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Development of a heat-mediated protein blotting method

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ABSTRACT

Western blotting is a significant tool employed for the detection of cell proteins. High-molecular-weight proteins have proven a challenge to detect by western blotting, but proteins even of 100 KDa can still present difficulties in detection. This work reports the development of a heat transfer method that is suitable for both low- and high-molecular-weight proteins. The procedure involves the use of a constant temperature at 78 °C in a dedicated heat transfer module. Through the use of this protocol the neuronal adaptor protein X11α (120 KDa), which prior to this methodology was undetectable endogenously in the neuroblastoma cell line (N2a), was successfully detected in the N2a cell line. The procedure provides a reproducible protocol that can be adapted for other high-molecular-weight proteins, and it provides the advantage that low-molecular-weight proteins are not sacrificed by the methodology.

Introduction

Electroblotting from a gel matrix after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to an immobilizing membrane such as nitrocellulose, nylon, or polyvinylidene fluoride (PVDF) is a significant tool for protein analysis [1–3]. The process relies on sufficient elution of the target protein from the gel matrix and adequate immobilization on a detection membrane [4]. Success of transfer, however, can be hindered by the protein and its size; in general transfer becomes more difficult as the protein size increases [5,6]. Proteins such as Titin, with a maximum subunit size over 4000 kDa [7] are known to be difficult to electroblot, but smaller proteins over 100 KDa can still pose problems. Standard electrotransfer techniques are not always suitable for all proteins, and this has led to the development of modified western blotting techniques [8,9] and to proteolytically cleaving the protein of interest out of gels [10] and even physically disrupting the gel matrix to release the protein [11] to enhance the process of immunoblotting.

One of the factors limiting the elution of larger proteins from acrylamide gels is the pore size of the gel. Low-percentage acrylamide gels (3–6%) can aid the elution process, but such gels can be impractical to use and can have low resolution [9]. Irrespective of this, the success of transfer is also influenced by the individual proteins' composition and affinity for the receptor membrane [12]. Approaches such as increased current and transfer times have been used to overcome the difficulties seen with large proteins, but modifying conditions to maximize transfer efficiency of large proteins can lead to the loss of smaller proteins that may pass through the receiving membrane, decreasing or eliminating their detection. A more recent adaptation is the application of heat to make the gel more permeable, to enable enhanced elution of high-molecular-weight proteins [5,9]. By the application of heat to western transfer, Kurien and Scolfield [9] observed efficient transfer of both high- and low-molecular-weight proteins within 15 min. Their methodology provided an efficient and rapid protein transfer method.

During the course of this work with a neuronal adaptor protein, X11α, a 120 kDa protein, we observed difficulties with its detection by standard western blotting techniques. Adding to the difficulties with its detection is that the protein has low expression levels in the cell line employed, the neuroblastoma cell line (N2a). Past studies on this protein within the N2a cell line have generally overexpressed the protein by transfection to enable detection by standard western blotting procedures [13]. Our work required its detection without transfection. As a consequence, various protocols were employed to detect X11α by western blot, including the short heat transfer technique of Kurien and Scolfield [9], but these failed to enable reproducible detection of the protein. Nonetheless, adaptation of the protocol of Kurien and Scolfield [9] and alteration of the western blotting apparatus facilitated effective and reproducible detection of the low levels of endogenous X11α in N2a cells. In this work we describe a technique based on heat transfer that

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can be adapted to improve detection of other high-molecular-weight proteins.

Materials and methods

Reagents and antibodies

Protran 0.45 μm nitrocellulose membrane was from Fisher Scientific. The primary antibody used for X11α detection was a rabbit polyclonal IgG (H-256) obtained from Santa Cruz Biotechnology Inc. The secondary HRP-conjugated Goat Anti-Rabbit IgG antibody was from Millipore. Rat cerebrum lysate was from BD Biosciences. Broad range prestained molecular weight markers were from New England Biolabs. All other reagents were supplied by Sigma–Aldrich.

Cell culture

N2a cells were grown in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% (v/v) fetal calf serum and 10 mM penicillin–streptomycin. The neuroblastoma cell line over-expressing the normal prion protein (PrP^C) N2a22L20Cr, which was reported previously [14], was grown in normal N2a media containing 300 μg/ml geneticin. Cells were lysed in cold RIPA-SDS lysis buffer (LB) (0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 1% (v/v) NP40, 0.1% (w/v) SDS, 50 mM Tris–HCl pH 6.8, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 2 mM EDTA). The total protein concentration was measured using the bicinchoninic acid protein assay kit (BCA assay) (Sigma). All samples were made up to 20 μl in loading dye (0.06 M Tris–HCl pH 6.8, 2% (w/v) SDS, 5% (w/v), 100 mM DTT, 10% (v/v) glycerol, 0.12 mg/ml bromophenol blue), heated to 100 °C for 5 min, and analyzed by 8% SDS-PAGE. Following electrophoresis, western blotting was carried out as indicated in the text.

Standard western blotting methods and conditions

Standard western blotting was carried out using the buffers indicated. Western transfers were carried out at room temperature (RT), with ice blocks for cooling per manufacturer instruction, at 400 mA for 1 h onto nitrocellulose membrane as indicated. Protein was detected by incubating immunoblots with the antibodies indicated followed by a horseradish peroxidase (HRP) secondary antibody and developed by enhanced chemiluminescence (ECL).

Long heat transfer western blotting method

Kurien and Schofield [9] advised the use of a western module from Hoeffer Scientific Instruments that could circulate heated water around the bottom of the tank; our adapted technique identified that stronger control of temperature was needed. For this we adapted a standard western blotting apparatus (Fig. 1). An electric mixer was attached to the base of the transfer apparatus lid to allow adequate heat transfer in the transfer buffer. A port was generated for a thermometer to monitor the temperature inside the tank during the transfer process. The tank was placed inside a temperature-controlled water bath set to 78 °C for the duration of the experiment and water within the water bath was constantly circulated. Heat transfer was run using a heat transfer buffer (HTB) (25 mM Tris, 192 mM glycine).

The following conditions are required for the long heat transfer western blotting method. In preparation and before carrying out gel electrophoresis, a water bath was set to 78 °C, and the HTB was then brought to 78 °C in the same water bath. The western sponges and filter paper were soaked in 1 L of HTB but at RT. The nitrocellulose membrane was activated by immersion in cold HTB containing 20% methanol for 1 h. After SDS-PAGE electrophoresis, the gel (0.75 mm width) was removed carefully and immersed in HTB at RT for 10 min. This reduced swelling during transfer and distortion of the gel. For thicker or higher-percentage-acrylamide gels, equilibration time was extended. The transfer cassettes with the sponges, filter papers, membrane, and gel were assembled together at room temperature. The HTB, preheated to 78 °C, was poured into the transfer tank and the cassette was immersed gently. The transfer was run at a constant 400 mA for 1.5 h with constant stirring, achieved by the mechanical mixer (Fig. 1). During transfer the temperature of the water bath was periodically checked, as was the temperature maintained inside the tank. The temperature was maintained at 78 °C without stopping transfer. Using a current over 400 mA will cause the temperature inside the tank to rise steadily as excess heat is generated; using a constant current of 400 mA should allow the temperature to remain constant throughout the run. At the end of the transfer, protein was detected, per the standard western blotting procedure.

Results

Detection of X11α by the standard western blotting procedure

During our work on X11α, it was noted that standard western blotting techniques did not allow the detection of endogenous X11α in the N2a cell line irrespective of varying buffer conditions (Fig. 2 A, B, and C). For this work we employed two cell lines, the N2a cell line and a transfected cell line, N2a22L20Cr. These cell lines were employed in a recent study [14] to examine the effect of the normal prion protein (PrP^C) on the expression of X11α. The N2a22L20Cr cell line stably overexpresses PrP^C, resulting in increased expression of X11α in the N2a cell line, which we could detect by standard western blotting, but full-length X11α could not be detected easily in N2a cells. As a result, we hypothesized that if a sensitive western blotting protocol was available then endogenous X11α in the N2a cell line would be possible.

X11α is a 120 kDa protein that is detectable in rat lysate by standard western blotting (Fig. 2, lane 1). In the N2a cell line X11α can be detected as two bands [14], and its full-length size is 120 kDa. The different banding seen with X11α has been proposed to be due to differences in phosphorylation [15] or to protein fragmentation [16]. To establish conditions for transfer, a number of buffers were tested for their ability to transfer the protein out of the gel. The most commonly used buffer in western blotting is the Tris–glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol) first used by Towbin et al. [17]. Using this original buffer, only slight levels of full-length X11α were observed in the N2a22L20Cr cell line (Fig. 2, lane 2), while in N2a cells only faint levels of the lower band were detected (Fig. 2, lane 3). Through SiRNA knockdown of X11α, both bands as observed in lane 2 were identified as X11α (data not shown). Initial attempts to increase detection looked at modification of the standard buffer. The methanol in the buffer causes stripping of complex SDS from the protein samples [18] and is used to facilitate protein transfer. However, it can partially fix proteins into gels, reducing the efficiency of transfer of large proteins [12,19]. As a consequence, we reduced the level of methanol to 10% (Fig. 2B). Reducing the methanol increased detection of the X11α upper band in the N2a22L20Cr cell line, but failed to increase detection in N2a cells (Fig. 2B, lane 4 and 5).

The final parameter adjusted alongside methanol was SDS content, which is known to affect release of protein from acrylamide gels [20]. Bolt and Mahoney observed SDS to significantly reduce the transfer of high-molecular-weight proteins [12], especially as levels increased to 0.1%. Both methanol and SDS were removed from the transfer buffer, but this did not significantly improve detection