Bio-Plex™ suspension array system tech note 3033

Application of the Bio-Plex Cell Lysis Kit in Western Blot Analysis of Phosphoproteins

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Introduction

The Alliance for Cellular Signaling (AfCS) is a multidisciplinary, multiinstitutional research collaboration to study G-protein-mediated and related signaling systems (www.signaling-gateway.org). The Alliance is charting interactions among cellular signaling molecules to produce theoretical models that will describe how cells respond to stimuli. One approach for producing these models is to quantitate changes in protein phosphorylation that occur in response to ligand binding to cell surface receptors. The assay currently used in the AfCS Antibody Laboratory is multiplex western blotting with phosphorylation site-specific antibodies. While this approach has proven useful (Hsueh et al. 2002, O'Connell 2003, Anon. 2003), our objective is to reduce the labor involved and substantially expand our capabilities by transitioning to Bio-Rad's Bio-Plex phosphoprotein assays, which employ Luminex xMAP technology and Cell Signaling Technology's phosphorylation site-specific antibodies.
The Bio-Plex cell lysis kit is used to prepare cell culture or tissue sample lysates for analysis by Bio-Plex phosphoprotein assays. We recently evaluated the Bio-Plex cell lysis kit to determine whether this protein extraction procedure yields western blotting results that are similar to those that we generate by routine cell lysis and protein extraction with the AfCS protocol. We were satisfied to find that western blots of both types of extracts yielded similar information about ligand-stimulated changes in protein phosphorylation.

Methods

AfCS protocols can be viewed online at www.signaling-gateway.org (registration allows free access).

Cell Culture and Ligand Stimulation

WEHI-231 cells (American Type Tissue Collection) were incubated at a density of 1 x 10^6 cells/ml in assay medium (RPMI 1640 medium with 1% fetal calf serum, AfCS protocol PS00000436) for 1 hr and then treated with anti-IgM (130 nM), anti-CD40 (65 nm), or IL-10 (0.35 nM) for 0, 2.5, 5, or 15 min.

Protein Extraction

A comparison was made between cells prepared with the Bio-Plex cell lysis kit and cells pelleted by centrifugation and then extracted according to AfCS procedure protocol PP00000117 with SDS-PAGE sample buffer (AfCS solution protocol PS00000050). A 4x buffer from AfCS protocol PS00000052 was added to the samples generated with the Bio-Plex cell lysis kit to a final concentration range of 1–1.2x. The protein concentration of all samples was determined by an Amido Black assay (AfCS protocol PP00000046).

Western Blotting

Protein samples (6–12 µg) were resolved by SDS-PAGE on duplicate Criterion™ 26-well, 4–20% acrylamide gradient gels (AfCS protocol PP00000003). A positive control sample (AfCS protocol PS00000468 or PS00000469) was included on each gel. The proteins were transferred to nitrocellulose in a Criterion blotter unit according to AfCS protocol PP00000005. The blots were processed with phosphospecific antibody mixtures 1 or 2 (AfCS protocol PS00000312 or PS00000334). The proteins are listed with sites of phosphorylation identified in parentheses: STAT6 (Tyr^{641}), p90RSK (Ser^{380}), Akt (Ser^{473}), ERK1/2 (Thr^{202}/Tyr^{204}), PKCµ (Ser^{916}), STAT3 (Tyr^{705}), NFκB p65 (Ser^{536}), JNK (Thr^{183}/Tyr^{185}), p38 MAPK (Thr^{180}/Tyr^{182}).

Quantitation of Phosphoproteins

The western blot signals for each phosphoprotein were quantitated (AfCS protocol PP00000007) and normalized to the signal for G protein b subunit (conventional antibody, not phosphospecific) to account for lane-to-lane variability. Fold change in phosphoprotein signal was calculated relative to the average of triplicate zero time points (fold change defined as 1).

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Results and Discussion

The protein concentration was 40% greater for WEHI-231 cell extracts prepared using the AfCS protocol than those prepared with the Bio-Plex cell lysis kit (1.4 ± 0.1 vs. 1.0 ± 0.1 mg/ml, respectively, n = 15). Protein from AfCS protocol extracts (12 µg) or Bio-Plex cell lysis kit extracts (6 µg) was analyzed by multiplex western blotting to quantitate fold changes in protein phosphorylation. Figure 1 shows examples of multiplex western blots with protein migrations identified. The signals for selected proteins were quantitated as fold changes in ligand-stimulated phosphorylation (Figure 2). It is satisfying to find that the magnitude of fold changes in phosphorylation quantitated for the Bio-Plex cell lysis kit extracts was greater than or equal to that calculated for our routine AfCS protocol extracts. We conclude that the Bio-Plex cell lysis method is adaptable to western blotting procedures for phosphoprotein detection. One obvious benefit of this finding is to enable direct comparisons between western blotting and Bio-Plex multiplex suspension array analysis of phosphoproteins with the same cell extracts.

Acknowledgements

We acknowledge strong technical support from Nicholas Wong, Christine Horvath, and Megan Smith. The AfCS is sponsored by the National Institutes of Health (NIGMS, NIAID, and NCI), the pharmaceutical industry (Eli Lilly and Company, The Merck Genome Research Institute, Aventis Pharmaceuticals, Johnson and Johnson, and Novartis Pharma AG), philanthropic foundations (The Agouron Research Institute and the Anonymous Foundation, Dallas, TX), and the University of Texas Southwestern Medical Center at Dallas.

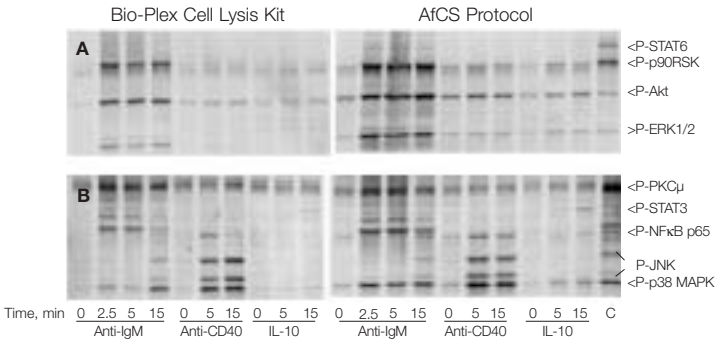

Fig. 1. Multiplex western blots of cell extracts obtained using the Bio-Plex cell lysis kit or by the AfCS procedure. Separate western blots of the same samples were processed with one of two mixtures of phosphospecific antibodies. A, western blots processed with antibodies specific to phospho-STAT6, -p90RSK, -Akt, and -ERK1/2. B, western blots processed with antibodies specific to phospho-PKCµ, -STAT3, NFκB p65, -JNK, and -p38 MAPK. The phosphoproteins are identified at the right of each blot. Lane C, positive control.

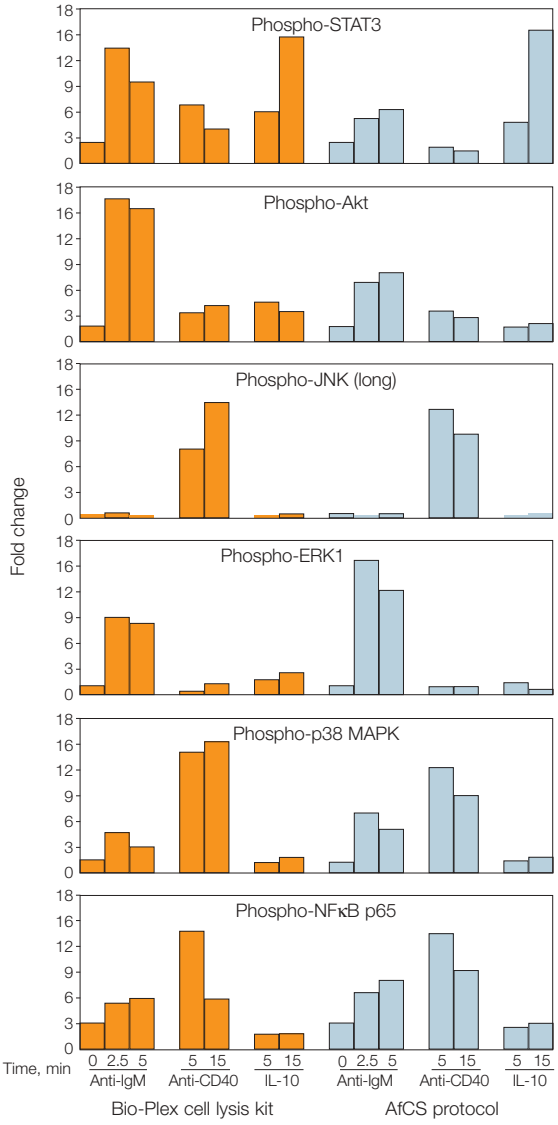

Fig. 2. Quantitation of fold changes in phosphorylation of phosphoproteins. WEHI-231 cells were extracted using the Bio-Plex cell lysis kit or by the AfCS protocol. Each panel represents quantitation of western blot results for a phosphoprotein in cells exposed to three separate ligands for the times indicated on the x-axis. Orange, cells extracted using Bio-Plex cell lysis kit; gray, cells extracted using AfCS protocol.

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