I. INTRODUCTION

The advent of modern molecular biology, including the development of gene array technologies, has resulted in an explosion of information concerning the specific genes activated during normal cellular development, as well as those associated with a variety of pathological conditions. These techniques have provided a highly efficient, broad-based screening approach for those specific genes involved in regulating normal cellular physiology and identifying candidate genes directly associated with the etiology of specific disease states. However, this approach provides information at the transcriptional level only and does not necessarily indicate that the gene in question is in fact translated into a protein or whether posttranslational modification of the protein occurs.

The critical importance of posttranslational modification (i.e., phosphorylation, glycosylation, sialylation) to protein function has been recognized with regard to a number of proteins involved in a variety of important disease states. For example, altered glycosylation of the β-amyloid precursor protein results in an increase in the amount of β-amyloid peptide generated and hence secreted as insoluble extracellular amyloid deposits (Georgopoulou et al., 2001; Walter et al., 2001), a pathological hallmark of Alzheimer’s disease. Abnormal phosphorylation of synapsin I has been linked to alterations in synaptic vesicle trafficking, leading to defective neurotransmission in Huntington’s disease (Lievens et al., 2002). Altered phosphorylation of the TAU protein involved in microtubule function has been linked to a number of neurodegenerative diseases, such as Alzheimer’s disease (Billingsley and Kincaid, 1997; Sanchez et al., 2001).

Aberrant sialylation of cell surface antigens has been detected in a number of different tumor cell types and has been linked to the acquisition of a neoplastic phenotype (Sell, 1990), whereas improper sialylation of sodium channels in cardiac tissue has been linked to heart failure (Ufret-Vincenty et al., 2001; Fozzard and Kyle, 2002). In addition to the limitations associated with the total lack of information provided regarding posttranslational modification of the encoded protein, gene analysis cannot provide information on the role of protein–protein interactions within the cellular milieu. For example, a combination of gene and protein analysis may indicate that a particular protein is unregulated at both mRNA and protein levels, respectively. However, the presence of a regulatory protein(s) in the cellular milieu may result in this overexpression being of no physiological importance. For example, cytokine stimulation leading to an elicited cellular response is not only associated with the action of a particular cytokine at its specific receptor, but also with a cascade of downstream signal transduction events that are under the control of a variety of negative regulatory proteins (Carra et al., 2000; Murphy et al., 2000). Such protein–protein interactions underscore the complexity of the interplay between proteins in modulating cell function and are an excellent example of the limitations of gene analysis techniques in probing “cause-and-effect” relationships between gene expression and cellular response. It is the study of the interaction among gene expression, modified transcript splicing, and posttranslational modification on protein function as it impacts cellular/tissue phenotype, which is now the modern research field of proteomics.

One approach to overcoming the limitations of genetic analysis is to insert a protein of interest into a...
cell and directly observe the effects on cell function. The direct “insertion” of a protein (in contrast to transfection of a genetic construct followed by protein expression) allows investigation of the functional role of the inserted molecule in determining cell phenotype in a fashion that also considers the role of protein–protein interactions in the cellular milieu. Insertion of individual or mixtures of purified proteins prepared in the native state also increases the possibility that any observed effects of the protein on cell phenotype/response are of true physiological relevance. As yet, such functional proteomic studies are limited by the availability of technologies that allow efficient insertion of native proteins directly into cells.

Microinjection (Bloom et al., 2003; Bubb et al., 2003), electroporation (Chow and Gawler, 1999; Ponsaerts et al., 2002), and lipid vesicle-mediated protein loading (Chen et al., 1993) all have been demonstrated to achieve protein insertion into cultured cells. Mechanical-based loading techniques such as biolistics (Maddelein et al., 2002), syringe loading (Clarke and McNeil, 1992), and scrape loading (McNeil et al., 1989) can also be used to load a variety of cell types with a range of proteins. However, apart from microinjection, which requires expensive equipment and skilled personnel to produce relatively few loaded cells, none of the aforementioned techniques have wide applicability to loading of either individual or mixtures of purified proteins into adherent primary cells in tissue culture. This article describes a technique, known as impact-mediated loading, that is capable of simultaneously loading a large number of adherent primary cells (>10,000 cells during a single procedure) with a variety of proteins at high efficiency. This approach is based on the production of transient plasma membrane wounds by particle impact with the cell membrane. We have used this technique previously in order to load dyes, antibodies, and plasmid constructs into the cytoplasm of a number of primary and established cell lines utilizing a crude, relatively uncontrolled experimental apparatus (Clarke et al., 1994). In addition, we have used a more refined version of this technique to study the effects of altered gravitational conditions on the membrane-wounding response of human primary myoblasts (Clarke et al., 2002). Based on observations made during these latter studies, we have developed a novel technology, known as the G-Loader (Fig. 1), which utilizes the effects of hypergravity at 200 g to enhance macromolecular loading into the cytoplasm of adherent cells via impact-mediated plasma membrane wounding (Clarke et al., 2001). The protocol described here specifically details the experimental approach used to load primary human skeletal myocytes with FITC-labeled IgG immunoglobulin as an example of the suitability of G-Loader technology for loading biologically active proteins into primary adherent cells.

II. MATERIALS AND INSTRUMENTATION

Dulbecco’s modified Eagle’s medium–F12 medium (X1 concentration) (DMEM/F12, Cat. No. 320-1885AG), bovine fetal calf serum (CS) (Cat. No. 200-6170AG), and penicillin–streptomycin solution (Cat. No. 600-5140AG) are obtained ready to use from Gibco BRL (Grand Island, NY). Alexa Fluor 488-labeled goat antimouse IgG (M, 150,000 kDa) (Cat. No. A-11001) is obtained from Molecular Probes (Eugene, OR). Tissue culture flasks (T-75, T25, and 35-mm diameter plates) (Cat. Nos. 10-126-41, 10-126-26, and 25050-35), and sterile polypropylene conical centrifuge tubes (50 ml capacity) (Cat. No. 05-538-55A) are from Fisher Scientific (Pittsburgh, PA). The G-Loader technology and associated consumables are from Peilier Technologies (Houston, TX). Living cells loaded with fluorescently labeled IgG are examined utilizing an Zeiss Axiophot inverted microscope in order to view them in the 35-mm tissue culture plate directly.

III. PROCEDURES

A. Preparation of Tissue-Cultured Cells for Impact-Mediated Loading Using the G-Loader

Stock Solutions and Media Preparation

1. Stock IgG solution: Stock Alexa Fluor 488 goat antimouse IgG (2 mg/ml) can be stored at 4°C in the dark for up to a month or aliquoted and frozen in the dark at ~80°C for storage up to a year. Prior to use on living cells, it is important to remove any preservative (e.g., sodium azide) from the immunoglobulin solution, as exposure of living cells to such contaminants can result in rapid cell death. Removal of such preservatives can be carried out using either a miniature dialysis device or buffer exchange using a 30,000 MW cutoff centrifugal miniature concentrator. One milliliter of stock IgG (2 mg/ml) solution should be dialyzed against a minimum of three changes of 100 ml of sterile DMEM/F12 medium over a period of 24 h prior to use. The total volume of dialyzed IgG solution should be adjusted to original volume prior to dialysis to maintain the overall IgG concentration. Buffer exchange using a centrifugal concentrator should
involve a minimum of three complete exchanges of the original volume of stock IgG solution with serum-free DMEM/F12 medium.

2. **IgG loading solution**: Add 125 μl of stock IgG solution to 0.875 ml of serum-free DMEM/F12 to obtain final concentrations of 250 μg IgG/ml. Equilibrate this loading solution to the correct temperature and pH by incubating in a standard 5% CO2 tissue culture incubator maintained at 37°C for a period of 30 min prior to loading cells with the IgG.

3. **10% FCS.DMEM/F12 tissue culture medium**: Add 5 ml of sterile penicillin/streptomycin solution and 50 ml of sterile fetal to 445 ml of sterile (X1) DMEM/F12 solution to obtain DMEM/F12 culture medium containing 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin (10% FCS.DMEM). Store at 4°C for up to 21 days.

**B. Culture of Human Primary Myoblasts**

1. Obtain primary human myoblasts from the Clonetics Corporation (Walkersville, MD) and culture to confluence in T-75 (75 cm²) culture flasks using 15 ml of 10% FCS.DMEM/F12 maintained at 37°C in a 5% CO2 humidified atmosphere with subculture every fourth day.

2. The day prior to the loading procedure, trypsinize, collect by centrifugation, and resuspend cells in 5 ml of 10% FCS.DMEM/F12 as a single cell suspension. Determine the number of cells in the suspension using a hemacytometer and adjust cell density in the solution to 50,000 cells/ml using 10% FCS.DMEM/F12. Place 2 ml of this cell suspension into a 35-mm tissue culture plate and incubate cells overnight to allow formation of a monolayer. Under

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**FIGURE 1** Impact-mediated loading apparatus known as the G-Loader. The G-Loader device itself is approximately 25 cm tall and 10 cm in diameter. A 35-mm tissue culture plate containing a monolayer of adherent cells is placed in the base of the G-Loader after being incubated with loading medium containing the macromolecule of interest. Prior to placing the cell sample into the G-Loader, a particle cartridge is inserted into the device consisting of a circular cartridge that supports a rupturable membrane on which is located a layer of 10-μm particles arranged in a specific pattern (see insert). In addition, the device has been charged with air to the required pressure and the on-board g-load accelerometer trigger has been set to the required g value using the G-Loader charging station (for benchtop operation there is a manual trigger button located on the side wall of the device). When the G-Loader is activated, either manually or by reaching a set g load, a metered volume of pressurized air is directed into the particle cartridge and the membrane ruptures and disperses particles into the airstream. The particles then impact the cell layer, inducing membrane wounding in a highly controllable and reproducible fashion.
these culture conditions the center of the 35-mm plate is between 60 and 80% confluent after 24 h of culture prior to loading.

3. Prior to loading, wash cells three times over a period of 5 min with warm, serum-free DMEM/F12 containing no antibiotics to remove any unattached cells and to wash away serum components that may interact with the IgG molecules being loaded.

C. Impact-Mediated Loading of IgG Using G-Loader Technology

1. Remove serum-free medium from the cell monolayer, replace with 400 µl of loading solution, and agitate the 35-mm plate gently so that the loading solution covers all of the culture surface. Agitate the loading solution for a minimum of 30 s to ensure that the dissolved macromolecule is in contact with the surface of the cells.

2. Remove the loading solution from the 35-mm plate by tilting the plate to one side and removing the loading solution with a 1-ml sterile pipette. Note: The loading solution can be reused on up to five additional 35-mm plates if used immediately after first centrifuging at 10,000 g for 1 min to remove any particulate/cellular material.

3. The cells are now ready to be loaded using the G-Loader technology. Place the 35-mm plate in the G-Loader and operate the firing mechanism in the tissue culture hood. Alternatively, the device and cells can be placed into the centrifuge and loading performed under hypergravity conditions (i.e., 200 g) (Fig. 1).

In the case of human primary skeletal myoblasts and IgG loading, highly efficient cytoplasmic loading of IgG can be achieved without the need for the use of hypergravity conditions during impact-mediated loading.

4. Remove the 35-mm plate from the G-Loader and immediately place 2 ml of warm serum-free DMEM/F12 medium (without antibiotics) into the 35-mm plate to prevent drying of the cells. Gently wash the cell layer three times over 2 min with three changes of serum-free DMEM/F12 medium to remove both excess IgG and particles impacted with the cell surface.

5. Replace washing medium with 10% FCS.DME/F12 tissue culture medium and incubate for the required time period. After this time (0–24 h depending on the goal of the project), wash once with warm D-PBS (pH 7.2), replace with fresh D-PBS, and view the living cell monolayer by inverted fluorescent microscopy using UV excitation and emission at 488 nm.

IV. COMMENTS

This technology provides a highly efficient means of loading large macromolecules into living adherent cells with little or no disruption of the adherent monolayer. As can be seen in Fig. 2, there is little or no visually discernible damage to the cell monolayer 1 h after impact-mediated cytoplasmic loading of a 2 × 10^6-Da-sized fluorescent dextran. The protocol detailed here describes the procedure required to load fluorescently labeled IgG into cultured primary skeletal muscle cells (Fig. 3). In addition, this technique has been applied to a large number of different cell types and a wide variety of different macromolecules, including

![FIGURE 2 Paired phase-contrast and FITC-fluorescent micrographs of a Swiss 3T3 fibroblast monolayer 1 h after impact-mediated cytoplasmic loading of 2 × 10^6-Da fluorescent dextran (FDx). The monolayer was washed three times with warm serum-free DMEM to remove excess FDx (200 kDa) and to remove the majority of the particles (arrows). Note the clear exclusion of the 200-kDa dextran from the nuclear region of the cells (arrowheads) and little or no visual damage to the cell monolayer (left). Complete bead removal can be achieved by washing the monolayer multiple times with medium over a period of 4 h.](image-url)
plasmid DNA constructs as a means of transfecting both primary and established cell lines (Fig. 4). Furthermore, this technique has the ability to simultaneously load two or more macromolecules directly into the cell cytoplasm of living cells, which are then localized to specific cellular compartments within the living cell based on size exclusion (Fig. 5). In addition, the use of hypergravity conditions during impact-mediated loading significantly increases the amount of fluorescently labeled IgG protein loaded into the cell cytoplasm (Fig. 6).

V. PITFALLS

The impact-mediated loading technology described here (i.e., The G-Loader) is a highly efficient means of simultaneously loading large numbers of living cells with large macromolecules. Under the conditions required for loading of macromolecules below $1 \times 10^6$ Da in size, the number of cells that are wounded, loaded, and survive the procedure is extremely high (approximately 70–80% of the starting population) (Fig. 1). One of the disadvantages of the technique described in this article for loading IgG is that not all cells in the 35-mm tissue culture dish are loaded. This is due to a limited particle impact area (approximately one-half of the culture area radiating from the center of the plate) generated by the device. This drawback can be overcome by growing cells only in the center region of the 35-mm plates utilizing a 20-mm-diameter cloning ring or plating cells on 20-mm-diameter glass coverslips located in the center of the 35-mm plates and removing the coverslips after loading. This approach yields a cell population in

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**FIGURE 3** Fluorescent micrographs of (A) control and (B) Alexa Fluor 488 goat antimouse IgG-loaded primary human myoblasts. Control monolayers are treated in an identical fashion to loaded cells (including incubation with IgG loading solution) except that they are not subjected to impact-mediated loading. Micrographs were photographed under identical conditions immediately after loading and washing of monolayers. Note the somewhat “perturbed” appearance of the cells, an appearance that disappears after 20 to 60 min of additional culture in serum-containing medium (see Fig. 6).

**FIGURE 4** Fluorescent micrographs of primary human skeletal myoblasts (HSKMC), Swiss 3T3 cells (3T3), and primary bovine capillary endothelial cells (BCEC) 24 h after impact-mediated loading of a plasmid construct encoding for a green fluorescent protein, Lantern Green (pLG).
which approximately 70–80% of the starting population is loaded. However, in those experiments where direct localization by microscopic techniques is utilized, the presence of unloaded cells in the region closest to the edge of the plate serves as an excellent internal negative control.

A second area of concern is the purity of the macromolecule that is being loaded. In initial experiments using commercially produced, fluorescently labeled IgG, IgM, and plant lectins, impact-mediated loading was observed at high efficiencies immediately after loading as determined by direct inspection of the cell layer by fluorescent microscopy. Subsequently, however, the majority of the cells died within 2 h of loading. After investigation, it was found that most commercially produced products had some form of preservative in the storage buffer that needed to be removed prior to contact with living cells during the loading procedure.

FIGURE 5 Matched fluorescent micrographs of NGF-differentiated PC-12 cells 20 min after impact-mediated loading of a mixture of fluorescently labeled dextrans. Two different-sized, different-colored dextran molecules (green, FDx–10 kDa; red, TRITC–30 kDa) were loaded simultaneously into monolayers of PC-12 cells using impact-mediated loading. Note exclusion of the larger dextran (red signal) from the nucleus of the cells (B), whereas the smaller dextran (green signal) is found in both the cytoplasm and the nucleus of the loaded cells (A). When a digital overlay of the images is generated, cells are colored predominantly orange in their cytoplasm (i.e., mixed red and green signal), whereas cell nuclei are green (C).

FIGURE 6 Fluorescent micrographs of Swiss 3T3 cell monolayers loaded cytoplasmically with Fluor 488 goat antimouse IgG employing impact-mediated loading at 1 g (B) and (C) 200 g in a benchtop centrifuge. Control cells (A) were exposed to IgG for the same period of time but were not impact loaded. Micrographs were taken at the same photographic conditions. Note the larger amount of IgG present in the cytoplasm of cells loaded at 200 g as compared to those loaded at 1 g and that IgG is excluded from the nuclear regions of the loaded cells.

The G-Loader technology utilizes a combination of particle impact-induced membrane wounding and hypergravity conditions to load macromolecules directly into the cell cytoplasm. This technology appears to have utility for loading in a wide range of cell types, both established cell lines (e.g., COS, 3T3, C2C12 cells) and primary cell types (e.g., human myoblasts, bovine endothelial cells, chick corneal fibroblasts, rat gut epithelium), as well as multicellular microorganisms (e.g., hydra, nematodes, *Xenopus* ova). However, in order to achieve optimal loading efficiency, a mechanistic approach to determining impact pressure, macromolecular concentration in the loading solution, and whether or not hypergravity conditions are required for efficient loading is suggested. In addition, the nature of the macromolecule being loaded is also important. Those compounds that are highly charged or may interact with cell surface components may need longer contact with the
cells prior to loading, a higher loading solution concentration, or higher g load (i.e., 200 g) (Fig. 5) in order to achieve the most efficient cytoplasmic loading possible.

References


