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actions in a 25-min electrophoresis run. One technician can therefore generate electrophoretic data for 600 genotypes per hour with due allowance made for gel loading and set-up time. The rate at which genotypes can be determined is limited by the PCR capacity and the time taken for image analysis, rather than electrophoresis.

MADGE (2) was invented to serve a need in human molecular genetic epidemiological studies for higher-throughput, microplate-compatible electrophoresis. For the examination of genetic influences on common complex diseases, it is often necessary to examine DNA banks of hundreds or thousands of families or individuals by association using SNPs. The development of a format of 192-well MADGE, accommodating separate allele-specific reactions, in combination with newly developed software for gel image analysis, is suit-

ed to detailed examination of candidate gene regions (multiple SNPs, perhaps 10–1000) within a single project. By continuing to use assays based on electrophoresis, a variety of well-established analytical molecular genetic methodologies such as ARMS retain their utility. This contrasts with higher-density set-ups such as microscope slide-based arrays and chips that demand sophisticated equipment.

Although a FluorImager was used here, a standard CCD camera would be sufficient for this MADGE application, so there is minimal capital expense. The approach is thus amenable to “distributability” and to centralized scale-up.

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Combining Enhanced Metabolic Labeling with Immunoblotting to Detect Interactions of Endogenous Cellular Proteins

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ABSTRACT

Metabolic labeling, immunoblotting and two-dimensional isoelectric focusing/SDS-PAGE are powerful techniques for characterizing endogenously expressed cellular proteins and their interactions. We achieved improved resolution and sensitivity for the detection of metabolically labeled proteins separated on two-dimensional gels by electroblotting the proteins onto polyvinylidene difluoride or nitrocellulose membranes and detecting the ³⁵S signal on a bio-image analyzer. We obtained independent detection of specific proteins from the same blot by subsequent rehydration of the membrane and immunoblot analysis. The combination of these enhanced detection techniques with immunoprecipitation and two-dimensional electrophoresis on precast minigels provides a simple, sensitive method for detecting interactions between endo-

Figure 3. ARMS-MADGE arrays: allele calling. The tracks numbered 54 to 59 correspond to microarray coordinates C7, B7, A7, G8, F8 and E8, flagged in Figure 2. The image of 96 double lanes in a single row is generated from the arrayed lanes selected in Figure 2. The A allele-specific tracks are above and the T allele-specific tracks are below. Wells carrying the T allele-specific products appear half way (12.25 mm) down each lane. Genotypes are designated by the presence or absence of an allele-specific band, provided that the slower migrating control PCR band is also present. Allele calling is numeric, the common A allele is 1 and the rare T allele is 2. PCR failure in the T allele-specific ARMS reaction prevents a genotype call in track 59.

genous proteins in the cell.

INTRODUCTION

Besides classical biochemical techniques, the yeast two-hybrid system (2) and phage-display (7) methods have been used extensively to identify protein-protein interactions. Another frequently used approach is to tag proteins differentially, co-express them by transfection and test to determine if they can be co-immunoprecipitated. Because such methods usually rely on overexpression, they often lead to artifactual observations. Alternative techniques for studying endogenous proteins, however, are usually hampered by insufficient sensitivity. Here, we show that the resolution and sensitivity for detection of metabolically labeled proteins can be enhanced by protein electroblotting from one- or two-dimensional (1-D, 2-D) mini-gels onto certain membranes and subsequent analysis by a bio-image analyzer (Fuji Photo Film, Greenwood, SC, USA). Individual protein bands or spots can then be identified on the same blots by immunodetection. We demonstrate this approach by applying it to the interaction between the death receptor CD95 (Fas/APO-1) and its apoptosis-signaling adaptor protein, FADD/MORT-1 (1).

MATERIALS AND METHODS

Metabolic Labeling

K50 cells (3×10^7) were washed twice with PBS and resuspended in 15 mL RPMI medium without cysteine and methionine (Life Technologies, Rockville, MD, USA). After a 30–60 min incubation at 37°C, 0.5 mCi ^{35}S -labeled mixture of cysteine and methionine (Pro-mixTM in vitro cell labeling mixture; Amersham Pharmacia Biotech, Piscataway, NJ, USA) was added to the media. The cells were labeled for up to 24 h at 37°C, washed once with PBS, lysed 30 min on ice with lysis buffer [1% Triton[®] X-100, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.57 mM PMSF, protease inhibitor cocktail completeTM (Roche Molecular Biochemicals, Mann-

heim, Germany)] and centrifuged at $15\,000\times g$ for 15 min at 37°C. Immunoprecipitation of this postnuclear supernatant was performed for 2–16 h at 4°C with protein A/G-beads (Pierce Chemical, Rockford, IL, USA), which were washed seven times with lysis buffer.

Electrophoresis

The first-dimension isoelectric focusing (IEF) was performed with the precast Immobiline[®] DryStrips in a IPGphorTM apparatus essentially following the manufacturer's instructions (Amersham Pharmacia Biotech). Briefly, the immunoprecipitation was mixed for 30 min at 30°C with reducing sample buffer (9.8 M urea, 4% NonidetTM P-40, 100 mM DTT, 2% IPG buffer and bromophenol blue) and electrofocused in 7-cm Immobiline DryStrips (pH 3–10 nonlinear) with the IPGphor. The following focusing protocol was used: 50 μA /strip at 20°C; (i) 30 V for 16 h (step and hold); (ii) 200 V for 3 h (step and hold); (iii) 3500 V for 1 h 30 min (gradient); and (iv) 3500 V for 1 h 30 min (step and hold). The settings resulted in a total of 9105 Vh. After electrofocusing, the strips were either stored at -70°C or shaken for 10 min at room temperature with equilibration buffer [50 mM Tris-HCl (pH 7.5), 6 M urea, 30% glycerol, 2% SDS, 10 mg/mL DTT] and subjected to SDS-PAGE.

The second dimension was performed with a precast NuPAGETM gel system (Novex, San Diego, CA, USA) following the manufacturer's instructions. The strips were trimmed outside the focusing area to fit into the 2-D well of 10% Bis Tris NuPage gels, sealed with agarose gel and run in NuPAGE 3-(N-morpholino) propane sulfonic acid SDS running buffer for 60 min at 200 V.

Transfer of Proteins

For the Western transfer, the Xcell IITM Blot Module (Novex) was assembled according to the manufacturer's instructions. A semi-dry transfer buffer [48 mM Tris-Base, 39 mM glycine, 0.0375% (w/v) SDS, 20% methanol], 200 mA (resulting in about 5 W) and a total of 2 h were used. The blot was air-dried for 2 h at 37°C, placed on a BAS-III imaging plate and exposed for the

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indicated time. The plates were analyzed with the BAS-2000 IP™ Scanner with the Image Reader V1.2 software and analyzed with the MacBAS V.2.4 software (all from Fuji Photo Film).

Immunodetection

The membrane was rehydrated in PBS containing 0.1% Tween®-20 (3 h for nitrocellulose and overnight for PVDF). The immunodetection was performed as described (3) using 1 µg/mL anti-FADD antibody (Transduction Laboratories, Lexington, KY, USA) as a primary antibody, 1:5000 diluted rat anti-mouse IgG₁ antibody conjugated with horseradish peroxidase (BD PharMingen, San Diego, CA, USA) as a secondary antibody and ECL™ (Amersham Pharmacia Biotech) as a detection reagent.

RESULTS AND DISCUSSION

The Immobiline DryStrip and IPG phor-based IEF technique gives reproducible results and is easy to use. The short Immobiline DryStrips have the advantage of compatibility with precast mini-SDS-PAGE gels for electrophoresis in the second dimension. However, the main drawback is that weak signals usually are obtained from metabolically labeled proteins on autoradiographic film. Therefore, we tested whether the detection of ³⁵S signals with Fuji Film's imaging plate (IP) might be more sensitive.

Figure 1 shows the results of a representative experiment. IP exposures taken from dried gels (Figure 1B) gave better results than autoradiography (Figure 1A); however, IP exposures of proteins that were electrotransferred onto a membrane (Figure 1D) gave substantially better results with respect to resolution, signal strength and dynamic range

(compare to Figure 1C). Notably, the electrotransfer itself increased the signal strength for autoradiography (Figure 1C), although the weak spots were better detected on IP exposures (Figure 1D).

Because we expect the signal to increase with more complete transfer, we attempted to optimize the electroblotting conditions. The transfer depends on various factors including membrane type, buffer composition, chamber type, field strength and transfer time (8). Because we chose a mini-gel approach, we used the same manufacturer's transfer chamber (Novex). We obtained near-complete electroelution of proteins from the gel with rather harsh conditions in terms of current (200 mA), time (2 h) and buffer composition (20% methanol and 0.0375% SDS), analyzed by silver staining (data not shown).

To compare different membrane types, we assessed the loss of protein

Figure 1. Improved sensitivity for metabolically labeled proteins by electrotransfer and detection by a bio-image analyzer. Lysates of K50 (10⁵) cells were analyzed on three 2-D gels. Exposures were taken for 5 h. (A and C): conventional autoradiographic film detection at -70°C of a dried gel (A) enhanced with Amplify™ (Amersham Pharmacia Biotech) and an electroblotted gel (C); (B and D): IP scans of a dried gel (B) and the same electroblotted gel as in C (D).

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Figure 2. Testing different membrane types for efficient electroblotting. Molecular weight protein markers (high molecular weight RainbowTM 1 $\times$ C-Marker; Amersham Pharmacia Biotech) (M) or lysates from metabolically labeled K50 cells (L) were resolved by 1-D SDS-PAGE, electroblotted on a first layer of membrane (A), followed by a second one (B), and visualized by IP scans. Lane 1: nitrocellulose 0.45 μm ; lane 2: nitrocellulose 0.2 μm ; lane 3: nylon⁺ 0.45 μm ; lane 4: PVDF 0.2 μm (all from Novex); lane 5: ImmobilonTM-P 0.45 μm (Millipore, Bedford, MA, USA). Exposure time was three days.

Figure 3. Combined ³⁵S-labeling analysis with subsequent immunodetection of a binding partner. Two-dimensional analyses of immunoprecipitations of CD95 by anti-CD95 antibody added after (A) or before (B) a lysis of ³⁵S-labeled K50 cells. Upper panels are IP scans and lower panels are immunoblots with anti-FADD antibody (3). Boxed spots represent CD95, and arrows indicate differentially phosphorylated forms of FADD.

that passes through them by capturing the protein on a second membrane layer (Figure 2B). Under conditions mentioned earlier, 0.2 μm nitrocellulose and 0.2 μm PVDF (both from Novex) performed best. These membranes had the most retardation of proteins and yielded the strongest signal (Figure 2A).

An additional advantage of electroblotting metabolically labeled proteins, besides the apparent improvement in detection of ³⁵S signals, is that the blot may then be analyzed by immunoblot detection. To assess this, we used the CD95 receptor system as a model. K50 cells were metabolically labeled, and an anti-CD95 antibody was added before (Figure 3B) or after (Figure 3A) lysis of the cells (3). The receptor was then immunoprecipitated with this antibody, the samples were resolved by 2-D gel electrophoresis, electrotransferred and subjected to IP analysis. Differentially glycosylated forms of CD95 were detectable (Figure 3, A and B, upper panels) and consistent with previous results (3).

Following receptor multimerization, CD95 transmits an apoptosis-inducing signal through interaction with the adaptor protein, FADD (3). This step can be mimicked by adding the anti-CD95 antibody to the cells before they are lysed (Figure 3B). Two of the spots seen in the autoradiogram correspond to differentially phosphorylated forms of FADD (Figure 3B, arrows), as reported previously (3). We confirmed the identity of these spots by immunoblot analysis of the identical blot with a monoclonal anti-FADD antibody (Figure 3, A and B, lower panel). The spot that corresponds to phosphorylated FADD (higher molecular weight) was less intense, both in autoradiographs and immunoblots, suggesting that the difference is antibody independent. Thus, unphosphorylated FADD may be recruited more effectively to the receptor than the phosphorylated form.

In conclusion, we have developed a simple, sensitive biochemical method that combines immunoprecipitation, 2-D electrophoresis on precast mini-gels and electroblotting with enhanced detection techniques. A similar approach was used by Puthalakath et al. (6) with 1-D gels; however, their technique was much less optimized and was not described in

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detail. Our data for CD95 and FADD demonstrate the usefulness of this approach for identifying interactions between known cellular proteins under physiological conditions. The method may also be useful for studying biologically relevant, novel interactions between known cellular proteins or between known proteins and unknown partners. Because the analysis of metabolically labeled proteins on 2-D gels yields the molecular weight and isoelectric point of the unknown binding partner(s), it is possible to narrow down the number of potential candidates. If the detected protein remains unidentified, a preparative reaction can be used to obtain sequence information by N-terminal sequencing or mass spectrometry (4,5).

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