Proteomic analysis of melanoma-derived exosomes by two-dimensional polyacrylamide gel electrophoresis and mass spectrometry

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Exosomes are 40–100 nm vesicles released by numerous cell types and are thought to have a variety of roles depending on their origin. Exosomes derived from antigen presenting cells have been shown to be capable of initiating immune responses *in vivo* and eradicating established tumours in murine models. Tumour-derived exosomes can be utilised as a source of tumour antigen for cross-priming to T-cells and are thus of interest for use in anti-tumour immunotherapy. Further exploration into the protein composition of exosomes may increase our understanding of their potential roles in *vivo* and this study has examined the proteome of exosomes purified from cell supernatants of the melanoma cell lines MeWo and SK-MEL-28. The vesicular nature and size (30–100 nm) of the purified exosomes was confirmed by electron microscopy and sucrose density gradient centrifugation. Western blotting demonstrated the absence of calnexin and cytochrome *c*, verifying the purity of the exosome preparations, as well as enrichment of MHC class I and the tumour-associated antigens Mart-1 and Mel-CAM. The two-dimensional polyarcylamide gel electrophoresis (2-D PAGE) protein profiles of exosomes from the two cell lines were highly comparable and strikingly different from the profiles of the total cell lysates. Mass spectrometric sequencing identified proteins present in 49 protein spots in the exosome lysates. Several of these have been identified previously in exosomes but some are novel, including p120 catenin, radixin, and immunoglobulin superfamily member 8 (PGRL). Proteins present in whole-cell lysates that were significantly reduced or excluded from exosomes were also identified and included several mitochondrial and lysosomal proteins, again confirming the proposed endosomal origin of exosomes. This study presents a starting point for future more in-depth protein studies of tumour-derived exosomes which will aid the understanding of their biogenesis and targeting for use in anti-tumour immunotherapy protocols.

Keywords: Exosomes / Melanoma / Mel-CAM / Two-dimensional polyacrylamide gel electrophoresis

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1 Introduction

Exosomes were first described in the early 1980s as exfoliated membrane vesicles released from various cell lines, with two populations of average diameter 500–1000 nm and 40 nm [1]. Now used to describe specifically those vesicles of 30–100 nm diameter with a characteristic cup-shaped morphology, they have been shown to be released by a variety of haematopoietic and non-haematopoietic cells including reticulocytes [2, 3] mast cells [4–6], T cells [7, 8], B cells [9, 10], platelets [11], dendritic cells (DCs) [12, 13], and several established epithelial cell lines [14–16]. Exosomes have also been identified *in vivo* being present in pleural and peritoneal tumour effusions [17], bronchoalveolar lavage fluid of

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Abbreviations: DC, dendritic cell; **FCS**, foetal calf serum; **Ig**, immunoglobulin; **Mel-CAM**, melanoma cell adhesion molecule; **TEM**, transmission electron microscopy

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healthy individuals [18], and bound to the surface of follicular dendritic cells [19]. Larger plasma-membrane derived vesicles sometimes referred to as "microvesicles" [11, 20], "ectosomes" [21], and "prostasomes" [22–24] have also been described but these tend to be $>$ 100 nm in size and their biogenesis and functions are distinct from those of exosomes.

The biogenesis of exosomes is initiated with the formation of multivesicular bodies (MVBs) as a result of inward budding of the limiting membrane of late endosomal compartments [3, 25, 26]. These MVBs then fuse with the plasma membrane, resulting in the release of the exosomes into the extracellular milieu. The function of exosomes *in vivo* is unclear and indeed may depend on the specific cell type from which they originate but exosomes derived from a variety of cell types are capable of delivering antigen to the immune system. This role as intercellular signalling devices may be important in immune defence and maintenance of T cell memory, and stimulation of T cells either directly *via* exosome MHC molecules or dependent on cross-presentation by antigen-presenting cells (APCs) has been described [6, 9, 18, 27]. Although exosomes from most cell types appear to be immunostimulatory, the situation may be more complex with those derived from intestinal epithelial cells ("tolerosomes") which contain both peptide-loaded MHC class I and II molecules, appearing to induce antigen-specific tolerance *in vivo* due to a lack of costimulatory molecules [28]. Similarly in rats it has been shown that pretreatment of heart allograft recipients with donor bone marrow-derived exosomes prolonged graft survival, decreasing anti-donor T cell reponses but increasing alloantibody production [29]. Interestingly the tolerogenic nonclassical class I HLA-G molecule is present in exosomes from the melanoma cell Fon, raising the possibility that such exosomes may also possess some negative modulatory effects on the host response to the tumour [16].

The recent interest in exosomes in cancer research has arisen due to their potential role in immunotherapy [30]. Exosomes derived from mast cells have been shown to be capable of stimulating both B-cell and T-cell proliferation [5] and murine DC-derived exosomes pulsed with tumour peptides are capable of suppressing or eradicating established tumours in mice [12]. More recently it has been shown that exosomes can be isolated from tumour cells and pulsed onto DCs *in vitro*, resulting in tumour antigen transfer. These DCs can subsequently prime anti-tumour immune responses against various established tumours in mice suggesting that they are a source of shared tumour antigens [15]. Protocols for developing clinical grade exosomes from DCs have now been developed [31] and phase 1 clinical trials are in progress [32].

Studies on exosomes isolated from different cell types have shown that although certain proteins are present irrespective of cell of origin, some proteins are cell type-specific [4, 8, 10, 13, 26, 33–37]. Additionally, although poorly understood, "sorting" of the plasma membrane proteins occurs with clear enrichment of some membrane proteins in exosomes and exclusion of others [8, 10] and indeed their composition can differ depending upon from which cell face they are released [14]. Studies characterising the protein content of exosomes have largely been based on Western blotting and fluorescence activated cell sorting (FACS) analysis to analyse particular previously described proteins of interest in an attempt to elucidate exosome biogenesis and function [4, 10, 16, 18, 38]. More general proteomic profiling approaches to identify components of exosomes are few and have utilised one-dimensional polyacrylamide gel electrophoresis (1-D PAGE) followed by mass spectrometric analysis of tryptic peptides [13, 14] with the most extensive of these being carried out on DC-derived [26] and B cell-derived [33] exosomes. In general, enrichment of MHC class I and II, tetraspanins, several heat shock proteins, cytoskeletal components such as actins and tubulins, proteins involved in intracellular membrane fusion such as annexins and Rab family members, signal transduction proteins including 14-3-3 and G proteins, and various metabolic enzymes have been described [10, 13, 26, 32, 33]. Analyses based on 2-D PAGE have been performed on prostasomes [24] and neutrophil ectosomes [39] but not yet on exosomes. In this study, we isolated exosomes from two human melanoma cell lines, SK-MEL-28 and MeWo, confirmed their purity by electron microscopy and Western blotting and analysed their proteomic profile in comparison with whole cell lysates using 2-D PAGE and mass spectrometry.

2 Materials and methods

2.1 Chemicals and reagents

General chemicals (Analar-grade or equivalent) were purchased from Sigma (Poole, UK) and VWR (Poole, UK). Other reagents were from the following sources: colloidal Coomassie blue, thiourea, DTT, and iodoacetamide from Sigma (Poole, UK); Pefabloc from VWR (Poole, UK); Hybond™ C super nitrocellulose membrane, Hyperfilm™ ECL™ film, Pharmalyte pH 3–10, IPG strips, Dry strip cover fluid, bromophenol blue, and PlusOne Silver Stain from Amersham Biosciences (Little Chalfont, UK); urea and Tris from ICN (Basingstoke, UK); glycine from Genomic Solutions (Huntingdon, UK); CHAPS from Calbiochem (San Diego, CA, USA); LMP agarose, RPMI-1640, fetal calf serum (FCS), L-glutamine, Harks Balanced Salt Solution (HBSS) from Invitrogen Life Technologies (Paisley, UK); acrylamide from National Diagnostics (Hull, UK); OWL

Silver Stain from OWL Separation Systems (Portsmouth, NH, USA); trypsin sequencing-grade from Promega (Southampton, UK); ACN from Rathburn (Walkerburn, UK); Complete™ mini protease inhibitor cocktail tablets from Roche (Lewes, UK), and West Dura Extended Substrate from Pierce (Tattenhall, UK).

2.2 Cell lines

Human melanoma cell lines SK-MEL-28 and MeWo were obtained from the American Type Culture Collection and European Collection of Animal Cell Cultures, respectively. Cells were cultured in RPMI 1640 supplemented with 10% v/v FCS and 2 mM L-glutamine in humidified air in 5% CO_2 at 37°C. For routine passaging, cells were washed in PBS followed by incubation in 0.1% w/v EDTA in PBS for a few minutes at 37°C.

2.3 Exosome isolation

The isolation procedure was based on that previously described for DCs with slight modifications [26]. Cells were taken from near-confluent starter cultures and transferred to 1700 cm^2 expanded surface roller bottles for maximal cell:supernatant ratio. Cells were seeded (2×10^7 per bottle) in 150 mL RPMI 1640 supplemented with 10% v/v depleted-FCS (FCS was pre-depleted of bovine exosomes by ultracentrifugation at 100 000 × *g* for 16 h at 4°C) and 2 mM L-glutamine. The roller bottles were gassed with 5% CO_2 and incubated at 37°C, rotating at 2.5 rpm. When cells reached ~60% confluency, the medium was changed and supernatants were collected after a further 48 h. Supernatants were centrifuged at room temperature at 720 × *g* (MSE Harrier 15/80) for 10 min to remove any dead cells, then subjected to 0.2 μm and 0.1 μm filtration steps using 0.2 μm and 0.1 μm Supor® membrane Vacucap® filters (Pall Gelman Labs, Ann Arbor, MI, USA) to remove cell debris and large vesicles. Exosomes were pelleted by ultracentrifugation at 100 000 × *g* (45Ti rotor, L-80 ultracentrifuge; Beckman Coulter, High Wycombe, UK) for 1 h at 4°C. Exosomes were pooled, washed twice in PBS pH 7.3, resuspended in 50–100 μL PBS and stored at −80°C. Four sequential 48 h exosome isolations were carried out per roller bottle by which time the cells were ~90% confluent. Protein concentration was determined by a modified Bradford assay using Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK). Typical yields of exosomes were 0.13 μg per 10^6 Mewo cells/48 h with 0.2 μm filtration and 0.09 μg with 0.1 μm filtration. After removal of supernantants for exosome isolation, total protein lysates were also prepared from the cells as described below.

2.4 Electron microscopy of exosomes

Exosomes equivalent to 50 μg protein content prepared as described were pelleted by ultracentrifugation at 100 000 × *g* (TLA-45 rotor, Optima™ TL Ultracentrifuge; Beckman Coulter) for 1 h at 4°C. Pellets were fixed by suspension in 50 μL 2% w/v paraformaldehyde in Sorensen's buffer (81% 0.1 M disodium hydrogen orthophosphate, 19% 0.1 M sodium dihydrogen phosphate) and 5 μL suspended exosomes were dropped onto a formvar-carbon coated grid and left to dry at room temperature for 20 min. After three 1 min washes in Sorensen's wash buffer (0.1 M sodium phosphate buffer, pH 7.4) the exosomes were fixed in 1% w/v gluteraldehyde in Sorensen's buffer for 5 min. Following 6 × 2 min water washes, the exosome samples were stained with saturated aqueous uranyl acetate for 10 min. Samples were then embedded in 0.4% w/v uranyl acetate, 1.8% w/v methylcellulose on ice (2 × 1 min, 1 × 10 min). The excess liquid was removed and the grid dried at room temperature then viewed in a JEOL transmission electron microscope (TEM).

2.5 Characterisation of exosomes by 1-D SDS-PAGE and Western blotting

To prepare total cell lysates, cell monolayers were washed with ice-cold PBS, pH 7.3, scraped off tissue culture flasks with a rubber policeman and lysed in RIPA buffer (PBS, 0.1% w/v SDS, 0.5% w/v sodium deoxycholate, 1% w/v Nonidet P40) for 30 min at room temperature. Extracts were microfuged at 13 000 rpm for 5 min at room temperature to remove insoluble material. Total cell lysates and exosomes (prepared as described in Section 2.3) were diluted to 1 × loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% v/v glycerol, 5% v/v β-mercaptoethanol, 2% w/v SDS, 0.0025% bromophenol blue) prior to loading and running on 1-D SDS-PAGE using the Bio-Rad Mini Protean II electrophoresis system as described by the manufacturer. For Coomassie blue staining, 5 μg protein was loaded per lane on 7.5%T and 12%T polyacrylamide gels. Gels were fixed in 40% v/v methanol, 7% v/v acetic acid for 30 min then stained with Coomassie blue for 1 h. Gels were destained in 25% v/v methanol, 10% acetic acid for 1 min then in 25% v/v methanol for up to 6 h. For immunoblotting 12% T minigels were loaded with 2 μg protein per well for Mart-1, MHC class I, annexin II, calnexin, and melanoma cell adhesion molecule (Mel-CAM) and 4 μg per well for cytochrome *c* and MHC class II. Protein was transferred to Hybond™-C Super nitrocellulose membrane using Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) for 1 h at 100 V. Western blotting was performed using the EnVision™ + system (Dako, Ely, UK) as previously described [40]. Primary antibody dilutions were: anti-MHC class I (clone HC10; Cancer Research UK

Monoclonal Antibody Production Unit) 1:100, anti-annexin II (clone 3D5, Cancer Research UK) 1:100, anti-Mel-CAM (clone MUC-18, Cancer Research UK) 1:20, anti-cytochrome *c* (clone 7H8.2C12, BD Biosciences, Cowley, UK) 1:5000, anti-Mart-1 (clone A103) 0.5 μg/mL and polyclonal rabbit anti-calnexin (Bioquote, York, UK) 1:2000. Peroxidase-labelled polymer conjugated to goat anti-mouse or anti-rabbit immunoglobulins from the rabbit and mouse Envision™ + kits (Dako, Ely, UK) were used at a dilution of 1:100. Blots were developed using Super-Signal West Dura Extended Duration Substrate and Hyperfilm™ ECL™ film. Negative control blots were probed with irrelevant matched antibodies and prior to probing blots with multiple antibodies, initial experiments were carried out with single antibodies to confirm their specificity and optimal concentrations.

2.6 Characterisation of exosomes by 2-D PAGE

To prepare total cell lysates, cell monolayers were washed three times with ice-cold PBS, pH 7.3, then with ice-cold isotonic sucrose (250 mM) and lysed into urea/thiourea lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 1% w/v DTT, 0.8% v/v Pharmalyte pH 3–10, 1 mg/mL Pefabloc) containing Complete™ mini protease inhibitor cocktail (1 tablet per 2.5 mL lysis buffer). After 2 min at room temperature, cells were scraped from the flask and incubated for a further 30 min at room temperature with intermittent vortexing. Following ultracentrifugation at 40 000 × *g* for 1 h at 15°C, lysate supernatants were aliquotted and stored at −80°C. Protein concentration was determined by a modified Bradford assay using Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK). Exosomes prepared as described in Section 2.3 were pelleted by centrifugation at 100 000 × *g* for 1 h at 4°C, resuspended in urea/thiourea lysis buffer and processed as described for the total cell lysates. Protein extracts were analysed by 2-D PAGE as previously described [41]. Isoelectric focusing was carried out using the IPGPhor system (Amersham Biosciences, Uppsala, Sweden). Samples (30 μg protein for analytical gels and 300 μg–1 mg for preparative gels) were diluted in reswelling buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.46% w/v DTT, 0.2% v/v Pharmalyte pH 3–10 with a trace of bromophenol blue) to give a final volume of 450 μL per strip and applied to 18 cm pH 3–10NL IPG strips by in-gel rehydration (30 V, 13 h). Focusing (65 000 Vh) was carried out at 200 V (1 h), 500 V (1 h), 1000 V (1 h), gradient incline to 8000 V (1 h), 8000 V to end. Separation in the second dimension was carried out using 10%T/3.3%C polyacrylamide gels with a 4%T stack using the ISO-DALT system (Amersham Biosciences). IPG strips were incubated in equilibration buffer (6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris, pH 6.8) containing 1% w/v DTT for 15 min followed by equilibration buffer containing 4% w/v iodoacetamide for 10 min. Strips were rinsed in running buffer (24 mM Tris, 200 mM glycine, 0.1% w/v SDS), and sealed in place on top of the gel using 1% w/v agarose in running buffer with a trace of bromophenol blue. Gels were electrophoresed overnight in running buffer (18 mA per gel) at 12.5°C. Analytical gels were fixed and stained using OWL Silver Stain. Preparative gels were stained with PlusOne Silver Stain using a modified staining protocol as previously described [42]. Gels were scanned as 12-bit images using a personal densitometer SI (Amersham Biosciences) and analysed using Melanie 3 software (GeneBio, Geneva, Switzerland).

2.7 Identification of proteins by mass spectrometry

Spots were excised from preparative gels, cut into small pieces and destained using 50 mM sodium thiosulphate, 15 mM potassium ferricyanide. Gel pieces were then washed with H_2O (20 min), equilibrated with 25 mM ammonium bicarbonate (20 min), dehydrated with ACN (20 min), rehydrated with 25 mM ammonium bicarbonate, dehydrated with ACN and dried in a SpeedVac. Gel pieces were rehydrated in 5 μL (200 ng) trypsin working solution (200 μL 0.1 mg/mL trypsin at 16 000 U/mg prepared in ice-cold resuspension buffer supplied with the enzyme and made up to 500 μL with 25 mM ammonium bicarbonate) for 45 min on ice and 30 μL 25 mM ammonium bicarbonate was added to each sample. Samples were incubated at 37°C for 4 h and digestion was stopped by placing samples on dry ice. Samples were defrosted, the supernatant was decanted to a 0.2 mL siliconised thin-walled tube (Bioquote, York, UK) and two extractions were performed with 30 μL 5% v/v formic acid, incubating in a sonicating water bath for 15 min. Peptides were pooled then dried in a SpeedVac. Peptides were reconstituted in 5 μL 50% v/v ACN/0.1% v/v TFA and 0.3 μL loaded onto the target plate with 0.3 μL α-cyano matrix (3 mg/mL) containing 25 fmol angiotensin I. Peptides were analysed using a 4700 Proteomics Analyzer (Applied Biosystems) in MS Reflector Positive mode. The collated MS and/or MS/MS data was used to search the NCBI database using Protein Prospector with a mass accuracy of 20 ppm.

3 Results

3.1 Purification and characterisation of exosomes

Exosomes were purified from cell culture supernatants by a combination of ultracentrifugation and filtration. Analysis by TEM of purified exosomes prepared using a single filtration

step with a 0.2 μm filter showed large numbers of membrane bound cup-shaped vesicles ranging from approximately 30–150 nm in diameter (Fig. 1A). Although the majority of the vesicles were within the expected size range for exosomes, < 10% of the vesicles ranged from 100 to 150 nm in diameter. Some of these larger vesicles were cup-shaped but they tended to be more irregular and more electron dense than the smaller vesicles. To remove any vesicles > 100 nm a second filtration step using a 0.1 μm filter was introduced as part of our standard protocol. When viewed by TEM, these exosome preparations were found to lack the large irregular membrane bound vesicles previously seen, with all the vesicles measuring 30–100 nm in diameter (Fig. 1B). Similar results were obtained for both MeWo and SK-MEL-28 derived exosomes.

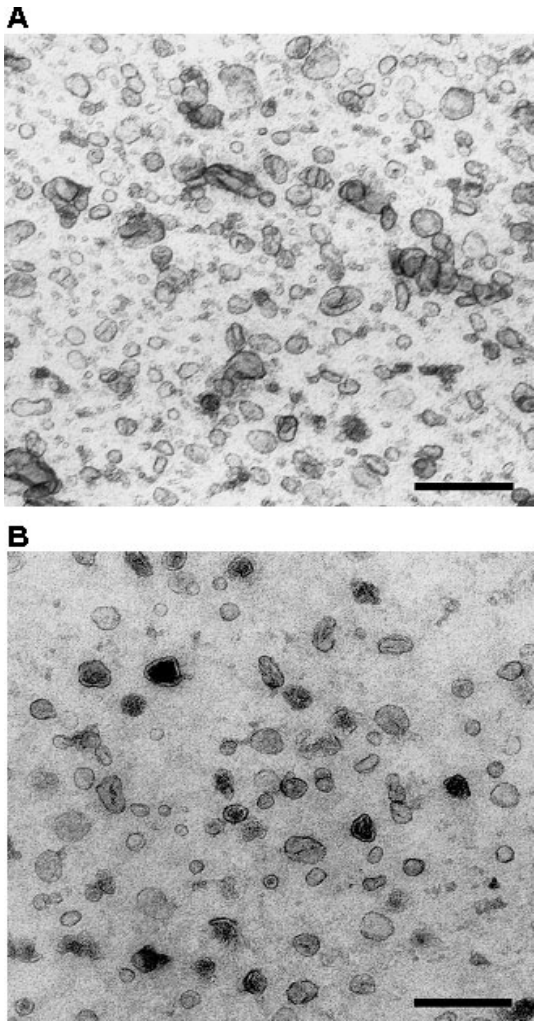

Figure 1. Electron micrograph of exosome preparations. Transmission electron micrographs of (A) exosomes isolated with a 0.2 μm filtration step, and (B) exosomes isolated following sequential filtration steps with both 0.2 μm and 0.1 μm filters to remove large contaminating vesicles. Scale bar = 200 nm.

Examination of exosomes by Western blotting showed clear enrichment of MHC class I compared to whole-cell lysates (Fig. 2A). The melanoma associated antigens Mart 1 and Mel-CAM (Muc18) were also strongly enriched in the exosome samples (Fig. 2B) and annexin II was present at moderately higher levels in exosomes compared to whole-cell lysates (Fig. 2B). Calnexin, a protein found in the endoplasmic reticulum, was readily detectable in whole-cell lysates but essentially absent in the exosome samples (Fig. 2C) indicating that the exosome preparations were not contaminated with other vesicles. A similar result was obtained for cytochrome *c* (Fig. 2D) demonstrating that there was little contamination with apoptotic vesicles. Essentially identical results were also seen with SK-MEL-28 whole cell lysates and exosomes (data not shown). The results obtained for MeWo-derived exosomes which did not undergo a second filtration step through the 0.1 μm filter were also similar suggesting that the larger vesicles seen *via* electron microscopy are either exosomes or are present at such low levels that they do not interfere with the analysis.

The vesicular nature of SK-MEL-28 derived exosomes was confirmed by floatation on sucrose density gradients, migrating at a density between 1.13 and 1.20 mg/mL as previously described for exosomes from B-lymphoblastoid cell lines and dendritic cells [13, 43] with concomitant enrichment of Mart-1, Mel-CAM, and annexin II in these fractions (data not shown).

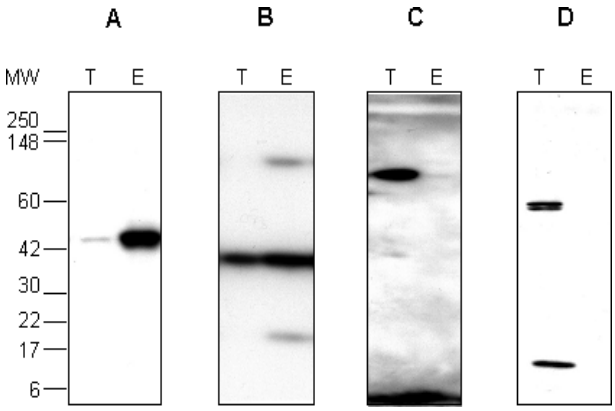

Figure 2. Western blot analysis of MeWo total cell lysates and MeWo-derived exosomes. Protein from whole-cell lysates (T) or exosomes (E) were separated by 12% SDS-PAGE and analysed by immunoblotting using antibodies to (A) MHC class I (50 kDa), (B) Mel-CAM (113 kDa), Mart-1 (18 kDa) and annexin II (36 kDa), (C) calnexin (90 kDa) and (D) cytochrome *c* (15 kDa). The second band at approximately 60 kDa observed with the anti-cytochrome *c* antibody has also been observed by other groups (Mauro Deli Esosti, personal communication).

3.2 Characterisation of exosomes by 1-D and 2-D PAGE

Clear differences could be seen between the 1-D PAGE protein profiles of exosomes compared to those of whole-cell lysates (Fig. 3). Many protein bands seen in the whole-cell lysates were absent or present in lower levels in the exosome samples, whilst several bands were clearly enriched in the exosome samples compared to the whole-cell lysates.

Representative 2-D gel images of exosomes and whole-cell lysates are shown in Fig. 4. The spot patterns of exosomes isolated from both melanoma cell line supernatants were less complex than those of the total cell lysates containing approximately 75% of the protein species (for example, an average of 1250 protein spots in exosomes compared with 1660 in whole-cell lysates for the MeWo cell line). Furthermore, there were very clear differences in the gel profiles of total cell lysates and exosomes. Gel comparisons indicated that 45–50% of proteins were shared between exosomes and whole-cell lysates with several hundred proteins being either strongly enriched or lost in the exosome samples. Importantly, two independent exosome preparations from SK-MEL-28 showed very good reproducibility and the profiles of 0.1 μm and 0.2 μm filtered exosomes were also largely identical (data not shown). Significant similarity was also observed between the profiles of MeWo and SK-MEL-28 derived exosomes with $>$ 80% of protein spots (percentage volume $>$ 0.02) being matched between the two exosome preparations.

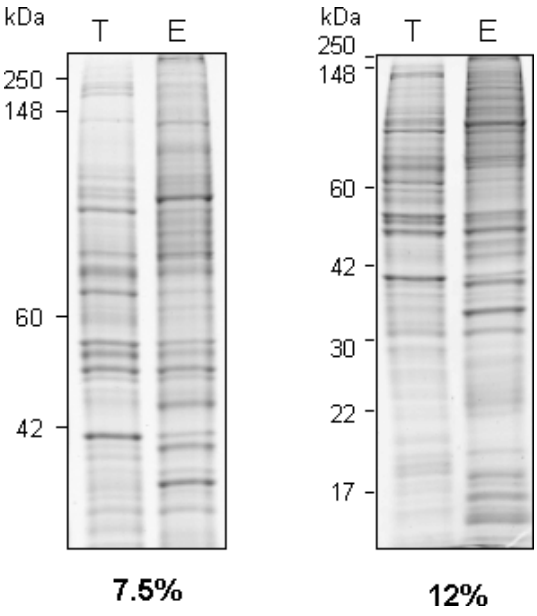

Figure 3. 1-D PAGE analysis of exosomes. Protein (5 μg) from whole-cell lysates (T) or exosomes (E) prepared from the MeWo cell line were separated by 7.5% or 12% SDS-PAGE and analysed by Coomassie blue staining to ilustrate the differences in protein profile.

The identities of 49 protein spots found in the exosome fraction (Table 1) and 13 protein species present in total cell lysates but absent from exosomes (Table 2) were successfully determined by mass spectrometry. The gel positions of the proteins identified are shown in Figs. 5A and B, respectively. The protein species identified from the 49 protein spots in the exosomal preparations corresponded to 41 different proteins (Table 1). These included 2 heat shock proteins, 9 enzymes, and 11 structural proteins with 15 proteins having a variety of functions including GTP-binding, tetraspanin binding, apoptosis-related and cell structure-related, and 4 proteins being bovine in origin. A large number of the proteins which were identified as being absent from exosomes were localised to subcellular organelles (mitochondria, lysosomes and the endoplasmic reticulum; Table 2).

4 Discussion

At present the precise function of exosomes *in vivo* are not fully understood and may in part be dependent on their cell of origin. However, there are clear indications of their potential therapeutic utility *via* antigen delivery or presentation properties. For example, tumour-derived exosomes have been demonstrated to elicit immune responses by delivering tumour antigens to APCs, resulting in rejection of tumours in mice both prophylactically and therapeutically [15]. Such exosomes may either be suitable for generic or patient-specific immunotherapies depending on their specific protein content and their utility may also be dependent on how representative of the original tumour the parental cell line is following extensive *in vitro* culture. Increased appreciation of the proteomic composition of exosomes may provide further confirmation and insight into their proposed endosomal biogenesis and may ultimately allow critical proteins to be identified and support the generation of synthetic exosome-like structures for use as tumour vaccines.

The protocol adopted for exosome isolation in this study was ultrafiltration following filtration through a 0.2 μm membrane. This strategy was initially developed for the isolation of exosomes from dendritic cells and is optimised to remove the contaminating larger vesicles shed from the plasma membrane [26]. Earlier studies employed the use of differential centrifugation rather than filtration to remove larger contaminating vesicles, however, the resulting exosomes have been reported to be quantitatively and qualitatively identical [26]. Electron microscopy of exosomes isolated *via* the standard protocol using fil-

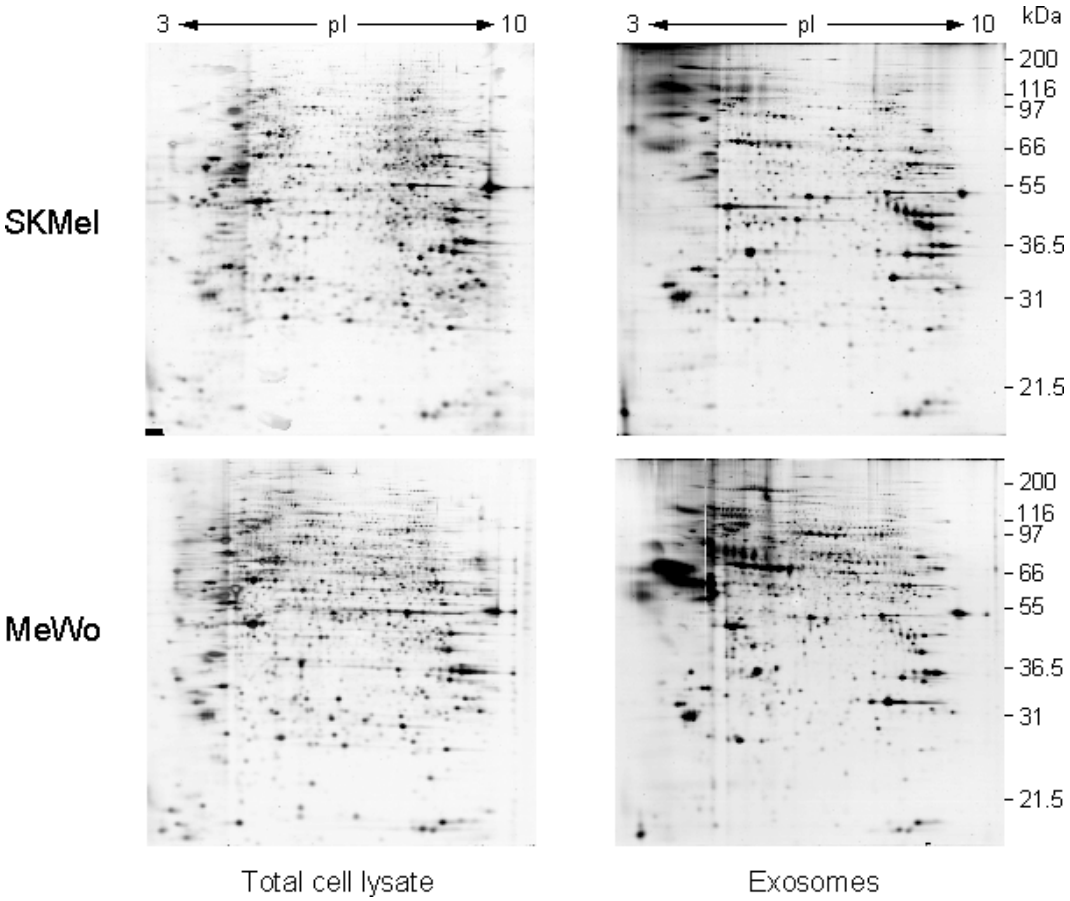

Figure 4. 2-D PAGE analysis of exosomes. 30 μg protein from whole-cell lysates or exosomes from the melanoma cell lines MeWo and SK-MEL-28 were separated by 2-D PAGE using pH 3–10NL IPG strips for the first dimension and 10%T SDS-PAGE for the second dimension. Proteins were detected by silver staining.

tration through a 0.2 μm membrane revealed the presence of largely 30–100 nm cup-shaped vesicles but 10% of the vesicles were 100–150 nm in size. These larger vesicles were removed with the addition of a 0.1 μm filtration step. The Western blotting results for the exosomal preparations using both these filtration protocols were identical as were the 2-D gel profiles suggesting that these larger vesicles may represent a population of larger exosomes and thus have similar protein profiles. The alternative explanation is that they may be contaminating plasma-membrane derived microvesicles [11] but present at such low levels as to have no gross impact on the 2-D PAGE profile. By Western blotting, clear enrichment of proteins such as MHC class I, and absence of the ER protein calnexin and the mitochondrial protein cytochrome *c* were also seen and by 2-D PAGE several proteins of mitochondrial or ER origin were seen to be present in the whole cell lysates but absent in the exosomes, again supporting the endosomal origin and infers a lack of extensive contamination with apoptotic or other vesicles [13, 26]. As the FCS used was depleted of bovine exosomes by overnight high-speed ultracentrifugation [13], the presence of bovine proteins probably reflects the adherence of some of the more abundant bovine serum proteins to exosomes after their release into medium. Similar contamination with bovine serum proteins has been previously reported [26] and indeed clinical grade exosomes prepared from human peripheral blood-derived dendritic cells have slight albumin contamination despite ultrafiltration of the media [31]. The use of serum-free media may avoid this but ultimately also increases the release of apoptosomes presumably due to the increased stress, thus potentially compromising both the purification and composition of exosomes, although gradual adaptation may overcome this.

The exosomes prepared and used in this study clearly fulfil the proposed minimal requirements to be described as exosomes [44] in that they have an appropriate size, density in sucrose gradients and are enriched in several of

Table 1. Identities of proteins found in MeWo-derived exosomes

Spot	Protein name	Acc. No.	Theoretical		Observed		Peptide No.	Sequence coverage (%)	Previous description in other exosome studies
			MW (kDa)	p*I*	MW (kDa)	p*I*			
Enzymes									
E6	ATP citrate lyase	P53396	120.8	6.95	113.8	7.2	27	33	
E15	Enolase 1 (α-enolase)	P06733	47.0	6.99	53.1	6.9	18	50	B cell [33], IEC [14]
E20	Lactate dehydrogenase	P07195	36.5	5.72	36.9	5.7	12	42	
E29	Glutathione *S*-transferase	P09211	23.2	5.44	28.4	5.6	8	61	
E35	Phosphoglycerate mutase 1	P18669	28.7	6.75	30.7	6.8	16	70	
E36	Triosephosphate isomerase	P00938	26.5	6.51	29.8	6.9	15	75	
E56	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	P00354	35.9	6.60	35.8	8.7	13	48	B cell [33], IEC [14]
E58	Cytosolic malate dehydrogenase	P40925	36.3	6.89	35.8	6.5	10	42	
	Aldose reductase	P15121	35.7	6.55			7	39	
Heat shock proteins									
E48–E50	Heat shock 70 kDa protein 8 isoform 1 (hsc71, hsc70, hsp73, hsp71)	P11142	70.9	5.37	67.1	5.5–5.6	20/26/21	43/51/50	B cell [33]. DC [13], reticulocytes [3], tumour [15], mast cell [4]
	Heat shock 70 kDa protein 1	P08107	70.1	5.48			25/26/30	58/60/71	
Structural proteins									
E22/E23	Actin (γI)	P02571	41.8	5.31	50.2	5.4	17–19	56–60	DC [13], mast cell [6], B cell [33], IEC [14]
	Actin (β)	P02570	41.6	5.29			14–16	53–54	
E30	F-actin capping protein β-subunit	P47756	31.4	5.36	31.5	5.6	13	57	
E42	Radixin	P35241	68.6	6.03	76.5	6.3	24	43	
E43	Moesin	P26038	67.7	6.09	78.1	6.2	30	50	B cell [33]
E44	Villin (ezrin)	P15311	69.3	5.95	75.0	6.2	28	42	
E33, E34	Syntenin 1 (melanoma differentiation-associated protein-9	O00560	32.4	7.06	32.1	6.7, 7.1	8/14	38/66	DC [26]
E32	Keratin 10	P13645	59.5	5.13	35.8	5.8	11	19	IEC [14]
E55, E57	Annexin A2	P07355	38.5	7.56	35.2/34.3	7.6, 8.2	24/25	62/64	DC [13]
E24, E25	Annexin A5	P08758	35.8	4.94	33.4	5.2, 5.1	20	65	DC [26]
E48, E49	Annexin A6	P08133	75.7	5.42	66.0	5.6	9/10	21/23	Mast cell [4]
Others									
E1	Eukaryotic translation elongation factor 2 (EEF2)	P13639	95.2	6.42	97.0	7.0	20	40	
E2, E3, E66	Ig superfamily member 8 (CD81 partner 3, EWI2, keratinocyte-associated transmembrane protein 4, PGRL)	Q969P0	65.0	8.23	69.4/70.7/70.7	6.9, 6.6, 6.5	13/14/20	41/37/42	
E4	Syntaxin-binding protein 1 (UNC18A, STXBP1)	Q64320	67.6	6.50	64.1	6.8	16	43	
E7, E8	p120 catenin (data consistent with multiple forms)	O60716	108.2	5.86	104.1	6.7, 6.5	43–45/32–34	53–58/46–52	

Table 1. Continued

Spot	Protein name	Acc. No.	Theoretical		Observed		Peptide No.	Sequence coverage (%)	Previous description in other exosome studies
			MW (kDa)	p*I*	MW (kDa)	p*I*			
E9–E12, E14	Programmed cell death interacting protein (ALIX, AIP1)	Q8WUM4	96.0	6.13	97.0/98.8/104.1	6.0–6.2	25/30/31/24/13	43/50/53/45/27	DC [26]
E16	RabGDI β	P50395	50.7	6.11	51.7	6.0	29	81	
E18–E20	G protein β1 subunit	P04901	37.4	5.60	36.1/36.1/36.9	5.5–5.7	9/13/37	37/63/57	
E21	G protein α subunit	P29992	42.1	5.51	43.2	5.5	6	25	
E20	Protein phosphatase 1	P08129	37.5	5.94	36.9	5.7	8	40	
E24, E25	Translin-associated factor X interacting protein 1	NP_060900[a)]	42.3	4.60	33.4	5.2, 5.1	7	20	
E27	Tyrsosine 3/tryptophan 5-mono-oxygenase activation protein (14-3-3ε, KCIP-1)	P42655	29.2	4.63	31.9	4.9	22	73	
E37	Ras-related nuclear protein (GTP-binding nuclear protein, Ran)	P17080	24.4	7.01	28.1	7.2	4	24	
E41	Septin 2 (Nedd5)	Q15019	41.5	6.15	47.0	6.1	20	60	
E62	WD repeat-containing protein 1 isoform 1	O75083	66.2	6.17	64.1	6.2	15	49	
	Syntaxin binding protein 2 (UNC18B, STXBP2)	Q15833	66.4	6.11			29	58	
Bovine									
E5	Plasminogen	P06868	88.4	7.39	104.1	7.2	9	19	DC [26]
E28	Apolipoprotein A-1	P15497	27.5	5.36	28.6	5.4	20	41	DC [26]
E46, E47	Serum albumin	P02769	66.4	5.60	66.0	5.7–5.8	22/8	42/17	DC [26]
E51	α-2-HS-glycoprotein	P12763	36.4	5.10	70.7	4.7	7	24	

a) Accession number in Swiss-Prot with exception of [a)] from NCBI
The spot numbers correspond to those indicated on the master map in Fig. 5A. Theoretical MW and p*I* are as calculated for the predicted mature, unmodified proteins with the observed values being those seen for the proteins separated by 2-D PAGE. The peptide number is the number of peptides found by MS to match the identified protein with the sequence coverage indicated.

the previously described molecules associated with endocytic pathways although tetraspanins *per se* were not seen, possibly due to their relatively poor solubility in lysis buffers containing the detergent CHAPS [33]. However, enrichment of the tetraspanin-associated protein PGRL was seen. The enrichment of MHC class I and the tumour-associated antigen Mart-1 in melanoma-derived exosomes is consistent with previously published results using cell lines Mel-888 and Fon [15]. Western blotting also identified the enrichment of Mel-CAM, (MUC-18), a membrane glycoprotein of the immunoglobulin superfamily involved in cell adhesion and a marker of tumour progression in melanoma and prostate cancer [43, 45]. Several adhesion molecules have been identified in exosomal preparations previously but this is the first report of the presence of Mel-CAM [26, 33]. This further confirms the enrichment of tumour antigens in exosomes which is of potential interest in their development as vaccine-based therapies.

The 2-D gel profiles of the exosome preparations from the two melanoma cell lines showed striking similarity and this study provides the starting point for the generation of a protein master map of melanoma-derived exosomes which ultimately will be compared with exosomes derived from other epithelial cell lines. As expected, extensive

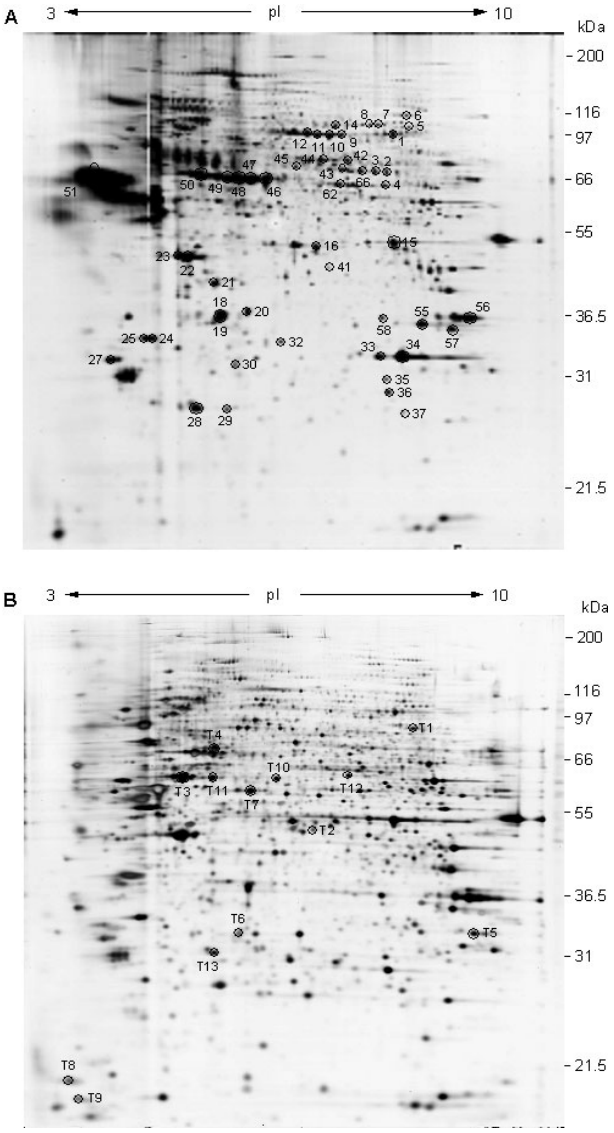

Figure 5. Master maps of proteins identified in MeWo-derived exosomes and MeWo total cell lysates. 2-D PAGE master maps of (A) MeWo-derived exosomes and (B) MeWo total cell lysates showing the locations of proteins identified by mass spectrometry. The numbers refer to the identities listed in Tables 1 (exosomes) and 2 (total cell lysates).

differences were seen between the gel profiles of exosomes and whole-cell lysates. Previous proteomic studies of exosomes have focused on the use of 1-D SDS-PAGE analysis with the most extensive of these finding at least 7 proteins to be strongly enriched in DC-derived exosomes compared with DC lysates [13]. These were the cytosolic proteins annexin II, heat shock cognate protein hsc73 and G protein Gi2a, and the integral or peripherally associated membrane proteins MHC class II, Mac-1, CD9, and MFG-E8. Several of these were also found in our study including ones implicated in the immunostimulatory function proposed for exosomes, such as MHC class I and many other members of the heat shock protein family. Subsequent expansion of this DC-based study [26] found a further 21 proteins identified as being associated with exosomes and likely to be implicated in their biogenesis including cytoskeletal-related proteins (cofilin, profilin-1, and elongation factor 1α) and proteins involved in membrane transport and signalling factors (annexins, Rab proteins, and syntenin). In addition, several proteins involved in apoptosis were also found including thioredoxin peroxidase II, Alix, 14-3-3, and galectin-3 and this was not due to contamination with apoptotic bodies [26]. It is readily apparent that the findings of our study using melanoma exosomes overlap with those of DC-derived exosomes, with either identical proteins being found as indicated in Table 1, or members of the same families, such as G proteins, Rab proteins, and 14-3-3 proteins. This therefore further supports the idea of distinct proteins being conserved in exosomes irrespective of their origin. In addition to the above proteins, several other proteins such as MHC class I and II, several members of the integrin and Ig families, tetraspanins such as CD63, CD81, CD9, and CD82, heat shock proteins and cytoskeletal proteins, such as actin and tubulin, appear to be conserved between exosomes of different origins [9, 10, 12, 13, 26, 33, 44]. In terms of exosome biogenesis, the absence of proteins of mitochondrial, ER or Golgi apparatus is confirmed in our study in support of an endosomal origin. The presence of nuclear proteins may reflect shuttling between the nucleus and cytoplasm.

Several proteins not previously described to be associated with exosomes were found in this study including ezrin, radixin, PGRL, p120 catenin, syntaxin-binding proteins 1 and 2, Septin 2 (Nedd5) and WD repeat-containing protein 1. Moesin has been described previously in B cell-derived exosomes [33] and together with ezrin and radixin, is a member of the ERM (ezrin-radixin-moesin) family which are thought to be involved in cell-cell and cell-substrate interaction and the association of actin filaments with the plasma membrane. Changes in expression of members of the ERM family have been found in some cancers including melanoma [46] and recently it has been shown that inactivation of ERM proteins are involved in disanchoring of the actin cytoskeleton from the plasma membrane in T cells leading to changes in cell rigidity and more effective formation of the immunological synapse between an antigen presenting cell and T cells [47].

PGRL is a novel member of the immunoglobulin (Ig) protein subfamily and associates specifically with the tetraspanin molecules CD9 and CD81 [48], both of which have been

Table 2. Identities of the proteins found to be present in MeWo whole-cell lysates but absent in exosomes

Spot	Protein name	Acc. No.	Theoretical		Observed		Peptide No.	Sequence coverage (%)	Subcellular localisation/ function
			MW (kDa)	p*I*	MW (kDa)	p*I*			
T1	Aconitase	Q99798	82.4	6.85	84.1	6.9	49	60	Mitochondrial TCA cycle.
T2	Ornithine aminotransferase	P04181	44.8	5.72	51.7	5.9	24	67	"
T3	Heat shock protein 60	P10809	58.0	5.24	60.3	5.2	37	72	Mitochondrial matrix. Molecular chaperone.
T4	GRP 75 (mortalin)	P38646	68.8	5.44	70.5	5.4	40	59	"
T5	VDAC-1 (outer mitochondrial membrane protein porin 1)	P21796	30.6	8.63	32.2	8.4	18	72	Mitochondrial membrane and plasma membrane ion channel.
T6	3-Hydroxyisobutyrate dehydrogenase	P31937	31.5	5.54	32.4	5.5	8	24	Mitochondrial enzyme.
	Cathepsin D	P07339	26.6	5.56			23	53	Lysosomal. Acid protease.
T7	ERp60	P30101	54.3	5.61	57.9	5.5	27	51	Endoplasmic reticulum.
T8	Ribosomal protein P1	P05386	11.5	4.26	17.5	3.8	Identified by MS/MS		Cytoplasm. Ribosomal subunit.
T9	Ribosomal protein P2	P05387	11.7	4.42	16.5	4.0	6	71	"
T10	TCP1, subunit α	P17987	60.3	5.8	59.8	5.7	30	67	Cytoplasm. Molecular chaperone.
T11	TCP1, subunit ε TCP1, subunit υ	P48643	59.7	5.45	60.3	5.6	54	80	"
		P50990	59.6	5.42			21	35	"
T12	TCP1, subunit ζ	P40227	58.0	6.24	60.8	6.1	36	70	"
T13	Prohibitin	P35232	29.8	5.57	31.3	5.4	21	83	Cytoplasmic. Inhibits DNA synthesis.

The spot numbers correspond to those indicated on the master map in Figure 5B. Theoretical MW and p*I* are as calculated for the predicted mature, unmodified proteins with the observed values being those seen for the proteins separated by 2-D PAGE. The peptide number is the number of peptides found by MS to match the identified protein with the sequence coverage indicated.

previously reported to be enriched in exosomes [10, 13]. In addition PGRL has been recently described to associate with the metastasis suppressor KAI1/CD82, functioning together to inhibit cell migration [49]. Tetraspanins form an extensive complex network of interactions, often called the tetraspanin web, associating with many proteins such as MHC molecules, integrins and growth factor receptors such as EGFR [50]. In many cases the exact specificity and functions are not clear but members such as CD9 and CD81 have been implicated in cell fusion, cell motility, and adhesion. Our study did not specifically detect members of the tetraspan family in the exosomes, possibly reflecting their poor solubility in CHAPS-based lysis buffers [33], although other membrane proteins are apparent amongst our profiles.

Several of the proteins identified here for the first time as being components of exosomes have been implicated in membrane fusion events, for example, syntaxin-binding proteins (STXBP) 1 and 2. The SNARE superfamily, which includes the syntaxins, plays a key role in regulating intracellular membrane fusion events [51]. It is likely therefore that STXBP1 and 2 have a regulatory role in exosome formation. A number of proteins found have been implicated in functional interactions with the cytoskeleton including WD-repeat protein 1 which interacts with cofilin and is involved in disassembly of actin filaments [52], the GTPase Septin 2 (Nedd5) which is member of the septin family [53, 54], and catenins which are part of a multiprotein complex linking cadherins to the actin cytoskeleton. The presence of a number of cytosolic

enzymes including several involved in glycolysis is interesting and presumably reflects their relative abundance, with some having been identified before [14, 33]. Little is known about the recently described translin-associated factor X (TRAX) interacting protein [55] which is cytoplasmic and normally colocalises with TRAX. Translin binds DNA particularly in recombination hotspots and translin-associated factor X (TRAX) is its cytoplasmic binding partner. The exact function of this protein is unknown but it is postulated to regulate the RNA and DNA binding activity of translin.

In conclusion, this study has shown for the first time the potential of global proteomic profiling using 2-D PAGE and mass spectrometry in examining the protein composition of exosomes, confirming previous reports and demonstrating the presence of some proteins for the first time. However, membrane proteins continue to be underrepresented on 2-D gels due to their relative hydrophobicity, although using combinations of urea with thiourea as used here partially overcomes this problem and the use of detergents of the sulfobetaine series is also being exploited [56, 57]. Many membrane proteins contained within exosomes have been shown to be resistant to CHAPS solubilisation introducing a further problem. In future studies it would be ideal to combine more extensive 2-D PAGE analysis with either 1-D SDS-PAGE or alternatively adopt a multidimensional chromatography and mass spectrometric approach as used successfully for "shotgun" proteomics with success in prostasomes [58]. The need for such proteomic studies has been highlighted [30] and the additional knowledge generated will assist in further determining the mechanisms of biogenesis of exosomes, provide insight into their potential functions *in vivo* and if incorporated into clinical trials, may determine the critical factors for activity. Ultimately this may result in the future generation of synthetic exosomes, thus avoiding the need for tedious purification schemes. Previous demonstrations [15, 17] and the results of this study showing clear enrichment for tumour antigens and tumour-associated proteins, such as Mart-1, Mel-CAM, Her2/neu, TRP, and gp100, may also make exosomes an attractive source for proteomic mining in the field of marker or antigen discovery.

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