levels per nanogram of cell lysates. The measurements were independent of the cell matrix or the cell lysis buffer and were not affected by different antibody affinities for their specific epitopes. We then applied this method to quantitate absolute levels of expression of protein kinase C (PKC) isoforms in LNCaP and U937 cells, two cell lines used extensively for probing the downstream biological responses to PKC targeted ligands. Our absolute quantitation confirmed the predominance of PKCδ in both cells, supporting the important functional role of this PKC isoform in these cell lines. The method described here provides an approach to accurately quantitate levels of protein expression and correlate protein level with function. In addition to enhanced accuracy relative to conventional Western analysis, it circumvents the distortions inherent in comparison with signal intensities from different antibodies with different affinities.

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Information about protein expression is important for understanding the mechanism of protein function as well as for formulating strategies to develop biomedicines [1,2]. Although messenger RNA (mRNA)[1] quantitation has been widely used as an indirect indication of protein expression, studies have found that mRNA and protein levels are not always correlated [3–5]. Protein quantitation methods have been established through both antibody-based and mass spectrometry-based technologies. Although those technologies provide good information regarding relative protein levels as well as protein modifications in response to stimuli, methods for precise and accurate quantitation of absolute protein levels in a complex biological matrix face many challenges [2,6]. For example, quantification standards are difficult to establish in enzyme-linked immunosorbent assays (ELISAs) and antibody array assays due to endogenous signal interference, and poor assay reproducibility has been observed in conventional Western systems. Although mass spectrometry provides a high-precision analysis platform, it may have low accuracy due to the prevalence of interference from other peptides and small molecules in the sample matrix. Mass spectrometry methods also require complicated sample preparation procedures [2,7].

In this article, we describe a method to precisely and accurately quantitate absolute protein expression in cell lysates by using the size-based Simple Western system. Simple Western is a gel-free, blot-free, capillary-based, automated Western blotting system recently developed by ProteinSimple (Santa Clara, CA, USA). In Simple Western analysis, all steps following sample preparation are fully automated, including sample loading, size-based protein separation, immunoprobing, washing, detection, and data analysis. The system greatly reduces the variability caused by manual processes in conventional Western systems. Data generated by the Simple Western system are highly quantitative, with good run-to-run reproducibility [8]. Using the Simple Western system, we developed a method for absolute quantitation of endogenous proteins in cell lysates by spiking the samples with glutathione *S*-transferase (GST)-tagged recombinant proteins as a standard

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[1] *Abbreviations used:* mRNA, messenger RNA; GST, glutathione *S*-transferase; PKC, protein kinase C; HRP, horseradish peroxidase; FBS, fetal bovine serum; ATCC, American type culture collection; cDNA, complementary DNA; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline solution; SDS, sodium dodecyl sulfate; ECL, enhanced chemiluminescence; CV, coefficient of variation; PMA, phorbol myristate acetate.

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for comparison, and we demonstrated the reliability and accuracy of the method for measuring absolute levels of Erk1 and Erk2 in total cell lysates. To illustrate the power of this approach, we then applied this system to quantitate protein kinase C (PKC) isoforms in LNCaP and U937 cells. We were able to compare the relative abundance of PKC isoforms and determine the absolute protein levels of the different PKC isoforms in the two cell lines quantitatively. This latter information is helpful in understanding the relative contributions of these various PKC isoforms to the signaling network activated on the addition of PKC ligands such as the phorbol esters and bryostatin 1, a compound currently in cancer clinical trials (http://www.clinicaltrials.gov).

PKC is a validated therapeutic target for cancer [9,10]. Intense efforts are under way to develop the next generation of bryostatin-related drugs through structural simplification while maintaining its biological specificity for PKC proteins [11–13]. LNCaP and U937 are ideal cell lines for this investigation because they are well-characterized systems that highlight the dramatic differences in biological response to the phorbol esters and bryostatin [11,14]. A critical part of this effort is to understand which PKC isoforms in these cells are important for response and how these individual PKC isoforms are differentially modulated by phorbol ester, by bryostatin 1, and by various structural analogues of bryostatin 1 [14–16].

Materials and methods

Materials

Recombinant proteins GST–PKCα, GST–PKCβII, GST–PKCδ, GST–PKCε, and GST–Erk1 were obtained from SignalChem (Richmond, BC, Canada), and GST–Erk2 was obtained from EMD Millipore (Billerica, MA, USA). Primary antibodies anti-PKCβII (sc-13149), anti-PKCδ (sc-937), and anti-PKCε (sc-214) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the anti-PKCα (cat. no. 1510-1) was obtained from Epitomics (Burlingame, CA, USA), and the anti-Erk1 (cat. no. 05-957) and anti-Erk1/2 (cat. no. 06-182) were obtained from Millipore. The secondary antibodies, goat-anti-rabbit and goat-anti-mouse horseradish peroxidase (HRP) conjugated, were obtained from Jackson ImmunoResearch (West Grove, PA, USA) for Simple Western analysis and from Bio-Rad (Hercules, CA, USA) for conventional Western analysis. The human prostate cancer cell line LNCaP, the human monocytic leukemia cell line U937, the human breast cancer cell line MCF7, the human cervical cancer cell line HeLa, fetal bovine serum (FBS), and RPM1-1640 medium were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

Cell growth

The cells were grown at 37 °C in a humidified atmosphere at 5% CO_2 with growth medium as recommended by the ATCC.

Cell lysate preparation for Simple Western analysis

Cells were lysed with M-Per buffer (Thermo Scientific, Waltham, MA, USA) or RIPA buffer (20 mM Hepes [pH 7.5], 150 mM NaCl, 1% NP-40 alternative, 0.25% [w/v] sodium deoxycholate, and 10% glycerol) containing phosphatase and protease inhibitors (EMD Millipore, Billerica, MA, USA). Protein concentration was measured on a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA) using the Pierce 660 protein assay and bovine serum albumin (BSA) standards (Thermo Scientific).

Real-time RT–PCR analysis

RNA was isolated from cultured cells with TRIzol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). For complementary DNA (cDNA) synthesis, 1.5 μg of total RNA was reverse transcribed using the iScript Advanced cDNA synthesis kit (Bio-Rad) as recommended by the manufacturer. Real-time quantitative polymerase chain reaction (qPCR) was performed on a MyiQ or iQ5 instrument (Bio-Rad) in a volume of 20 μl using iQ SYBR Green Supermix (Bio-Rad) on 150× diluted cDNA. The primers used were predesigned and validated primers from Qiagen (Valencia, CA, USA) for PKCα and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or from Origene (Rockville, MD, USA) for the other genes. Relative gene expression levels were calculated using the $2^{-(\Delta Ct)}$ formula, where ΔCt represents the cycle difference between the gene of interest and GAPDH, used as internal control. The efficiency of the qPCRs was between 99 and 109% when tested on serially diluted (1:5) "universal" RNA samples prepared to contain all transcripts of interest.

Western blot analysis

Total cell lysates were prepared by vortexing and sonication in ice-cold phosphate-buffered saline solution (PBS) containing 1% Triton X-100 and supplemented with protease and phosphatase inhibitors (Roche, Branford, CT, USA). Cell debris was removed by centrifugation at 2600*g* for 5 min in a cooled microcentrifuge (Eppendorf). Samples containing the same amounts of protein (protein concentration measured using the Bio-Rad DC protein assay) were added to sodium dodecyl sulfate (SDS) and β-mercaptoethanol containing sample buffer (Quality Biological), incubated at 100 °C for 5 min, separated on 10% SDS–polyacrylamide gels (Invitrogen), and transferred to nitrocellulose membranes (GE Healthcare, Waukesha, WI, USA) at 100 V (constant) for 75 min using a Mini Trans-Blot Electrophorectic Transfer Cell (Bio-Rad). The membranes were blocked with 5% nonfat dry milk (Bio-Rad) diluted in PBS, incubated overnight at 4 °C with primary antibodies (dilutions empirically determined to give optimal results), washed (3 times for 10 min in PBS containing 0.5% Tween 20), and incubated for 1 h at room temperature with secondary antibodies. After washing (3 times for 10 min in PBS containing 0.5% Tween 20), the signal was developed by enhanced chemiluminescence (ECL) and detected on high-performance chemiluminescence film (GE Healthcare). The scanned films were edited using Adobe Photoshop CS3 (Adobe Systems, McLean, VA, USA).

Simple Western analysis

Simple Western analyses were performed according to the ProteinSimple user manual. In brief, cell lysate samples were mixed with a master mix (ProteinSimple) to a final concentration of 1× sample buffer, 1× fluorescent molecular weight markers, and 40 mM dithiothreitol (DTT) and then heated at 95 °C for 5 min. The samples, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies, chemiluminescent substrate, and separation and stacking matrices were also dispensed to designated wells in a 384-well plate. After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated. Simon–Simple Western analysis is carried out at room temperature, and instrument default settings were used except as specified below. Capillaries were first filled with separation matrix, followed by stacking matrix and approximately 40 nl of sample loading. During electrophoresis, proteins were separated on the basis of molecular weight through the stacking and separation matrices at 250 V for 40 to 50 min and then immobilized on the capillary wall using proprietary