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Ubiquitinated Proteins in Exosomes Secreted by Myeloid-Derived Suppressor Cells

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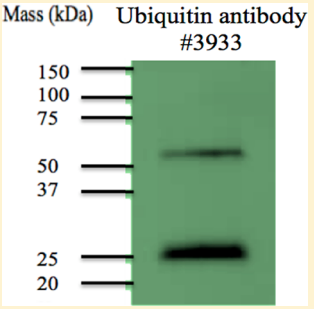
Supporting Information

ABSTRACT: We provide evidence at the molecular level that ubiquitinated proteins are present in exosomes shed by myeloid-derived suppressor cells (MDSC). Ubiquitin was selected as a post-translational modification of interest because it is known to play a determinant role in the endosomal trafficking that culminates in exosome release. Enrichment was achieved by two immunoprecipitations, first at the protein level and subsequently at the peptide level. Fifty ubiquitinated proteins were identified by tandem mass spectrometry filtering at a 5% spectral false discovery rate and using the conservative requirement that glycinylglycine-modified lysine residues were observed in tryptic peptides. Thirty five of these proteins have not previously been reported to be ubiquitinated. The ubiquitinated cohort spans a range of protein sizes and favors basic pI values and hydrophobicity. Five proteins associated with endosomal trafficking were identified as ubiquitinated, along with pro-inflammatory high mobility group protein B1 and proinflammatory histones.



KEYWORDS: *Exosomes, ubiquitinated proteins, proinflammatory against, MDSC, endosomal pathway, aberrant tryptic digestion, immunoaffinity enrichment, LC−MS/MS*

INTRODUCTION

Exosomes are extracellular vesicles 30−100 nm in diameter that are shed by most cells.[1,2] They were first observed in 1987 in maturing reticulocytes,[3] and recently they have gained attention as agents of intercellular communication[4] and as potential prognostic tools.[5] In previous studies, we interrogated the content of myeloid-derived suppressor cells (MDSC) and exosomes shed by MDSC. We focused on these cells because of their widespread presence in most cancer patients and their critical role in promoting tumor progression through their inhibition of innate and adaptive antitumor immunity.[6] We have reported that exosomes shed by MDSC contain proinflammatory molecules that drive the accumulation and immune suppressive potency of MDSC and macrophages, respectively, and have identified the specific proteins responsible for these bioactivities.[7] On the basis of these and other potential functions, there is global interest in the nature of the protein cargo carried by exosomes.[8−12]

Exosomes are formed by a series of intracellular events initiated by the invagination of the plasma membrane to form endosomes.[1,2] Within endosomes, proteins are sorted into luminal vesicles to form late endosomes or multivesicular bodies.[13,14] These luminal vesicles and their cargo are then incorporated into the lysosome for degradation, recycled to the plasma membrane, or exocytosed as exosomes. Ubiquitination has been shown to signal both the internalization of surface proteins and the sorting of endosomal proteins into luminal vesicles.[13,15−17]

Given the strategic role of ubiquitination in intracellular protein trafficking, the present study seeks to confirm the presence and identify ubiquitinated proteins in exosomes derived from MDSC. A previous study using western blot analyses indicated that exosomes and their parental cells contain distinct populations of ubiquitinated proteins;[18] however, the conjugated proteins were not identified. Identification of the conjugated proteins allows assignment of their original locations in the parent cell, their original cell functions, and the range of protein sizes and pI values and may contribute to understanding the complexity of the endosomal pathway. In addition, enriching for ubiquitinated proteins allows detection of proteins that may be relatively low in abundance but play an important role in exosome structure and function and that contribute to MDSC function.

In the present work, ubiquitinated proteins have been recognized in MDSC-derived exosomes by identifying peptides that carry glycinylglycine-modified lysine residues as remnants of the ubiquitin carboxyl terminus. Additionally, we have used Gene Ontology annotations and the UniProt database to look for trends in the source, function, size, and pI values of the ubiquitinated species.

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Mass spectrometry-based bottom-up proteomics has proven to be a powerful tool for recognizing ubiquitinated tryptic peptides and identifying sites of ubiquitination on these peptides and their related proteins. Tryptic digestion of ubiquitinated proteins cleaves ubiquitin at R74, which leaves two glycine residues on the modified lysine of the substrate peptide.[19] Enrichment of ubiquitinated proteins from MDSC-derived exosomes was accomplished using two immunoprecipitation steps: immunoprecipitation of ubiquitinated proteins followed by tryptic digestion and immunoprecipitation of peptides containing glycinylglycine-modified lysine residues. In a parallel workflow, immunoprecipitation of ubiquitinated proteins was followed by 1D gel electrophoresis and in-gel digestion. In each case, enriched peptides were analyzed using LC−MS/MS and a bioinformatic search program that allowed for the variable modification of glycinylglycine-modified lysine residues (K_{GG}).[20] Here, we identify 50 ubiquitinated proteins carried by MDSC-derived exosomes. These ubiquitinated proteins constitute a small subset of proteins in these exosomes, originate from a diversity of subcellular locations, and have a variety of functions.

EXPERIMENTAL SECTION

Myeloid-Derived Suppressor Cells

BALB/c mice were injected in the mammary fat pad with approximately 7000 wild-type 4T1 mammary carcinoma cells stably transfected to express interleukin-1β (IL-1β). When tumors were greater than approximately 8 mm in diameter (about 3−4 weeks after initial inoculation), MDSC were harvested from the blood, stained with fluorescently labeled monoclonal antibodies against markers of MDSC (Gr1 and CD11b), and analyzed by flow cytometry.[21] Cell populations that were greater than 90% $Gr1^+CD11b^+$ were used in all experiments.[21] For each experiment, a total of about 1×10^8 MDSC were pooled from 2 to 3 mice. The UMBC and UMCP Institutional Animal Care and Use Committees approved all procedures with animals and animal-derived materials.

Exosomes

MDSC were plated in serum-free HL-1 medium (BioWhittaker, Walkersville, MD) and maintained at 37 °C with 5% CO_2. After 18 h, the cultures were centrifuged at 805*g* for 5 min (Eppendorf 5810 rotor, Eppendorf, Hamburg), the pellets were discarded, and the supernatants were centrifuged at 2090*g* for 30 min (Sorvall RC5C, SS34 rotor, DuPont, Wilmington, DE). The supernatants were then ultracentrifuged at 100 000*g* for 20 h at 10 °C (Beckman L8, SW40Ti rotor, Beckman, Pasadena, CA). The supernatants were discarded, and the pellets containing the exosomes were resuspended in PBS. Absorbances were measured at 260 and 280 nm. Exosomes were stored at −80 °C until use.

Exosomes were lysed in an optimized lysis buffer of 8 M urea in 50 mM ammonium bicarbonate with 50 μM of deubiquitinase inhibitor PR-619 (LifeSensors, Malvern, PA) and 1% of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). They were centrifuged at 14 000*g* for 30 min with a 3 kDa molecular weight cut off filter, and the supernatants were discarded. This process was done three times. After lysis, the buffer was diluted to 0.8 M urea in 50 mM ammonium bicarbonate. Protein content before and after immunoprecipitation was measured by the Quick Start Bradford Assay (Bio-Rad, Hercules, CA).

Immunoprecipitation of Ubiquitinated Proteins

Ubiquitinated proteins were enriched using Protein A-Sepharose 4B beads (Invitrogen, Carlsbad, CA) that had been incubated with anti-ubiquitin antibody 3933 (Cell Signaling Technology, Danvers, MA) in a 1:600 dilution with rotation for 4 h at 4 °C. Excess antibody was removed from the beads by washing with 0.8 M urea in 50 mM ammonium bicarbonate and centrifuging three times at 3000*g* for 2 min. One-hundred micrograms of exosome lysate was added to the Sepharose bead slurry and incubated with rotation overnight at 4 °C. The unbound fraction was collected via centrifugation at 500*g* for 5 min. The Sepharose bead slurry was washed with 50 mM ammonium bicarbonate and centrifuged at 1000*g* for 5 min to remove nonspecifically bound proteins. Bound proteins were eluted by incubating the Sepharose bead slurry in 0.2 M glycine, pH 2.6, for 1 h at 4 °C and collected via centrifugation at 13 000*g* for 5 min. The elution was repeated, and the two elution fractions were combined.[22] Enriched fractions of ubiquitinated exosomal proteins were subsequently processed either by tryptic digestion in gel or in solution and immunoprecipitation of peptides with glycinylglycine-modified lysine residues.

In-Gel Tryptic Digestion of Ubiquitinated Proteins

Proteomic studies were conducted on exosomal proteins enriched for ubiquitin conjugates by immunoprecipitation. Three biological replicates were resuspended in 2% SDS, 5% β-mercaptoethanal, and 62.5 mM Tris HCl and reduced at 90 °C for 5 min. The samples were then loaded onto 8−16% polyacrylamide gels (Bio-Rad) and subjected to electrophoresis for approximately 50 min at 200 V, 15 mA, and 50 W. The gels were stained using Coomassie blue (40% methanol, 20% acetic acid, 0.1% m/v Coomassie blue reagent 250; Thermo Scientific, San Jose CA) stain and then cut into 13 slices. After destaining, tryptic digestion was performed on each gel slice overnight at 37 °C.[23] The extracted tryptic peptides were resuspended in 0.1% formic acid for injection into the LC−MS/MS. (See below for instrumental conditions.)

Tryptic Digestion and Immunoprecipitation of Glycinylglycine-Tagged Peptides

Enriched fractions of ubiquitinated exosomal proteins from five biological replicates were frozen, lyophilized, and resuspended in 50 mM ammonium bicarbonate. Proteins were reduced with 20 mM dithiothreitol for 30 min at 56 °C and alkylated with 10 mM methylmethanethiosulfonate for 45 min. One microgram of trypsin was added to each fraction, and digestion was performed overnight at 37 °C. As a positive control, a ubiquitin dimer linked with an isopeptide bond at K48 (Life Sensors, Malvern, PA) was also digested with trypsin under these conditions.

Peptides with glycinylglycine-modified lysine residues were enriched using Protein A-Sepharose 4B beads coupled to anti-diglycyl-lysine antibody GX41 (Millipore, Billerica, MA) using the same procedure as that with the anti-ubiquitin antibody, except the anti-diglycyl-lysine antibody was prepared at a 1:1000 dilution. The fractions of immunoprecipitated ubiquitinated proteins were added to the Sepharose bead slurry and incubated with rotation overnight at 4 °C. The unbound fraction was removed via centrifugation at 500*g* for 5 min. The Sepharose bead slurry was washed with 50 mM ammonium bicarbonate and centrifuged at 1000*g* for 10 s to remove nonspecifically bound peptides. Bound peptides were eluted by incubating the Sepharose bead slurry in 0.2 M glycine, pH 2.6,

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for 1 h at 4 °C and collected via centrifugation at 13 000*g* for 5 min. The elution was repeated, and the two elution fractions were combined. Prior to LC−MS/MS analysis, all fractions were desalted with C18 TopTip spin columns (Glygen, Columbia, MD) and resuspended in 100 μL of 0.1% formic acid.

Western Blotting

All fractions were subjected to one-dimensional gel electrophoresis on an 8−16% Criterion precast gel (Bio-Rad) at 200 V, 50 mA, and 15 W for 56 min, followed by transfer to a PVDF membrane (EMD Millipore, Billerica, MA) at 100 V, 350 mA, and 35W for 1 h. Free ubiquitin, polyubiquitin, and ubiquitinated proteins were detected by blotting with anti-ubiquitin antibody 3933 (Cell Signaling Technology) followed by anti-mouse IgG-HRP (Cell Signaling Technology). Protein bands were visualized with an Image Lab System (Bio-Rad, Hercules, CA) using the Gel-Doc program (Kodak Molecular Imaging Systems) and the SuperSignal West Dura chemiluminescent substrate (Thermo Fisher Scientific, Waltham, MA).

Extraction of Histones

Exosomal histones were extracted using the EpiQuick Total Histone Extraction Kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions and analyzed for ubiquitination via western blotting with anti-ubiquitin antibody 3933 as previously described.

LC−MS/MS and Bioinformatics Analysis

LC−MS/MS analyses were performed on a Shimadzu Prominence nano HPLC (Shimadzu Scientific Instruments, Columbia, MD) in-line with an LTQ Orbitrap XL (Thermo Fisher Scientific). A 10 μL aliquot of tryptic peptides was injected onto an Acclaim PepMap 300 C18 precolumn (Dionex, Sunnyvale, CA) followed by desalting with 10% solvent A (97.5% H_2O, 2.5% CAN, and 0.1% formic acid) for 20 min. Peptides were fractionated on a C18 analytical column (150 × 0.15 mm, 300 Å, Grace Davidson Discovery Sciences, Deerfield, IL) with a linear gradient increasing from 0 to 40% solvent B (97.5% ACN, 2.5% H_2O, and 0.1% formic acid) in 85 min, followed by an increase from 40 to 85% solvent B in 20 min. The flow rate was 500 nL/min. Precursor scans were acquired in the orbitrap with a resolution of 30 000 at m/z 400. In each cycle, the nine most abundant ions were selected for fragmentation by collisional induced dissociation, and product ion scans were acquired in the LTQ. A dynamic exclusion of 1 repeat count over 180 s was used.

Peptide and protein identifications were made by the PepArML[24,25] meta-search engine against the UniProt mouse database (July 2014). For the in-gel digestion, all peptide identifications were filtered at 10% spectral FDR, and proteins were required to be supported by at least 2 unshared peptides, bounding a protein FDR at 1%. For the two-step immunoaffinity enrichment of glycinylglycine-modified lysine-containing peptides, all peptide identifications were filtered at a 5% spectral FDR. Fixed modifications listed methylthio modification of cysteine, and variable modifications included oxidation of methionine and glycinylglycine modification of lysine. Proteins with at least one peptide with a lysine residue tagged with glycinylglycine were considered to be ubiquitinated. When a protein identification was based on a single peptide from the double immunoaffinity workflow, spectra are presented in the Supporting Information. Subcellular location and function assignments of the identified proteins were made using the Protein Information Resource GO Slim (http://pir.georgetown.edu) using UniProt Gene Ontology annotations (July 2014).

RESULTS AND DISCUSSION

Western blotting was used to determine if MDSC-derived exosomes contain ubiquitinated proteins. A general ubiquitin antibody was used as well as antibodies that recognize K48 and K63 linkages in ubiquitin chains. Figure 1 confirms the presence of ubiquitinated proteins, including proteins with K48- and K63-linked branched ubiquitins. Consequently, mass spectrometry-based proteomic strategies were used to identify conjugated proteins. In-gel digestion of immunoprecipitated ubiquitin-conjugated proteins was performed and evaluated. Table 1 lists 16 ubiquitinated proteins identified from glycinylglycine-tagged peptides recovered from tryptic digestion in gel. Table 2 lists 38 proteins identified from glycinylglycine-modified peptides isolated after tryptic digestion in solution. The experimental design, with immunoaffinity isolation of glycinylglycine-modified peptides, leads to several protein identifications that are each based on a single tagged peptide and thus are less reliable than those listed in Table 1. These are listed in Table 2, and annotated spectra are provided in Supporting Information.

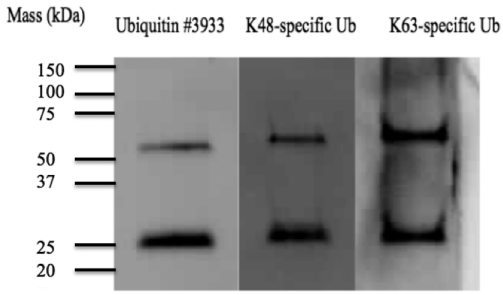

Figure 1. Western blots of lysates from MDSC-derived exosomes. Antibodies used are (left) anti-ubiquitin 3933, (middle) anti-K-48-linked polyubiquitin, and (right) anti-K-63-linked polyubiquitin.

To report a protein as being ubiquitinated, reliable identification was required of at least one peptide containing a K_{GG} residue. Initial identifications were made by the PepArML meta-search engine (see Experimental section), and all candidate tandem mass spectra were confirmed manually (Supporting Information Figure S1). A combined total of 65 tryptic peptides containing modified lysines correspond to 50 ubiquitinated proteins (Supporting Information Table S1). Only 10 of the 50 proteins identified were previously reported in an MDSC-derived exosome lysate,[7] demonstrating that enrichment for ubiquitinated proteins enabled the identification of low-abundance proteins in exosomes. As suggested by the western blots in Figure 1, a polyubiquitin fragment was characterized with multiple branch sites. Several histones were also observed to be conjugated at multiple unique and non-overlapping sites, and the pro-inflammatory high mobility group protein B1 (HMG B1) was found to be ubiquitinated. The glycinylglycine-lysine sites identified experimentally were compared to ubiquitination sites predicted in silico by a ubiquitination prediction tool, UbiProber.[26] Of the 65 peptides identified, the glycinylglycine-lysine sites in 42 peptides are ubiquitination sites predicted with probabilities > 0.7, UbiProber's confidence level.

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Table 1. Ubiquitinated Proteins and Peptides Identified from In-Gel Digestion of Exosomal Proteins with Glycinylglycine-Modified Lysine Residues

protein accession	protein name	no. of nonoverlapping peptides identified	protein FDR	peptide sequence
F6XI62	60S ribosomal protein L7 (Fragment)[a]	2	2.450×10^{-5}	REKKKKVATVPGTLKKKVPAGPKTLK(GG)K
P61161	Actin-related protein 2	3	6.486×10^{-4}	VVVCDNGTGFVK(GG)
P26040	Ezrin	3	6.490×10^{-4}	EELMLRLQDYEQK(GG)TKR
P17156	Heat shock-related 70 kDa protein 2	5	4.860×10^{-6}	HWPFRVVSEGGK(GG)PK(GG)
P63158	High mobility group protein B1	4	5.614×10^{-4}	WK(GG)TMSAK(GG)
P10922	Histone H1.0	4	5.610×10^{-5}	AAKPKKAASK(GG)APSK
				K(GG)KPAATPK(GG)K
				KAKKPK(GG)VVK
				ASK(GG)PKKAKTVKPK
P15864	Histone H1.2	11	3.920×10^{-14}	K(GG)ATGAATPKKAAK
				AKKPAAAAVTK(GG)K
				K(GG)VAKSPK
				KAK(GG)VTKPKK
				AAK(GG)PKVAK
P43277	Histone H1.3	9	2.738×10^{-10}	TPVKK(GG)KAK(GG)
				SPKKVKAAK(GG)PK
				KAAKSPAKAK(GG)
				AKASK(GG)PKASKPK
P43274	Histone H1.4	4	1.250×10^{-10}	AKKPAGAAK(GG)
				TVKPKAAKPK(GG)TSK(GG)
P43276	Histone H1.5	10	3.040×10^{-18}	AKK(GG)TGAAKAK
				AKKPAGATPKKPKK(GG)
				K(GG)PAAAGVK
				VTKPKTAKPK(GG)AAKAK
Q07133	Histone H1t	3	6.486×10^{-4}	GKGK(GG)KSASAK(GG)
				TK(GG)AVKKPKATPTK(GG)
P27661	Histone H2A.x	5	4.860×10^{-6}	K(GG)SSATVGPK(GG)APAVGKK
P62806	Histone H4	6	4.207×10^{-7}	GKGGK(GG)GLGK(GG)GGAK
Q6IFX2	Keratin, type I cytoskeletal 42[a]	9	2.729×10^{-10}	NK(GG)ILAATIDNASIVLQIDNAR
P08071	Lactotransferrin	29	1.522×10^{-31}	GDADAMSLDGGYIYTAGK(GG)
P52480	Pyruvate kinase isozymes M1	7	3.640×10^{-8}	GPEIRTGLIKGSGTAEVELK(GG)K

[a]Indicates that the protein has been reported previously to be ubiquitinated.

Tables 1 and 2 also present the glycinylglycine-tagged peptides, where it can be seen that 15 of the 65 peptides have been formed by tryptic cleavage at GG-derivatized lysine residues. This unexpected tryptic cleavage has been observed previously by others,[27,28] and a control experiment was also carried out to confirm its occurrence under the conditions of the present investigation. A commercial ubiquitin dimer linked with an isopeptide bond at K48 was subjected to tryptic digestion as described in the Experimental Section. Both the expected peptide LIFAGK$_{GG}$QLEDGR and the irregular peptide LIFAGK$_{GG}$ were identified by tandem mass spectrometry in approximately a 3:1 ratio (data not shown). This further supports the assignments in Tables 1 and 2.

Several comparisons were made between the ubiquitinated proteins and a larger set of 412 proteins identified in an earlier study of MDSC exosome lysates.[7] Figure 2 presents a comparison of the UniProt-derived locations of exosome proteins referenced to parental MDSC and illustrates an increased percentage of nuclear proteins in the ubiquitinated cohort and significantly lower percentages of cytosolic and plasma membrane proteins. The ubiquitinated nuclear proteins include nine histones and isoforms as well as other nucleic acid binding proteins (transcription factor A, mitochondrial, density regulated protein, transcription initiation factor TFIID subunit 3, and protein Bodl1) (Tables 1 and 2). It should be noted that histones, especially linker histones such as the histone H1 family, have been reported to be located in the cytoplasm and cell surface as well as the nucleus[29] and that several histones are already known to be ubiquitinated, e.g., refs 30−32. The observation of ubiquitinated histones was confirmed by western blotting using anti-ubiquitin antibody 3933 on the histone fraction recovered using a total histone extraction kit (see Experimental Section) (data not shown). Figure 2 also indicates that 12 of the ubiquitinated proteins have no assigned cellular location.

Gene Ontology annotations and the UniProt database were used to compare distributions of protein sizes (without ubiquitin), grand average of hydropathicity (GRAVY) scores, and isoelectric points (pI) of the 50 ubiquitinated proteins identified. The intact masses of the proteins are somewhat evenly distributed between 11 and 327 kDa (excluding ubiquitination). The GRAVY scores and pI distributions are shown in Figure 3, panels a and b, respectively. Although both sets of data illustrate a wide range of GRAVY scores (−0.081 to −1.627) and pI values (4.72 to 11.71), proteins that are ubiquitinated in MDSC-derived exosomes tend to cluster as hydrophilic and basic proteins. Seventy-two percent of the proteins identified have a GRAVY score less than −0.5, and 50% of the proteins have a pI greater than 9.00. (Ubiquitin is not included in these calculations; its monomer has a GRAVY score of −0.489 and a pI value of 6.56.) The bias toward a high pH is consistent with observations by Chen and co-workers,[26]

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Table 2. Ubiquitinated Proteins and Peptides Identified Following Immunoaffinity Enrichment of Tryptic Peptides with Glycinylglycine-Modified Lysines

protein accession	protein name	no. of peptides identified	no. of nonoverlapping K(GG) containing peptides	peptide FDR	peptide sequence
F6XI62	60S ribosomal protein L7 (Fragment)[a,b]	2	1	7.83×10^{-2}	REKKKKATVPGTLKKKVPAGPKTLK(GG)K
Q5SWU9	Acetyl-CoA carboxylase 1	1	1	4.48×10^{-2}	FGGNKVIEKVLIANNGIAAVK(GG)CMRSIR
E0CYH9	Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein	1	1	5.10×10^{-2}	KKKVSIMVSVDGVKVILK(GG)KKKKLLLLQK
Q6P925	Cysteine-rich perinuclear theca 4	1	1	5.10×10^{-2}	AK(GG)RSKLKKKRNPRSKLPK(GG)RSRHSLIR
Q9CQJ6	Density-regulated protein	2	1	5.10×10^{-2}	QKK(GG)K(GG)TVPQKVTIAKIPRAKKKYVTR
P08113	Endoplasmin	2	1	3.63×10^{-2}	LLKVIRK(GG)KLVR
P43275	Histone H1.1	10	2	5.10×10^{-2}	KTVK(GG)TPKKPKKPAVSKKTSKSPKKPKVVK
				5.10×10^{-2}	AKKVAKSPAKAKAVKPKASKAKVTKPK(GG)TPAKPK
P15864	Histone H1.2[b]	11	5	8.66×10^{-2}	K(GG)ATGAATPKKAAK
				8.66×10^{-2}	AKKPAAAAVTK(GG)K
				8.66×10^{-2}	K(GG)VAKSPK
				8.66×10^{-2}	KAK(GG)VTKPKK
				8.66×10^{-2}	AAK(GG)PKVAK
P43274	Histone H1.4[b]	4	2	8.66×10^{-2}	AKKPAGAAK(GG)
				8.66×10^{-2}	TVKPKAAKPK(GG)TSK(GG)
P43276	Histone H1.5[b]	10	7	8.66×10^{-2}	AKK(GG)TGAAKAK
				8.66×10^{-2}	AKKPAGATPKKPKK(GG)
				8.66×10^{-2}	K(GG)PAAAGVK
				8.66×10^{-2}	VTKPKTAKPK(GG)AAKAK
P15975	Inactive ubiquitin carboxyl-terminal hydrolase 53	1	1	4.48×10^{-2}	MAWVK(GG)FLRKPSGNLGK
B2RXC2	Inositol 1,4,5-trisphosphate 3-kinase B	1	1	2.15×10^{-2}	GTPASPRCGSPTPMETDK(GG)RVAPSLER
Q61781	Keratin type I cytoskeletal 14	2	1		TIEDLKSK(GG)ILAATVDNANVLLQIDNAR
Q6IFX2	Keratin, type I cytoskeletal 42[a,b]	9	1	8.66×10^{-2}	NK(GG)ILAATIDNASIVLQIDNAR
Q924L1	LETM1 domain-containing protein 1	1	1	8.52×10^{-3}	MKGIQMLWADGKK(GG)AR
Q0P5X1	Leucine-rich repeat and IQ domain-containing protein 1	1	1	5.10×10^{-2}	KLRKKLEPSVRLALFKKAK(GG)NK(GG)VSVTK
P51960	Myb-related protein A	1	1	8.52×10^{-3}	WSLIAK(GG)HLK(GG)GR
E9Q5F6	Polyubiquitin-C (Fragment)	4	2	1.87×10^{-3}	TLSDYNIQK(GG)ESTLHLVLR
				5.10×10^{-2}	MQIFVK(GG)TLTGK
Q9Z100	Probable carboxypeptidase X1	2	1	2.60×10^{-3}	LRVIKKKKIVVKKRK(GG)KLR
H3BKN5	Probable global transcription activator SNF2L2	1	1	9.13×10^{-3}	VLGRK(GG)LPKKKRVRKKAMK(GG)KR
H3BL88	Protein 9930021J03Rik	2	1	5.10×10^{-2}	K(GG)LKLTKMRAKKKKKKK
E9Q6J5	Protein Bod1l	2	1	3.99×10^{-3}	IKEVLKERKVLEKKV(GG)ALSKRRRK
J3QQ16	Protein Col6a3	1	1	3.63×10^{-2}	DLK(GG)IMVLMLTGDMQR
A2AU83	Protein GM14124	1	1	1.02×10^{-2}	AFSSPSGFLYHK(GG)R
E9PZM7	Protein Scaf11	1	1	5.10×10^{-2}	RK(GG)SVRRGRK(GG)PPLLKKKLRR
G3UWJ2	Protein Zfp69	1	1	2.93×10^{-2}	GEGPCMAESQGPEDPILDVKNKLETK(GG)
F6SB18	RNA-binding protein 28	2	1	5.10×10^{-2}	KVLALPSHRGPKIRRLKERLRRIRQK(GG)
Q8C4U3	Secreted frizzled-related protein 1	2	1	1.29×10^{-5}	IVPKKKKPLKLGPIKKK(GG)ELKRLVLFLK
Q9CZ91	Serum response factor-binding protein 1	2	1	4.05×10^{-4}	KEVKRIRVLVIRK(GG)LVRSVGRLKSKK
Q9CXH7	Shugoshin-like 1	2	1		EKRNKNLAGIGK(GG)
G5E861	Sodium channel and clathrin linker 1	1	1	3.77×10^{-2}	LQQENEQLQKETEDLRKVALEAQK(GG)
Q6PHS6	Sorting nexin-13	1	1	5.28×10^{-3}	DDQVK(GG)GTAEDLVETFFEVEVEMEK
D3Z1Z3	Sphingosine-1-phosphate lyase 1	1	1	5.10×10^{-2}	KKLFKLIRKMPFIGRKVSKAK(GG)KDLVK(GG)
Q9CSP9	Tetratricopeptide repeat protein 14	1	1	5.10×10^{-2}	TK(GG)K(GG)IETRAEKLRKLLKEEKRLKKK
P40630	Transcription factor A, mitochondrial	2	1	2.45×10^{-5}	QRRLKKKALVKRRELILLGKPK(GG)R
Q5HZG4	Transcription initiation factor TFIID subunit 3	2	1	5.10×10^{-2}	LPSSVDVKKKLKKELKTKLKK(GG)KEKQR
Q6ZPJ3	Ubiquitin-conjugating enzyme E2 O	1	1	5.10×10^{-2}	KKSIPLSIKNLK(GG)RK(GG)HKRKKNKVTR

[a]Indicates that the protein has been reported previously to be ubiquitinated. [b]Indicates that the protein was also identified in the in-gel digestion.

who report a greater abundance of positively charged amino acids in ubiquitinated proteins.

Among the combined cohort of 50 proteins, 34 have not been previously reported to be ubiquitinated (Tables 1 and 2). Among these, sorting nexin 13 has been observed to participate in endosomal trafficking of ubiquitinated proteins.[33] Identification of two ubiquitinated keratins is consistent with the proposed role of protein aggregation in invagination, the initial step of exosome formation.[34,35] Other ubiquitinated proteins that are thought to play important roles in endosome and exosome formation include leucine zipper EF hand-containing transmembrane protein 1 (LETM1) and endoplasmin. Although the

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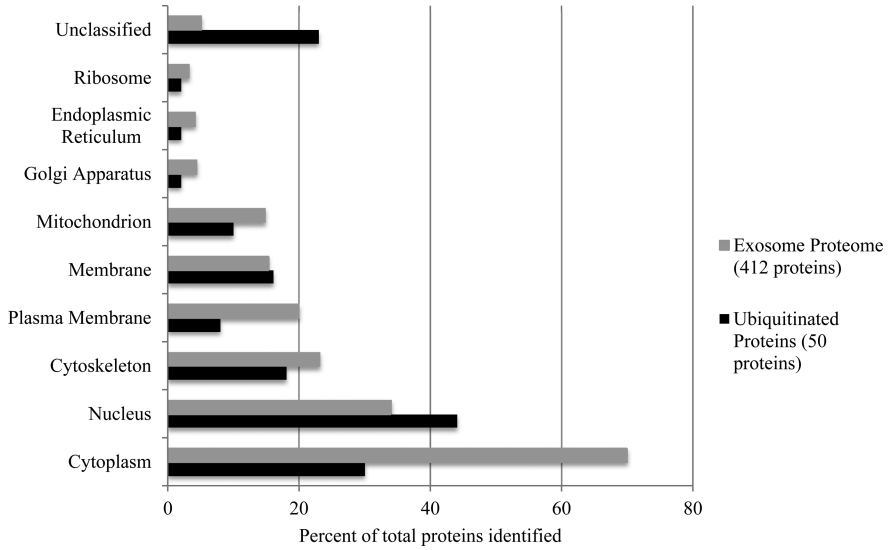

Figure 2. Protein locations assigned to MDSC-derived exosomal lysate (412 proteins) in gray and the ubiquitinated cohort (50 proteins) in black. Some proteins have multiple locations.

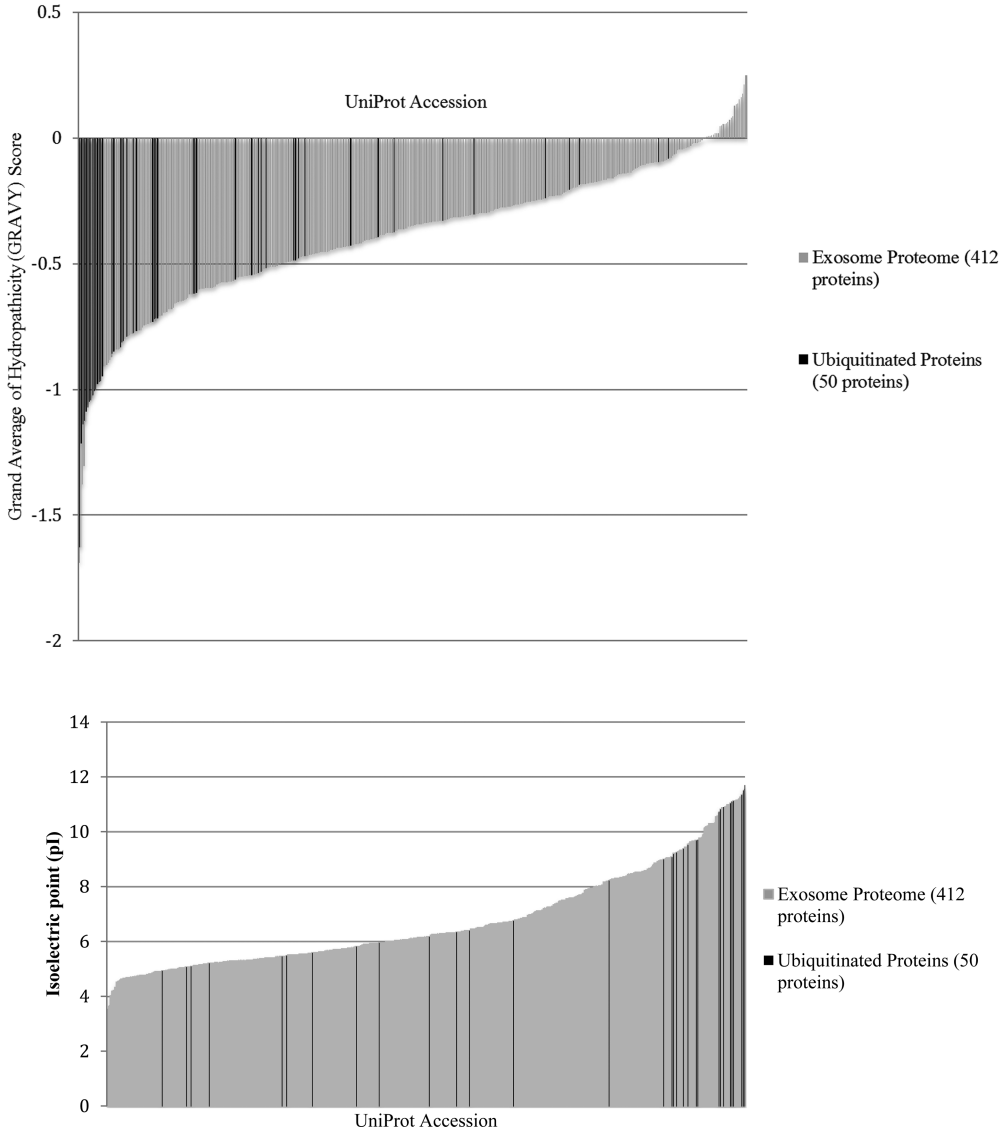

Figure 3. Distribution of (top) grand average of hydropathicity score (GRAVY) and (bottom) isoelectric point of MDSC-derived exosomal lysate (412 proteins) in gray and the ubiquitinated cohort (50 proteins) in black.

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functions of the ubiquitinated proteins are not known, these two unconjugated proteins participate in transporting and maintaining the high luminal concentration of Ca^{2+} required for optimal exocytosis of exosomes.[36]

CONCLUSIONS

On the basis of protein assay results, approximately 10% of the MDSC-derived exosome lysate comprises ubiquitinated proteins. Tandem mass spectrometry coupled with immunoprecipitation has been used successfully to isolate and identify 50 ubiquitinated proteins from MDSC-derived exosomes and to determine their positions of conjugation. Five of these are associated with formation of endosomes and exosomes, consistent with earlier proposals. The skew of pI values toward basicity in the conjugated cohort of exosomal proteins may contribute to their concentration and retention by the progressive acidification that has been documented[37] along the endosomal pathway. The presence of ubiquitinated histones in these exosomes should be considered in the context of heightened interest in extracellular histones and their proinflammatory activity.[38−40] HMG B1 is another pro-inflammatory mediator of particular interest since it was recently established as a driver of MDSC accumulation and suppressive potency.[41] Interestingly, S100 A8 and S100 A9, exosomal pro-inflammatory proteins previously demonstrated[7] to contribute to the bioactivity of MDSC, have not been identified with a glycinylglycine-lysine modification, even though they are quite abundant and readily identified in the exosome data sets.

ASSOCIATED CONTENT

Supporting Information

Table S1. Peptides and proteins identified. Figure S1. Annotated MS/MS spectra for all single peptide protein identifications. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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