Expression Profiling of Lymphocyte Plasma Membrane Proteins* [S]

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The physicochemical properties of plasma membrane proteins of mammalian cells render them refractory to systematic analysis by two-dimensional electrophoresis. We have therefore used in vivo cell surface labeling with a water-soluble biotinylation reagent, followed by cell lysis and membrane purification, prior to affinity capture of biotinylated proteins. Purified membrane proteins were then separated by solution-phase isoelectric focusing and SDS-PAGE and identified by high-pressure liquid chromatography/tandem mass spectrometry. Using this approach, we identified 42 plasma membrane proteins from a murine T cell hybridoma and 46 from unfractionated primary murine splenocytes. These included three unexpected proteins; nicasin, osteoclast inhibitory lectin, and a transmembrane domain-containing hypothetical protein of 11.4 kDa. Following stimulation of murine splenocytes with phorbol ester and calcium ionophore, we observed differences in expression of CD69, major histocompatibility complex class II molecules, the glucocorticoid-induced TNF receptor family-related gene product, and surface immunoglobulin M and D that were subsequently confirmed by Western blot or flow cytometric analysis. This approach offers a generic and powerful strategy for investigating differential expression of surface proteins in many cell types under varying environmental and pathophysiological conditions. Molecular & Cellular Proteomics 3:56–65, 2004.

The plasma membrane (PM) 1 constitutes the interface between eukaryotic cells and their external environment. Consequently, the functions of proteins embedded in this membrane include cell/cell and cell/extracellular matrix recognition, the reception and transduction of extracellular signals, and the transport of solutes and water molecules into and out of the cell. Abnormal PM protein expression has profound biological effects and may, for example, underlie phenotypic and functional differences between normal and tumor cells (1). For these reasons, profiling of PM protein expression is an area of intense interest (2).

Although about 25% of open reading frames in fully sequenced genomes are estimated to encode integral membrane proteins (3), global analysis of membrane protein expression has proved problematic (4). Two-dimensional gel electrophoresis (2DE) can resolve over a thousand proteins on a single large format gel, but PM proteins are often severely underrepresented in 2DE protein patterns because of their large size, low abundance, and hydrophobicity (5). Although the introduction of improved chaotropes such as thiourea (6) and the development of novel zwitterionic detergents (6–8) has improved matters, particularly for prokaryotic organisms, the detection of integral membrane proteins from higher eukaryotes by 2DE remains unsatisfactory (9).

One strategy to increase the representation of membrane proteins on two-dimensional gels would be to pre-enrich the membrane proteins prior to electrophoresis. Several groups have attempted this using the established technique of cell surface biotinylation (10) and affinity capture with avidin (11–13). However, because the approach does little to address the intrinsic limitations of 2DE, it is unsurprising that the number of membrane proteins characterized remains disappointing; 18 in a recent study of the gastric pathogen Helicobacter pylori (11) and 14 in an investigation of human tumor cell lines (13), which, in the latter study, were accompanied by a large number of heat shock proteins, not normally thought of as PM proteins.

An alternative approach is to omit the isoelectric focusing (IEF) step and apply an enriched membrane protein fraction directly to SDS-PAGE, thus overcoming the solubility and transfer problems intrinsic to 2DE (14, 15). Because the high resolution of 2DE is sacrificed, most bands on the gel contain multiple co-migrating proteins, but the ability of high-pressure liquid chromatography; PM, plasma membrane; 2DE, two-dimensional gel electrophoresis; MS/MS, tandem mass spectrometry; ICAT, isotopic affinity tag; IEF, isoelectric focusing; SA, streptavidin; sulfo-NHS-LC-biotin, sulfo-NHS-LC-biotin; sulfo-NHS-SB-biotin, sulfo-NHS-SB-biotin; GAVY, grand average of hydropathy; GRP78, glucose-regulated proteins; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; LAT, linker for activation of T cells; 1DE, one-dimensional gel electrophoresis; IPG, immobilized pH gradient; HPLC, high-pressure liquid chromatography; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase; Ig, immunoglobulin; MHC, major histocompatibility complex.

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1 The abbreviations used are: PM, plasma membrane; 2DE, two-dimensional gel electrophoresis; MS/MS, tandem mass spectrometry; ICAT, isotope coded affinity tag; IEF, iso-electric focusing; SA, streptavidin; sulfo-NHS-LC-biotin, sulfo-NHS-LC-biotin; sulfo-NHS-SB-biotin, sulfo-NHS-SB-biotin; GAVY, grand average of hydropathy; GRP78, glucose-regulated proteins; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; LAT, linker for activation of T cells; 1DE, one-dimensional gel electrophoresis; IPG, immobilized pH gradient; HPLC, high-pressure liquid chromatography; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase; Ig, immunoglobulin; MHC, major histocompatibility complex.

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liquid chromatography (HPLC) tandem mass spectroscopy (MS/MS) to analyze complex mixtures of peptides enables the use of a conventional strategy of tryptic digestion and mass spectrometric identification. A recent study using this approach identified over 500 proteins in breast cancer cell membranes, including several putative tumor-specific markers (14).

“Shotgun” methods take this approach a stage further, and proteolytically digest the sample without prior electrophoretic separation. The resulting mixture of thousands of peptides are then separated by sequential strong cation exchange and reverse-phase chromatography before identification by HPLC MS/MS (16, 17). This approach offers considerable advantages for membrane protein characterization because (provided efficient digestion can be effected) separation is performed at the peptide rather than protein level, so most of the solubility problems associated with hydrophobic proteins are obviated.

When used in conjunction with the isotope-coded affinity tagging (ICAT) strategy (18), it is possible to determine quantitative protein expression profiles. Thus, Aebersold and colleagues identified 491 proteins in a microsomal membrane fraction of HL-60 cells. Of these, 55 (11.2%) were known cell surface antigens, receptors, and membrane proteins and 24 (4.9%) were channel proteins. Furthermore, the expression of eight PM proteins differed more than 2-fold between phorbol ester-treated cells (19).

The disadvantage of most shotgun strategies is that computationally intensive analysis of the entire dataset is always required, even when only a few proteins display changes in expression levels. Furthermore, no information on the charge or apparent mass of the intact proteins is obtained, and all connectivity between parent proteins and their peptide digestion products is lost, which hinders characterization of post-translational modifications.

Multidimensional chromatography is therefore best regarded as a complement rather than a replacement for gel-based proteomics, because it appears that there are substantial subsets of cellular proteins that remain undetected unless both methodologies are applied in tandem. For example, in a recent comprehensive analysis of the proteome of Oryza sativa (rice), 47% of the leaf proteins identified from two-dimensional gels were not detected by multidimensional chromatography, while a substantial number of proteins identified by the latter technique were not observed on the gels (20).

In the present report, we describe an improved cell-surface biotinylation/streptavidin (SA) affinity capture methodology for profiling membrane proteins. The resulting mixture of surface-derived proteins can in principle be analyzed either by shotgun or conventional methods. However, we adopted a gel-based approach because this technology is available to the majority of the proteomics community and is more readily integrated into biochemical purification strategies. We have circumvented the major limitations of conventional 2DE by adopting solution-phase IEF and one-dimensional gradient SDS-PAGE prior to trypsinolysis and HPLC MS/MS of recovered proteins. With this approach, we have identified 74 PM proteins from a T cell clone and primary murine splenocytes. Of these, 12 appeared to be differentially expressed by cells activated with phorbol ester and ionomycin. Six of these putative activation-induced changes were selected for further study and independently verified by immunoblotting or flow cytometry. This technique should provide a useful complement to existing proteomic approaches for cataloguing and comparing PM protein profiles in diverse cell types.

EXPERIMENTAL PROCEDURES

Cell Culture—The mouse T cell hybridoma clone 11A2 was derived and propagated in RPMI 1640 containing 10 mm glutamine and 25 mm Hepes (Biowhittaker, Wokingham, UK) further supplemented with: 10% fetal calf serum (Labtech, Ringmer, UK); 10 mm sodium pyruvate (Sigma, Poole, UK); 10 U/ml penicillin/streptomycin (Biowhittaker), and 10 mm β-mercaptoethanol (Life Technologies, Inc., Paisley, UK) as previously described (21).

Cell Isolation—Splenocytes were derived from 8–12-week-old male or female DBA1/J H-2d mice. Single-cell suspensions were prepared by standard techniques prior to culture at a density of 10⁷ cells/ml in complete medium in the presence or absence of phorbol 12-myristate 13-acetate (PMA) (30 ng/ml) and ionomycin (30 ng/ml), both purchased from Sigma.

Cell Surface Biotinylation—Cells were washed three times and resuspended (2·5 × 10⁷/ml) in BBS (10 mm boric acid, 2.3 mm sodium tetraborate, 115 mm NaCl, pH 8.1) in which biotinylation of lysines proceeds more efficiently than in phosphate-buffered saline (PBS) (22). Cells were biotinylated (20 min, 4 °C) using 0.1 mg/ml sulfo-succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin) or, on occasion, sulfo-succinimidyl-6-(biotinamido)hexanoate (sulfo-NHS-LC-biotin; Perbio, Tattenhall, UK), and then washed a further three times in BBS.

Preparation of a PM-enriched Membrane Fraction—Biotinylated cells were lysed in hypotonic lysis buffer (20 mm Tris-HCl, pH 7.5, 5 mm EDTA, 1 μl/10⁶ cells protease inhibitor mixture) and subjected to 30 strokes of a tight fitting Dounce homogenizer. A PM-enriched fraction was obtained from the hypotonic lysate according to the method of Johnstone and Crumpton (23), with minor modifications. Briefly, the Dounce homogenate was clarified by centrifugation (4,000 × g, 15 min) to generate a postnuclear supernatant (PNS). The PNS was centrifuged again (20,000 × g, 30 min) to yield a PM-enriched 20,000 × g pellet (p20). Recovery of PM in the p20 was between 50 and 80% as judged by the presence of the PM protein linker for activation of T cells (LAT) or CD45 (data not shown). Aliquots of the supernatant of the 20,000 × g spin (s20) and other cell fractions were retained for protein analysis (see below). The p20 fraction was solubilized (30 min) in hypotonic lysis buffer supplemented with 150 mm NaCl and 1% v/v Triton X-100. The choice of Triton X-100 on economic grounds did not preclude the detection by Western blot of the lipid raft markers, Thy1 (Fig. 2A) and LAT (data not shown) in the detergent. The mouse T cell hybridoma clone 11A2 was derived and propagated in RPMI 1640 containing 10 mm glutamine and 25 mm Hepes (Biowhittaker, Wokingham, UK) further supplemented with: 10% fetal calf serum (Labtech, Ringmer, UK); 10 mm sodium pyruvate (Sigma, Poole, UK); 10 U/ml penicillin/streptomycin (Biowhittaker), and 10 mm β-mercaptoethanol (Life Technologies, Inc., Paisley, UK) as previously described (21).
Protein-MS Analysis of Plasma Membrane Proteins

sample buffer (125 mM Tris-HCl, pH 6.8, 0.025% w/v bromphenol blue, 4% w/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol). All procedures were performed at 4 °C unless otherwise stated. Cell fractions were assayed for protein content prior to one-dimensional PAGE and Western blotting using a bicinchoninic acid assay kit (Pierbio).

Solution-phase IEF—Aliquots (50 μl of 1–2 ml) of affinity-purified PM preparations were retained for analysis by one-dimensional gel electrophoresis (1DE). The remainder was subjected to solution-phase IEF in a Rotofor cell (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer’s instructions using a gradient of pH 3–10. The buffer used was extraction buffer supplemented with 1% pH 3–10 ampholytes (Bio-Rad). Twenty fractions (~2 ml each) were collected under vacuum and concentrated through 10-kDa molecular mass filter tubes (Vivascience, Hanover, Germany). Samples were further concentrated using an SDS-PAGE “clean-up” kit (Amersham Biosciences, Little Chalfont, UK; catalogue no. 80-6484-70) comprising an acid precipitation step and organic solvent wash in conjunction with proprietary reagents. Solubilized proteins were boiled in SDS-PAGE sample buffer containing 5% β-mercaptoethanol and separated over laboratory-cast gradient gels (4–12% acrylamide) and silver stained as described by Shevchenko et al. (24). Comparative one-dimensional analyses of precipitated and unprecipitated samples demonstrated greater than 90% protein recovery (data not shown).

Two-dimensional PAGE—Protein samples were dissolved in a two-dimensional sample buffer (7 M urea, 2 M thiourea, 1% w/v am-photolytes, pH 3–10) (all Amersham Biosciences); 1% w/v ASB-14 (Cal-Biochem, Nottingham, UK); 1% w/v DTT, 10 mM Tris-HCl, pH 7.5) utilized by Molloy et al. (25) for analysis of bacterial outer membranes. The chaotropes (urea and thiourea) and novel aminosulphobetaine twizzerionic detergent (ASB-14) in this buffer have been reported to enhance solubilisation of membrane proteins (6, 7). Solubilized proteins were loaded into 18-cm linear pH 3–10 immobilized pH gradient (IPG) strips by in-gel rehydration (overnight, room temperature). Strips were subjected to IEF (70,000 Volt hours) in a Multiphor II unit and resolved in the second dimension with 12% acrylamide) and 10% Tris/glycine polyacrylamide gels using a Hoeffer DALT horizontal gel apparatus (Amersham Biosciences), originally described by Anderson and Anderson (46).

Mass Spectrometry—Tandem electrospray mass spectra were re-corded using a quadrupole time-of-flight hybrid quadrupole/orhog-onal acceleration time-of-flight spectrometer (Micromass, Manchester, UK) interfaced to a Micromass CapLC capillary chromatograph. Samples were dissolved in 0.1% aqueous formic acid, and 6 μl was injected onto a Pepmap C18 column (300 μm × 0.5 cm; LC Packings, Amsterdam, NL), and washed for 3 min with 0.1% aqueous formic acid (with the stream select valve diverting the column effluent to waste). The flow rate was then reduced to 1 μl/min, the stream select valve was switched to the data acquisition position, and the peptides were eluted into the mass spectrometer with an acetonitrile/0.1% formic acid gradient (5–70% acetonitrile over 20 min).

The capillary voltage was set to 3,500 V, and data-dependant MS/MS acquisitions were performed on precursors with charge states of 2, 3, or 4 over a survey mass range 540–1200. Known trypsin autolysis products and keratin-derived precursor ions were automatically excluded. The collision voltage was varied between 18 and 45 V depending on the charge and mass of the precursor. Product ion spectra were charge-state de-encrypted and de-isolated with a maximum entropy algorithm (MaxEnt; Micromass). Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TREMBL using ProteinLynx Global Server (versions 1 and 1.1; Micromass). One missed cleavage per peptide was allowed, and the fragment ion tolerance was set to 100 ppm. Carbamidomethylation of cysteine was assumed, but other potential modifications were not considered in the first pass search. All matching spectra were reviewed manually, but in cases where the score reported by ProteinLynx global server was less than 100, additional searches were performed against the NCBI nonredundant database using Mascot, which utilizes a robust probabilistic scoring algorithm (47). Identifications based on a single matching peptide were verified manually using the MassLynx program Pepseq (Micromass).

Western blotting—Matched amounts of protein from cell fractions of resting or activated cells (5–20 μg/lane) were separated over 4–12% Bis/Tris pre-cast gels (Invitrogen, Paisley, UK) and transferred to nitrocellulose membranes. Membranes were blocked (overnight, 4 °C) in Tween/Tris-buffered saline (TTBS; 0.05% v/v Tween-20, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl) supplemented with 4% bovine serum albumin (TTBS-BSA). Membranes were probed with appropriate primary antibodies (1 h, room temperature) diluted in TTBS-BSA. Membranes were washed (4 × 15 min) in TTBS and incubated (1 h, room temperature) with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies in TTBS-BSA. Membranes were washed (4 × 15 min in TTBS) and proteins detected by enhanced chemiluminescence.

Two-color Fluorescence-activated Cell Sorting (FACS)—Cells were washed twice in FACS buffer (10 mM PBS, pH 7.2, 2% w/v BSA, 0.05% w/v sodium azide) and incubated in the same buffer supplemented with mouse immunoglobulin (lg) G (100 μg/ml, 1 h, 4 °C). Cells were then incubated (1h, 4 °C, 1/1000 dilution) with phycocerythin-conjugated monoclonal antibodies to B220 or CD3 alone or in combination with biotinylated antibodies (1/100) to either major histocompatibility complex (MHC) class II-(I-a^+), CD45, or CD69 (all from BD Biosciences, Cowley, UK) or a control antibody, biotinylated mouse IgG. Cells were then washed and resuspended in SA-conjugated fluorescein isothiocyanate (1/1000) for 1 h at 4 °C, then fixed in 2% formaldehyde in 10 mM PBS, pH 7.2. Cells were acquired using a Becton Dickinson LSR benchtop flow cytometer and data analyzed using CellQuest software (BD Biosciences).

Antibodies—A rabbit polyclonal antibody to murine osteoclast inhibitory lectin (OCIL) was a generous gift from Dr. Matthew Gillespie (St. Vincents Institute of Medical Research, University of Melbourne, Australia). A rabbit anti-nicarastin antibody was purchased from Chemicon International (Harrow, UK). A polyclonal goat antibody to the product of the murine glucocorticoid-induced tumor necrosis factor receptor-related gene (GITR) was purchased from R&D Systems (Abingdon, UK). Mouse monoclonal and rabbit polyclonal antibodies to glucose-regulated protein of 78 kDa (GRP78) were ob-tained from Bioquote Ltd. (York, UK). A rat monoclonal antibody to Thy1.2 was prepared in-house from a hybridoma generated in the laboratory of Dr. H. Waldmann (26). A rabbit polyclonal anti-LAT (UBI, Milton Keynes, UK) and a goat polyclonal antibody to CD45 (Santa Cruz Biotechnology Inc., Wiltshire, UK) were also used on occasion. HRP-conjugated secondary antibodies to mouse, rabbit, goat, and rat immunoglobulins were obtained from DAKO (Ely, UK).

Confocal Microscopy—Hybridoma cells were biotinylated as de-scribed above or left unlabelled. Cells were washed twice (BBS) and once in PBS and allowed to adhere to quadruplicate wells of a glass adhesion slide (Bio-Rad). Adherent cells were fixed in 3% formalde-hyde in PBS (15 min, room temperature), rinsed in PBS, and, where indicated, permeabilized in 0.1% Triton X-100 in PBS (30 min, 4 °C). Cells were rinsed in PBS and blocked (10 mg/ml BSA in PBS, 30 min, 4 °C), then incubated with SA-Alexa (1/1000 in PBS/BSA, 30 min, 4 °C) from Molecular Probes (Leiden, The Netherlands). Cells were rinsed in PBS and visualized using an UltraView LCI confocal micros-copy suite (Perkin Elmer, Beaconsfield, UK).
RESULTS AND DISCUSSION

Our strategy was to label with biotin solvent-exposed lysine residues on the surface proteins of intact cells (10) prior to affinity purification of tagged proteins using SA-agarose beads. To optimize the biotinylation conditions, we compared two reagents, sulfo-NHS-LC-biotin (LC-biotin), recently used in several similar studies (11–13), and a more water-soluble analogue, sulfo-NHS-SS-biotin (SS-biotin). A SA-conjugated fluorescent dye, Alexa 488 was used to visualize the subcellular distribution of labeled proteins (Fig. 1). In cells not treated with biotinylation reagent (Fig. 1, a and b), no binding of SA-Alexa was detected. In nondetergent-treated cells (Fig. 1, c and e), biotinylation was confined to the plasma membrane. However, in cells permeabilized to enable internalization of SA-Alexa, biotinylation was confined to the plasma membrane (Fig. 1, d and f). Adherent cells were permeabilized or not, as indicated, using 0.1% Triton X-100. Biotin labeling was tagged using SA-Alexa 488. Confocal and bright-field images were generated using a Perkin Elmer UltraView LCI microscopy suite. The data shown are representative of three similar experiments.

Because some intracellular biotinylation was observed even when the SS-biotin reagent was used it was not surprising that SA affinity purification recovered a significant proportion of abundant housekeeping proteins from whole-cell extracts (data not shown). Such proteins (e.g. GRP78, actin) were not detected by Western blotting if SA beads were incubated with extracts of nonbiotinylated cells, confirming that their recovery was attributable to biotin/SA interactions (data not shown). To minimize this contamination, we prepared a PM-enriched fraction (p20) from biotinylated cells by differential centrifugation. This fraction contained only 1–3% of the total protein in a PNS but more than 50% of the PM markers CD45 and LAT (~25-fold enrichment, data not shown).

Biotinylated proteins were affinity-purified from this material using SA-agarose beads, and equal amounts of protein (8 μg total protein/lane) of the various fractions were probed for biotin with SA-HRP (Fig. 2a) for the presence of PM proteins with anti-Thy-1 (Fig. 2c) and for non-PM proteins with an antibody to GRP-78, a highly abundant endoplasmic reticulum lumen-located heat shock protein (Fig. 2b). Compared with the PNS (lane 1) and p20 (lane 2) fractions, the SA affinity-purified material (lane 3) was enriched in biotinylated proteins and Thy-1 but depleted of GRP78. After affinity purification, biotinylated proteins and Thy-1 were largely absent from the p20 fraction, whereas GRP78 content was unchanged (lane 4). Some proteins in the
supernatant from the 20,000 × g spin (s20) were biotinylated, though in many cases these appeared different to those enriched in the p20 and affinity-purified fractions (e.g. bands arrowed in Fig. 2), suggesting that they may not be PM proteins. Indeed, the PM marker Thy-1 was undetectable in this fraction while GRP78 remained prominent. A similar distribution was observed using CD45 and actin as markers for PM and non-PM proteins, respectively (data not shown).

These data demonstrate enrichment of PM proteins by affinity purification, with concomitant depletion of intracellular contaminants and suggest that almost all the p20 fraction-associated Thy-1 (Fig. 2) and CD45 (data not shown) are biotinylated under these conditions and are completely recovered by affinity purification.

The observation that some intracellular proteins (Fig. 2a, lane 5), including GRP78 (Fig. 2b, lane 3), were biotinylated was consistent with the confocal microscopy results shown in Fig. 1. However, we cannot exclude the possibility that the small amount of biotinylated GRP78 detected was genuinely exposed at the cell surface as has been reported (13).

We next used 2DE and MS/MS to catalogue the proteins recovered in the affinity-purified fraction (27, 28). Equal amounts of total protein from the PM-enriched p20 fraction (5 × 10⁷ cell equivalents) or the SA affinity-purified material from the same cells (10⁹ cell equivalents) were analyzed by 2DE. In each case, all detectable spots were excised and subjected to MS/MS. In the former, 160 proteins were identified (molecular mass range 20–150 kDa) of which seven (4.4%) were PM proteins. Of the 17 proteins (molecular mass range 25–180 kDa) identified in the SA affinity-purified material, five (29.4%) were PM proteins (see supplementary data Fig. 1). In agreement with a similar recent study (13), several of the non-PM species detected were heat shock proteins (HSP70 and GRP78). One-dimensional SDS-PAGE of 20-fold fewer cell equivalents (5 × 10⁷) of the same SA affinity-purified sample led to the identification of 20 proteins (molecular mass range 24–180 kDa), of which 11 (55%) were PM proteins, some of which were not detected in either 2DE experiment (see supplementary data Fig. 1). These latter included proteins that might be expected to perform poorly in 2DE, either because of their high molecular mass (e.g. CD45) or basic pI (e.g. CD4, galectin 9). Thus, compared with 2DE, one-dimensional SDS-PAGE appeared to favor identification of PM proteins.

Most of the problems encountered in analyzing hydrophobic proteins by conventional 2DE are attributable to poor solubility during IEF (which is incompatible with ionic detergents such as SDS), resulting in precipitation and inefficient transfer from first to second dimension.

We therefore performed solution-phase IEF as the first dimension of separation using, instead of a polyacrylamide IPG strip, a self-generating solution-phase gradient of pH 3–10. Thus, affinity-purified PM proteins were focused into 20 discrete fractions, which were combined pairwise prior to SDS-PAGE on 4–12% gradient gels (Fig. 3a), after which resolved protein bands were excised and characterized by HPLC MS/MS. The proteins so identified are listed in supplementary data Table I (PM proteins) and supplementary data Table II ("non-PM" proteins). A representative tandem mass spectrum (b) of a peptide derived from CD18 is shown.

Many of the proteins identified were not detected by conventional 2DE and include high molecular mass (CD45, PTPα), hydrophobic (CD47, grand average of hydrophobicity (GRAVY) score 0.563; tetraspanin 4-related protein, GRAVY score 0.385), and basic species (galectin 9, CD4, EV12). This contrasts with the 2DE analyses of the same samples in which
these proteins were not observed even though several (CD45, CD4, galectin 9) were readily detected in one-dimensional analyses of 20-fold fewer cell equivalents of the same material (data not shown). Second, the use of solution-phase IEF amplified the capacity of one-dimensional analyses from $5 \times 10^7$ to $>2 \times 10^9$ cell equivalents of affinity-purified protein, increasing the number of PM proteins identified in 11A2 cells from 11 to 42.

The recovery of SIT, a type I membrane protein lacking extracellular lysine residues, suggests that biotinylation of the N-terminal amino group alone enables affinity capture. Thus, theoretically, any N-terminally unblocked type I PM protein could be isolated using this method.

While PM proteins constituted more than half of the total identified, we also recovered a substantial number of proteins normally associated with other cellular locations, (supplementary data Table II) as was predictable from earlier experiments (Figs. 1 and 2). Given the punctate appearance of the intracellular staining depicted in Fig. 1, it is possibly noteworthy that many of these “non PM” proteins have a role in protein synthesis (translocon-associated protein δ, oligosaccharyl transferase, ribophorin, S60 ribosomal protein), folding (GRP78, HSP70, HSP60, calreticulin, protein disulphide isomerase), or trafficking (rab7, rab18, rab1B, SNAP23, SNAP25). This is consistent with the biotinylating reagent accessing the endoplasmic reticulum–Golgi complex and/or secretory vesicles, possibly via endocytosis notwithstanding the low rate of endocytosis predicted at the temperature at which labeling took place. Alternatively, the presence of some “non-PM” proteins may reflect in vivo interactions with the intracellular domains of PM proteins (e.g. Lck with CD4). There are also reports that a small fraction of actin (29) and heat shock proteins (13), including several identified here (e.g. HSP60, HSP70, GRP78), may be expressed at the cell surface. Thus, it is possible that their presence is not artifactual and simply reflects their heterogeneous subcellular localization. However, other proteins (e.g. prohibitin, Bax, Bak, metaxin 2) are mitochondrial. Their recovery probably reflects the contamination of the PM-enriched p20 fraction with mitochondrial components (as documented in preliminary studies using prohibitin as a mitochondrial marker, data not shown) exposed to the low levels of the biotinylating reagent gaining intracellular access (see Fig. 1f).

Some of the proteins listed in supplementary data Table I (e.g. CD98, basigin, lutheran antigen) have previously been detected in proteomic studies of myeloid (19) or tumor cells (13). Others, such as the “hypothetical” TM domain-containing protein of 11.4 kDa, had not previously been identified at the protein level. Studies to examine its expression, localization, and function are now underway.

Several known proteins were detected in unfamiliar contexts in this study. Expression of nicastrin, for example, has only recently been reported in human Jurkat T cells (30) and its presence in primary splenocytes is, to our knowledge, novel. Its expression was confirmed by Western blotting of murine splenocytes (Fig. 4a) and 11A2 hybridomas (data not shown). Nicastrin is a component of γ-secretase (31, 32), which generates the β-amyloid precursor protein implicated in Alzheimer’s disease (33, 34). However, it is also involved in the Notch signaling pathway (31, 35, 36), which may regulate T and B cell development (37). Interestingly, the transmembrane protease ADAM 10, also detected in our study (Table I), has likewise been implicated in signaling by Notch (38).

Also noteworthy was the identification of a member of the OCIL family of C-type lectins related to CD69 (39), originally identified as an osteoblast-derived factor capable of potent attenuation of osteoclast differentiation (40). Its expression on murine splenocytes was confirmed by Western analysis (Fig 4b). OCIL may thus represent a potentially important component of the emerging regulatory circuit between the immune system and the regulation of bone integrity, complementing other regulatory molecules such as osteoprotegrin (41).

The rationale of these studies was to develop methods for investigating differential expression of cell surface components. Thus, PM proteins were affinity-purified from primary murine splenocytes with or without PMA/ionomycin treatment (30 ng/ml, 16h), and equal amounts of protein were analyzed by solution-phase IEF/SDS-PAGE. Levels of most proteins were unchanged in resting and activated cells. However,
quantitative comparisons were complicated by the presence of a given protein in several Rotofor fractions and by the presence of several proteins in some silver-stained bands.

TABLE I
Proteins regulated by cell activation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Regulation by PMA/ionomycin</th>
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<tbody>
<tr>
<td>IgM (membrane-bound form)</td>
<td>Down*</td>
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<tr>
<td>IgD (membrane-bound form)</td>
<td>Down*</td>
</tr>
<tr>
<td>Ig κ chain</td>
<td>Down*</td>
</tr>
<tr>
<td>MHC class 2 α chain (I-Aq)</td>
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<tr>
<td>MHC class 2 β chain (I-Aq)</td>
<td>Up*</td>
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<tr>
<td>GITR</td>
<td>Up*</td>
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<tr>
<td>Complement receptor-related protein</td>
<td>Up</td>
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<tr>
<td>CD21 (complement receptor 2)</td>
<td>Up</td>
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<tr>
<td>CD22</td>
<td>Up</td>
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<tr>
<td>CD26</td>
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Notwithstanding these caveats, semiquantitative comparisons were possible between some bands that were clearly differentially represented in the resting and activated samples (Fig. 5 and Table I). The membrane-bound forms of IgM and IgD (Fig. 5a), as well as Ig κ chain (Fig. 5e), presumably derived from IgM and IgD, were down-regulated following PMA/ionomycin challenge. In contrast, the Toll-like receptor, CD180/RP105 (Fig. 5b), complement receptor-related protein (Fig. 5c), GITR (Fig. 5d), and both the α and β chains of MHC class II (Fig. 5e) were all up-regulated in the activated cells.

Because of the complicating factors alluded to above, a selection of these changes were corroborated by Western blotting (Fig. 4). Equal amounts of protein from extracts of the same resting or activated cells were probed for four PM proteins, nicastrin (Fig. 4a), OCIL (Fig. 4b), Ig κ chain (Fig. 4d), and GITR (Fig. 4e). GRP78 (Fig. 4c) was used as a loading control. As expected, all four PM proteins were enriched in the p20 fraction compared with the PNS and further enriched in the affinity-purified fraction. Levels of GRP78, OCIL, and nicastrin in the affinity-purified PM preparation were unaffected by the PMA/ionomycin stimulus. In contrast, GITR was clearly up-regulated and Ig κ chain was down-regulated by activation, consistent with data in Fig. 5. The up-regulation of MHC class II in activated cells was also confirmed (data not shown).

FACS analysis was used independently to verify other...
changes observed in the affinity purification experiments. Activation-induced up-regulation of CD69 (Fig. 6a) and MHC class II (I-A^q; Fig. 6b) were documented, whereas CD45 expression was unaffected (Fig. 6c). The Western and FACS data together suggest that 3–5-fold expression differences were detectable using the approach described here. Because OCIL, GITR, and CD69 were each identified by MS/MS on the bases of a single matching spectrum, expression of each molecule was independently verified by Western or FACS experiments.

Several of these observations (see Table I), for example, the up-regulation of MHC class II expression on murine B cells and monocyte/macrophages, GITR on T cells, and CD69 on both B and T cells, are known activation-induced changes. Activation-associated increases in the expression of CD40 (42), CD166 (activated leukocyte cell adhesion molecule, ALCAM) (43), CD26 (dipeptidylpeptidase IV) (44), and CD180 (45) have also been described. However, some of these changes (e.g. complement receptor-related protein, a murine homologue of human complement receptor 1) have not to our knowledge been reported previously. While the biological significance of these observations require further investigation, the fact that six of 12 apparent activation-induced changes in protein expression were corroborated by Western and FACS analyses demonstrates that this method can be used to detect alterations in PM protein expression even when specific reagents are unavailable.

Currently, this approach (in common with others that rely on mass spectrometric detection) requires relatively large numbers of cells, which somewhat limits its application to in vivo systems. However, because the method can detect markers for given cell types within mixed populations (e.g. Thy-1 for T lymphocytes, Ig receptor chains for B lymphocytes, CD11c for monocytes), it could in principle be used to determine PM protein expression profiles in mixed cell suspensions derived from diseased tissues, such as tumors. In addition, the technique readily lends itself to nonsample-limited systems such as analyses of PM protein expression on tumor cell lines.

In conclusion, by combining an optimized cell surface biotinylation procedure with solution-phase IEF, we have developed a generic and powerful strategy for profiling membrane proteins that is easily implemented in most biochemical laboratories. In initial studies, we identified one unknown and 74 known transmembrane proteins, which is comparable to the numbers detected in recent studies using multidimensional liquid chromatography (14, 15, 19). We were also able to detect differential protein expression in primary murine splenocytes activated by treatment with PMA/ionomycin. Thus, while we have not performed parallel shotgun analyses, comparison of our results with published data suggest that gel-based proteomic methods remain competitive with shotgun approaches. Both methods are capable of determining differential expression profiles of membrane proteins in a range of cell types under varying environmental and pathophysiological conditions.

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‡ The on-line version of this article (available at http://www.mcponline.org) contains supplemental data.

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REFERENCES


42. Stamenkovic, I., Clark, E. A., and Seed, B. (1989) A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J. 8, 1403–1410