research articles Journal of proteome research

Proteomic Analysis of Ubiquitinated Proteins from Human MCF-7 Breast Cancer Cells by Immunoaffinity Purification and Mass Spectrometry

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Received August 16, 2005

Post-translational modification of proteins via the covalent attachment of Ubiquitin (Ub) plays an important role in the regulation of protein stability and function in eukaryotic cells. In the present study, we describe a novel method for identifying ubiquitinated proteins from a complex biological sample, such as a whole cell lysate, using a combination of immunoaffinity purification and liquid chromatography-tandem mass spectrometry (LC−MS/MS) analysis. We have demonstrated the applicability of this approach by identifying 70 ubiquitinated proteins from the human MCF-7 breast cancer cell line after treatment with the proteasome inhibitor MG132. This method will aid the study of protein ubiquitination and may be used as a tool for the discovery of novel biomarkers that are associated with disease progression.

Keywords: ubiquitin • MCF7 • immunoaffinity purification • mass spectrometry • MG132

Introduction

Ubiquitin (Ub) is a small 8.5 kDa protein present in all eukaryotes that participates in a host of critical cellular functions by mediating the selective degradation of regulatory proteins. The progression of the cell cycle,[1] the heat shock response,[2] and antigen presentation,[3] are among the many processes regulated by Ub-dependent proteolysis. Not surprisingly, aberrations in this pathway are implicated in the pathogenesis of many diseases including cancer.[4] Ubiquitination also regulates certain processes by mechanisms that, although poorly understood, do not appear to involve proteolysis. These include ribosomal function,[5] DNA repair,[6] and the activation of protein kinases such as IκB kinase.[7]

Post-translational attachment of Ub to a target protein typically occurs via covalent attachment of its C-terminal glycine to a lysine residue on the target protein by a multi-enzymatic system consisting of activating (E1), conjugating (E2), and ligating (E3) enzymes.[8] Since Ub itself carries seven lysine residues, this initial modification is often followed by the glycine-lysine ligation of additional Ub molecules to the first forming di-, tri-, tetra-, or poly-Ub chains. Recent evidence suggests that for several proteins, initial covalent attachment of Ub may occur via the amino group of the N-terminal residue.[9]

Tetra- and poly-Ub chains serve as consensus signals for protein degradation by the 26S proteasome, which is a multi-subunit protease with activity specific for ubiquitinated proteins. Within the lumen of the barrel-shaped proteasome, the target protein is cleaved into peptides of 3−20 residues that are further hydrolyzed to amino acids by cytosolic peptidases.[10] The liberated poly-Ub chains are not degraded by the proteasome, but are reused for modification of other proteins (see Figure 1a).

Several types of low-molecular weight proteasome inhibitors that can readily enter cells are commercially available. The most widely used are peptide aldehydes, such as Cbz-leu-leu-leucinal (or MG132) and Cbz-leu-leu-norvalinal (or MG115). These agents are substrate analogues and act as potent transition-state inhibitors.[11,12] For example, the K_i of MG132 in vitro is a few nanomolar and its IC_{50} is in the micromolar range for inhibition of proteolysis in cultured cells.[13]

Enzymatic digestion of ubiquitinated proteins with trypsin results in a signature peptide containing a di-glycine remnant derived from the C-terminus of Ub that is covalently attached to a lysine residue on the target protein.[14,15] This signature peptide has a mass addition of 114.1026 Da as well as a missed cleavage site because trypsin is unable to cleave after the modified lysine residue (see Figure 1b). Such a uniquely tagged peptide provides a platform for the identification of ubiquitinated proteins by mass spectrometry because the di-glycine remnant is detected as a variable modification on lysine residues after MS/MS sequencing and database searching.

Previous studies in yeast have shown that an affinity purification step prior to MS/MS analysis offers significant advantages for studying ubiquitinated proteins. Peng et al. identified 72 Ub-protein conjugates from cells expressing 6 × His-Ub while Hitchcock et al. identified 211 membrane-associated ubiquitinated proteins from cells expressing 6× His-Myc-Ub.[15,16] In both cases, large quantities of yeast were cultured and epitope-tagged Ub conjugates were isolated. More recently, Kirkpatrick et al. adapted this strategy to HEK293

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10.1021/pr050265i CCC: $30.25
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Published on Web 10/22/2005

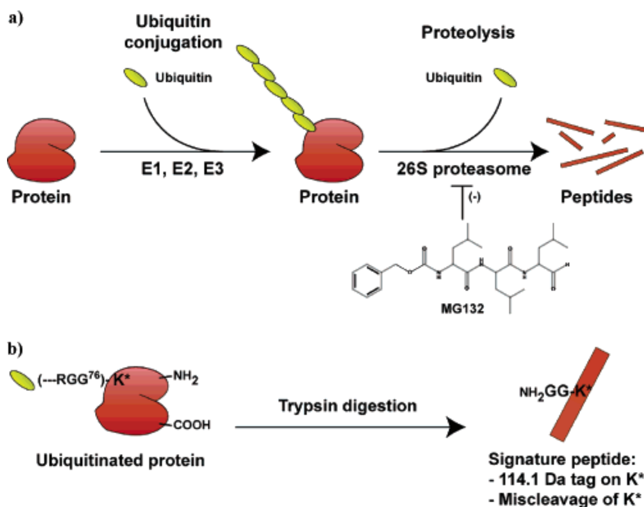

Figure 1. Ubiquitin-Proteasome pathway and tryptic digestion of ubiquitinated proteins. (a) Post-translational modification of proteins by Ub occurs via covalent attachment of its C-terminal glycine to a lysine on the target protein by 3 enzymes (E1, E2, E3). Ubiquitination serves as a consensus signal for protein degradation by the 26S proteasome complex. (b) After tryptic digestion, ubiquitinated proteins contain a di-glycine remnant attached to a lysine residue. A signature peptide with a 114.1026 Da mass increase and a missed cleavage site can be identified by MS/MS.

embryonic kidney cells expressing 6× His-Ub-GFP. Results from a series of experiments enabled the identification of 22 Ub-associated proteins, nearly half of which were identified by a single peptide match.[17]

In the present study, we describe a novel method for identifying ubiquitinated proteins using a combination of immunoaffinity purification and LC−MS/MS analysis. Application of this method enabled the confident identification of 70 ubiquitinated proteins from the human MCF-7 breast cancer cell line after overnight treatment with the proteasome inhibitor MG132. Proteins identified include Ub-ligating enzymes, subunits of the 26S proteasome complex, heat shock proteins, transport proteins, DNA repair proteins, transcription and translation elongation factors, and several proteins involved in cell cycle regulation, apoptosis, and signal transduction. Our findings represent the first proteomic analysis of native ubiquitinated proteins from a genetically unmodified mammalian cell line, and we expect our method to be adapted for the study of other biologically relevant samples soon.

Materials and Methods

Antibodies and Reagents. The mouse monoclonal anti-Ub antibody (clone FK2) was obtained from BIOMOL Inc. (Plymouth Meeting, PA). Purified mouse $IgG_1\kappa$ immunoglobulin was obtained from Sigma-Aldrich (St. Louis, MO). Goat anti-mouse HRP-conjugated secondary antibody was obtained from DAKO (Glostrup, Denmark). MG132 was purchased from Sigma-Aldrich. One mM stock solutions of MG132 were prepared reconstituting in dimethyl sulfoxide. All other reagents, unless specified, were obtained from Fisher Scientific (Burlington, ON).

Cell Culture. Human MCF-7 breast epithelial cancer cells were cultured at 37 °C in 5% CO_2 in DMEM medium (GIBCO−BRL, Burlington, ON) supplemented with 10% fetal calf serum. After reaching 80% confluence, cells were treated overnight with 5−50 μM MG132 in DMEM medium. Cells were harvested, washed twice with phosphate buffered saline (PBS), and resuspended in modified RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 1 mM EDTA) containing a cocktail of protease inhibitors (Roche Diagnostics, Laval, QC). Cell lysates were centrifuged for 10 min at 14 000 rpm to pellet cell debris. Supernatants were collected and protein concentration was assessed using a Bradford protein assay kit (Bio-Rad, Hercules, CA).

Immunoaffinity Purification. Anti-Ub beads were prepared by coupling 2 mg of anti-Ub Ab to 1 mL of protein G-agarose beads (Roche) with dimethyl pimelimidate according to manufacturer's instructions (Pierce, Rockford, IL). 0.8 × 4 cm Poly-Prep chromatography columns (Bio-Rad) were then filled with 0.5 mL of anti-Ub beads. Prior to each purification, cell lysates were pre-cleared with columns containing only protein G-agarose beads for 10 min. at 4 °C. Pre-cleared lysates containing 12.5 mg of total protein were then loaded onto immunoaffinity purification columns and allowed to incubate for 2 h at 4 °C. Columns were washed 10× with 5 mL of modified RIPA buffer. Bound material was eluted with 2% SDS for 20 min at 37 °C. Eluates from three successive purifications were pooled together and concentrated with an Amicon Ultra-4 10 000 MWCO centrifugal filter (Millipore, Nepean, ON). Five times sample buffer (2% SDS, 10% glycerol, 40 mM Tris pH 6.8, 715 mM β-mercaptoethanol when diluted to 1×) was added to the concentrated samples and proteins were separated on a 10% SDS-PAGE gel and visualized with a Colloidal Coomassie (Invitrogen).

LC−MS/MS. Protein bands were excised from SDS-PAGE gels and subjected to in-gel tryptic digestion as previously described.[18] Digestions were carried out for 8 h at 37 °C with sequencing grade trypsin (Promega, Madison, WI). Peptides from each gel band were extracted, brought to a final volume of 10 μL with 5% formic acid, and analyzed by LC−MS/MS. An Agilent 1100 series HPLC system (Agilent Technologies, Palo Alto, CA) was used to load peptides at 2 μL/min onto a 75 μm × 50 mm precolumn packed with 5 μm YMC ODS-A C_{18} beads (Waters, Milford, MA). Following a desalting step, the flow was split and peptides were eluted through a second 75 μm × 50 mm column packed with the same beads at approximately 200 nL/min using a 5−80% gradient of acetonitrile with 0.1% formic acid for 1 h. The LC effluent was electrosprayed into the sampling orifice of a QSTAR Pulsar quadrupole-TOF mass spectrometer (ABI/MDS Sciex, Concord, ON). MS/MS data was then analyzed and matched to human protein sequences in the MSDB database using the Mascot search engine. Peptide and MS/MS mass tolerances were set at ±100 ppm and 0.2 Da, respectively.

Western Blotting and Immunoprecipitation. Proteins were separated on NuPage 4−12% Bis-Tris pre-cast gels (Invitrogen) and transferred onto nitrocellulose. Membranes were blocked with 2% BSA (BioShop, Burlington, ON) overnight at 4 °C, incubated with 1° Ab for 1 h at RT, washed 4× with 0.1% Tween-20 in PBS for 5 min, and incubated with 2° Ab for 1 h at RT. After another round of washing, bands were visualized with ECL reagent (Amersham Biosciences, Oakville, ON) and BioMax XAR imaging film (Kodak, New Haven, CT). For immunoprecipitations, pre-cleared cell lysates were incubated with 10 μL of anti-Ub beads (described above) for 2 h at 4 °C. After washing 3× times with modified RIPA buffer, bound material was eluted by heating the beads in 2× sample buffer for 5 min at 95 °C.

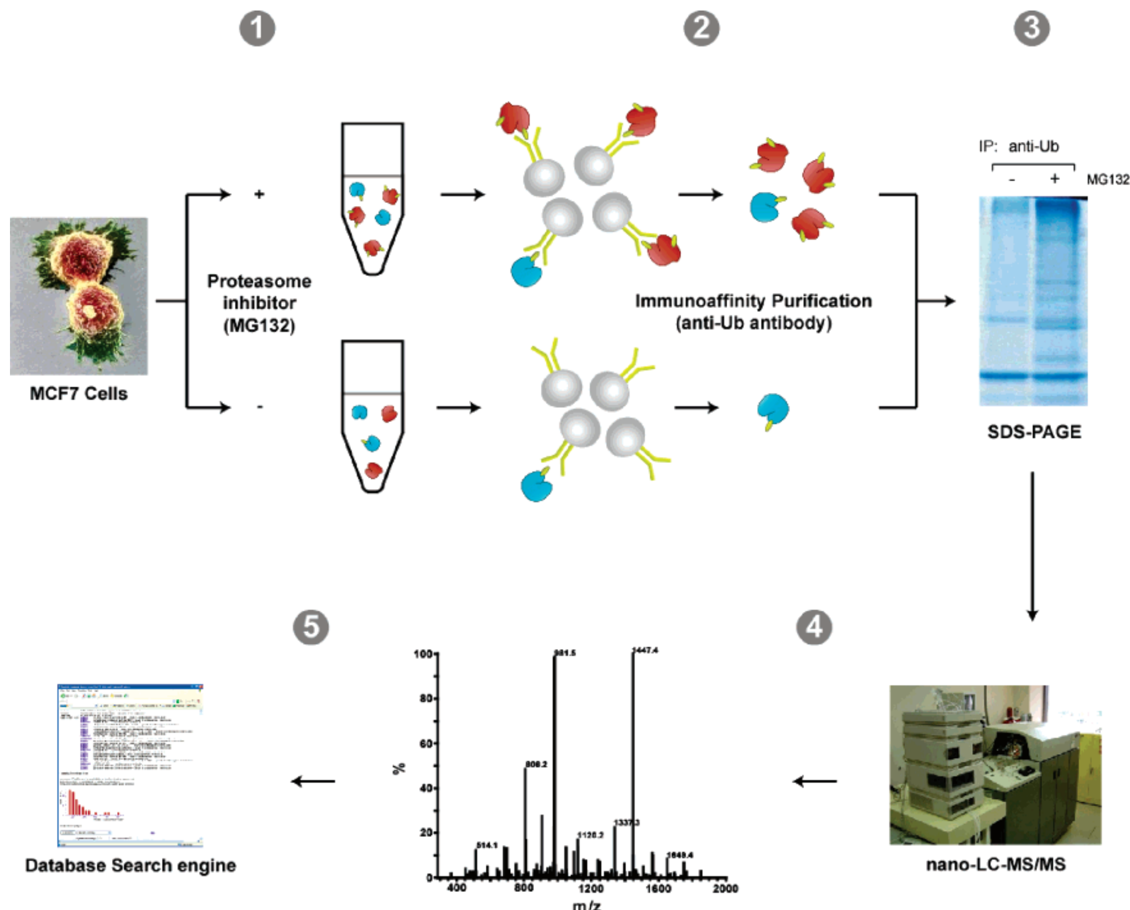

Figure 2. Experimental method. (1) MCF7 cells were treated with MG132, or were left untreated. (2) Ubiquitinated proteins were isolated by immunoaffinity purification. (3) Proteins were separated by SDS-PAGE and visualized with Colloidal Coomassie. Gel bands were excised and subjected to in-gel tryptic digestion. (4) Peptides were analyzed by LC−MS/MS. (5) MS/MS spectra were matched to human protein sequences after database searching and were manually verified to confirm ubiquitination sites.

Results and Discussion

Although many substrates of the Ub-proteasome pathway have been individually characterized by biochemical and genetic approaches,[19,20] only a few large-scale studies of ubiquitinated proteins have been reported primarily due to the difficulties encountered in isolating these low-abundance proteins using conventional purification methods. Several groups have reported that when Ub is tagged at its N-terminus with the Myc epitope or poly-Histidine, the isolation of Ub-conjugates is greatly facilitated.[21−23] Recently, Gygi and coworkers have adapted this strategy for the purification of 6× His- and 6× His-Myc tagged Ub-conjugates from yeast cells for mass spectrometry analysis.[15,16]

Epitope tagging strategies, however, are limited to cells that have been genetically modified to express tagged Ub and it is known that such molecules do not completely function as their wild-type counterparts.[21] In addition, many false positives are likely to be obtained because expression levels of these molecules may differ significantly compared to normal physiological conditions. Furthermore, when Nickel (Ni)-affinity chromatography is used to purify His-tagged Ub-conjugates, numerous proteins containing multiple His residues in their primary structure, or that have an alignment of His within their secondary or tertiary structures, are co-purified nonselectively.

Identification of ubiquitinated proteins, whether in cell lysates, tissues, or biological fluids, may enable the discovery of relevant protein-based disease and drug biomarkers. This is because degenerative diseases that destroy specific cells, or diseases in which there is a certain level of necrosis, are known to release ubiquitinated proteins into the blood.[24] In addition, drugs that induce apoptosis may also cause the accumulation and release of ubiquitinated proteins into biological fluids.[25] With this in mind, it was our goal to develop a novel proteomics method for the analysis of native ubiquitinated proteins from a complex biological sample (i.e., a human whole cell lysate) using a combination of immunoaffinity purification, SDS-PAGE separation, and LC−MS/MS analysis. For immunoaffinity purification, a mouse monoclonal anti-Ub antibody (clone FK2) that recognizes both mono- and poly-ubiquitinated species, but not free Ub, would be employed. This antibody has been successfully used to investigate the dynamics of Ub-conjugation and for the development of immunoassays permitting the quantification of intracellular and serum Ub chains.[26−28] A schematic representation of the method is outlined in Figure 2.

MCF-7 cells, which serve as a model system for breast cancer cell transformation and proliferation,[29,30] were treated overnight with the proteasome inhibitor MG132 prior to immunoaffinity purification. Biochemical studies have shown that many regulatory proteins are degraded by the Ub-proteasome pathway and because of their rapid degradation, such proteins are often very hard to isolate.[13] Therefore, their stabilization with proteasome inhibitors is a valuable means to enhance cell content and to facilitate their isolation from mammalian cells. As shown

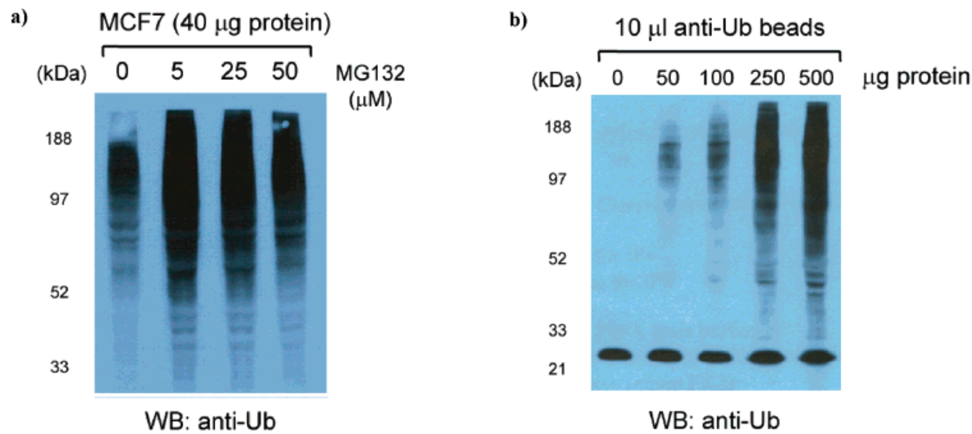

Figure 3. Treatment of MCF-7 cells with the proteasome inhibitor MG132. (a) MCF7 cells were treated overnight with various concentrations (5−50 μM) of MG132. Cell lysates containing 40 μg of protein were analyzed by Western blotting with an anti-Ub antibody to monitor differential levels of ubiquitination. (b) Small-scale immunoprecipitations with anti-Ub beads (see materials and methods) and MCF7 cell lysates were performed and analyzed by Western blotting.

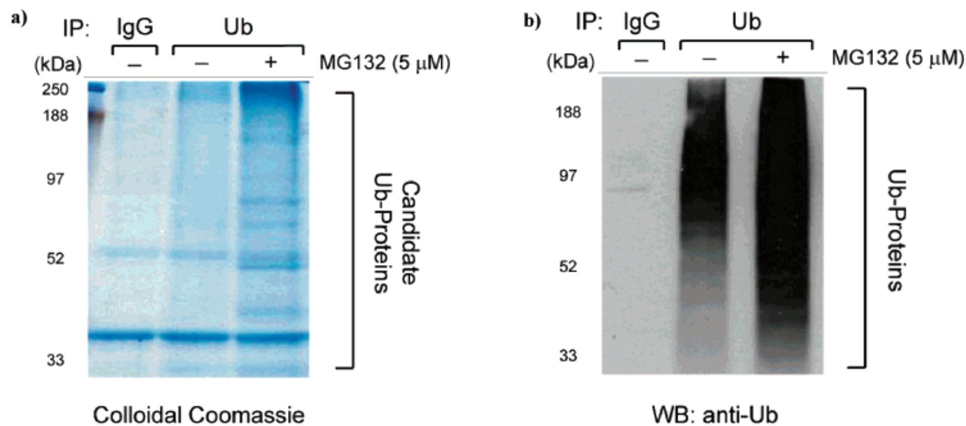

Figure 4. Immunoaffinity purification of ubiquitinated proteins from MCF-7 cells. (a) Ubiquitinated proteins from untreated MCF7 cells (lane 2) and MG132-treated MCF7 cells (lane 3) were purified by immunoaffinity chromatography, separated by SDS-PAGE, and visualized with Colloidal Coomassie. (b) Aliquots containing 2% of the purified samples were also analyzed by Western blotting.

in the Western blot in Figure 3a, treatment of MCF-7 cells with various concentrations of MG132 resulted in increased levels of ubiquitination. A 5-μM concentration was determined to be optimal based on the signal intensity strength and the number of ubiquitinated species that were detected compared to the untreated control. Concentrations above 50 μM were not assessed due to the low survival rate of cells after overnight treatment.

A series of small-scale immunoprecipitations was performed using the anti-Ub antibody to confirm its ability to purify ubiquitinated proteins from MCF-7 cells and to optimize a number of conditions. This included the ratio of anti-Ub beads for a given amount of starting material, the necessary incubation time to ensure strong antibody−antigen interactions, the number of washing steps to eliminate the bulk of co-purifying background proteins, and the most efficient type of elution buffer. (Anti-Ub beads were prepared by covalently coupling the anti-Ub antibody to Protein G-agarose beads (see Materials and Methods).) The Western blot in Figure 3b, for example, clearly shows that when 10 μL of anti-Ub beads was incubated with increasing amounts of protein from MCF-7 cell lysates, there was a proportional increase in the signal intensities of the bands detected. This indicated that the anti-Ub beads displayed specificity toward ubiquitinated proteins and enabled the determination that 250 μg of protein per 10 μL of anti-Ub beads was an optimal ratio that could be scaled-up for subsequent purifications. This optimal ratio was deduced as increasing the amount of protein up to 500 μg or above (data not shown) did not result in increases in the intensities of the bands.

To identify ubiquitinated proteins from MCF-7 cells, sufficient amounts of material needed to be purified for LC−MS/MS analysis. Initially, we attempted to pool material from several immunoprecipitations for visualization on a SDS-PAGE gel. Despite a considerable enrichment, as observed by Western blotting, this proved to be inadequate (data not shown). The pooling of multiple samples did little to improve the yield of ubiquitinated proteins and only resulted in an increased amount of nonspecific background. As a result, we decided to employ a large-scale purification approach based on immunoaffinity chromatography to overcome these constraints. In a recent study, such an approach enabled the successful identification of several low-abundance protein complexes by LC−MS/MS.[31]

Poly-Prep chromatography columns were filled with anti-Ub beads and MCF-7 cell lysates were loaded and allowed to incubate at 4 °C. Prior to loading, cell lysates were subjected to a pre-clearing step by passing them through chromatography columns containing only Protein-G agarose beads. After a 2 h incubation period, which allowed sufficient time for ubiquiti-

Table 1. Ubiquitinated Proteins from MCF7 Cells Identified by LC−MS/MS

band	protein	accession no.	Mascot score	no. of peptides	M_W (kDa)
1	PolyUbiquitin	Q9UEG1	297	4	68.4
1	E3 ubiquitin protein ligase	Q8NG67	94	4	372.5
2	Ubiquitin protein ligase EDD	O95071	769	19	312.3
2	FLJ32377 *Ubiquitin-like	Q96MH4	681	12	43.6
2	PolyUbiquitin	Q9UEG1	624	11	68.4
2	FLJ00343	Q8NF52	258	6	283.9
2	Fatty acid synthase	P49327	203	5	275.5
2	E3 ubiquitin protein ligase	Q8NG67	201	5	372.5
2	DNA-dependent protein kinase catalytic subunit	P78527	143	3	470.0
3	Fatty acid synthase	P49327	753	17	275.5
3	PolyUbiquitin	Q9UEG1	495	9	68.4
3	Glutamine-dependent carbamoyl-phosphate synthase	P27708	128	2	245.1
4	Fatty-acid synthase	P49327	856	23	275.5
4	Filamin A	P21333	520	13	283.3
4	PolyUbiquitin	Q9UEG1	376	6	68.4
4	Filamin B	Q9UEV9	80	3	280.1
5	Ubiquitin	P62958	272	5	8.5
5	Eukaryotic translation initiation factor 4-gamma 1	Q8N102	59	2	176.1
6	Ubiquitin	P62958	475	7	8.5
6	PolyUbiquitin	Q9UEG1	472	7	68.4
6	Myosin VI	Q9UM54	208	5	149.9
6	co-atomer protein complex, subunit alpha	Q8IXZ9	144	5	140.8
6	Splicing factor 3B, subunit 1	O75533	93	2	146.4
7	PolyUbiquitin	Q9UEG1	367	5	68.4
7	HLA-B associated transcript 3, isoform b	Q9BCN4	190	3	119.0
8	KIAA0090	Q8N766	443	10	112.1
8	Ubiquitin	P62958	375	5	8.5
8	26S proteasome non-ATPase regulatory subunit 1	Q86VU1	165	4	94.1
9	26S proteasome non-ATPase regulatory subunit 2	Q13200	707	17	100.8
9	Transitional endoplasmic reticulum ATPase	P55072	627	14	89.9
9	Polyubiquitin	Q9UEG1	304	5	68.4
10	26S proteasome non-ATPase regulatory subunit 2	Q13200	1398	26	100.8
10	Transitional endoplasmic reticulum ATPase	P55072	702	15	89.9
10	Ubiquitin	P62958	204	3	8.5
10	Glycogen phosphorylase	P11216	109	2	97.3
11	Heat shock protein HSP 90-beta	P08238	662	15	83.4
11	Heat shock protein HSP 90-alpha	P07900	571	12	84.8
11	Ubiquitin	P62958	327	4	8.5
12	Ubiquitin	P62958	342	5	8.5
12	ATP-dependent helicase DDX1	Q92499	168	3	83.3
12	Werner helicase interacting protein, isoform 2	Q8WV26	138	3	70.1
12	Ewing sarcoma breakpoint region 1, isoform EWS	Q96FE8	100	2	68.6
12	DNA replication licensing factor MCM5	P33992	99	3	83.0
12	Signal transducer and activator of transcription 1	*P*42224	60	2	83.5
13	Ubiquitin	P62958	371	6	8.5
13	Werner helicase interacting protein, isoform 2	Q8WV26	354	7	70.1
13	DNA replication licensing factor MCM7	P33993	213	6	81.8
13	Ewing sarcoma breakpoint region 1, isoform EWS	Q96FE8	193	3	68.6
13	Vacuolar protein sorting 35	Q96QK1	136	3	92.4
13	Heat shock protein HSP 90-beta	P08238	136	3	83.4
13	6-phosphofructokinase, type C	Q01813	92	3	86.4
13	Endoplasmin	P14625	85	2	92.6
13	Junction plakoglobin	P14923	59	2	82.2
14	Werner helicase interacting protein, isoform 2	Q8WV26	275	6	70.1
14	Ubiquitin	P62958	227	4	8.5
14	Ewing sarcoma breakpoint region 1, isoform EWS	Q96FE8	136	2	68.6
14	DNA replication licensing factor MCM7	P33993	119	4	81.8
14	6-phosphofructokinase	Q01813	48	2	86.4
15	BiP protein	Q9UK02	298	8	71.0
15	Ubiquitin	P62958	283	5	8.5
15	Heat shock 70kD protein 9B	Q8N1C8	182	3	74.0
15	Heat shock 70 kDa protein 1	P08107	129	4	70.2
15	Heat shock 70 kDa protein 6	P17066	96	3	71.2
16	Heat shock cognate 71 kDa protein	P11142	814	17	71.0
16	Stress-70 protein	P38646	574	12	73.9
16	Heat shock 70 kDa protein 6	P17066	249	5	71.2
16	Heat shock 70 kDa protein 1	P08107	235	5	70.2
16	Ubiquitin	P62958	204	3	8.5
17	Heat shock 70 kDa protein 1	P08107	1003	18	70.2
17	Heat shock 70 kDa protein 1L	Q8NE72	364	7	70.7
17	Heat shock 70 kDa protein 6	P17066	338	5	71.2
17	Ubiquitin	P62958	189	3	8.5
17	Heat shock cognate 71 kDa protein	P11142	151	3	71.0
17	Similar to ribophorin I	Q96HX3	120	3	64.6
17	Heat shock-related 70 kDa protein 2	P54652	114	3	70.2
18	Heat shock 70 kDa protein 1	P08107	396	8	70.2
18	Heat shock cognate 71 kDa protein	P11142	257	5	71.0
18	Ubiquitin	P62958	245	5	8.5

Table 1. (Continued)

band	protein	accession no.	Mascot score	no. of peptides	M_W (kDa)
18	Heat shock 70 kDa protein 6	P17066	238	5	71.2
18	Similar to ribophorin I	Q96HX3	82	2	64.6
18	Programmed cell death protein 8	O95831	71	2	67.1
19	Ubiquitin	P62958	202	4	8.5
19	T-complex protein 1, alpha subunit	P17987	100	3	60.8
19	60 kDa heat shock protein	P10809	98	3	61.1
20	Pyruvate kinase, isozymes M1/M2	P14618	351	8	58.3
20	Ubiquitin	P62958	227	4	8.5
21	PolyUbiquitin	Q9UEG1	272	4	68.4
21	26S protease regulatory subunit 4	P62191	197	4	49.3
21	P60 protein	Q13446	171	4	48.3
21	T-complex protein 1, delta subunit	P50991	167	3	58.3
21	Pyruvate kinase, isozymes M1/M2	P14618	142	3	58.3
21	T-complex protein 1, eta subunit	Q99832	77	2	59.8
22	26S protease regulatory subunit 4	P62191	491	11	49.3
22	Pyruvate kinase, isozymes M1/M2	P14618	467	9	58.3
22	P60 protein	Q13446	449	8	48.3
22	Phosphotyrosine independent ligand p62B	Q13502	272	5	46.4
22	T-complex protein 1, delta subunit	P50991	249	7	58.3
22	Ubiquitin	P62958	198	4	8.5
22	FBP-interacting repressor	Q9NZA0	77	2	58.3
23	26S protease regulatory subunit 4	P62191	608	11	49.3
23	Ubiquitin	P62958	259	4	8.5
23	Glucose-6-phosphate 1-dehydrogenase	P11413	142	4	59.7
23	UDP-glucose 6-dehydrogenase	O60701	123	3	55.6
23	T-complex protein 1, beta subunit	P78371	91	2	57.7
23	UV excision repair protein RAD23 homologue B	P54727	73	2	43.2
24	26S protease regulatory subunit 6A	P17980	617	12	55.2
24	26S protease regulatory subunit 6B	P43686	572	13	47.4
24	Eukaryotic translation elongation factor 1 alpha 1	Q6IPN6	445	9	50.4
24	Ubiquitin	P62958	272	4	8.5
24	Alpha enolase	P06733	251	5	47.3
24	52 kDa Ro protein	P19474	101	2	55.1
24	26S protease regulatory subunit 7	P35998	73	2	48.8
24	Gamma enolase	P09104	68	2	47.4
24	Chromosome 1 open reading frame 26	Q8NEK9	67	2	
25	26S protease regulatory subunit 6A	P17980	363	7	55.2
25	26S protease regulatory subunit 6B	P43686	227	5	47.4
25	Alpha enolase	P06733	89	2	47.3
25	Eukaryotic translation elongation factor 1 alpha 1	Q6IPN6	81	2	50.4
26	26S protease regulatory subunit 7	P35998	474	9	48.8
26	26S protease regulatory subunit 8	P62196	473	10	45.7
26	Ubiquitin	P62958	225	4	8.5
26	Elongation factor Tu	P49411	116	2	49.8
26	Eukaryotic translation elongation factor 1 alpha 1	Q6IPN6	93	2	50.4
27	26S protease regulatory subunit 8	P62196	366	9	45.7
27	Ubiquitin	P62958	232	4	8.5
27	Elongation factor Tu	P49411	205	5	49.8
27	Calcium-binding transporter	Q9P129	95	2	46.0
28	Ubiquitin	P62958	261	4	8.5
28	26S protease regulatory subunit 8	P62196	190	4	45.7
28	Ubiquinol-cytochrome-c reductase complex core protein 2	P22695	98	2	48.5
28	26S proteasome non-ATPase regulatory subunit 6	Q15008	97	2	45.7
28	Isocitrate dehydrogenase	P48735	84	2	51.3
29	26S protease regulatory subunit S10B	P62333	501	11	44.4
29	Ubiquitin	P62958	226	4	8.5
29	Guanine nucleotide-binding protein G−s-alpha-3	Q14433	134	3	44.6
30	Ubiquitin	P62958	176	3	8.5
30	Fructose-bisphosphate aldolase A	P04075	125	3	39.8

Table 2. Co-Purified Background Proteins

band	protein	accession no.	M_w (kDa)
1	Class IVb beta tubulin	Q8IWP6	50.1
1	Tubulin beta-2 chain	P07437	50.0
1	Tubulin alpha-ubiquitous chain	P68363	50.8
2	Actin, beta	Q96HG5	41.3
2	Actin-like protein 2	P61160	45.0

nated proteins to bind with high-affinity to the anti-Ub beads, a series of stringent washing steps was performed. Bound material was then eluted with a solution containing a low percentage of ionic detergent.

Eluates from three successive purifications were pooled, concentrated, and fractionated by SDS-PAGE (see Figure 4a). Staining the gel with Colloidal Coomassie revealed that numerous proteins from the two anti-Ub purifications (lanes 2 & 3) were not present in the IgG isotype control (lane 1). Visual inspection of the two anti-Ub purifications also revealed the presence of additional proteins specific to MCF-7 cells that were treated overnight with 5 μM MG132. A corresponding Western blot analysis of 2% of the final eluates is shown in Figure 4b. As expected, no ubiquitinated proteins were detected when the mouse anti-IgG antibody was used for purification, thus confirming the specificity of the anti-Ub antibody.

For the present study, it is necessary to point out that it was not our intention to perform a comparative or differential analysis of ubiquitinated proteins from MG132-treated and

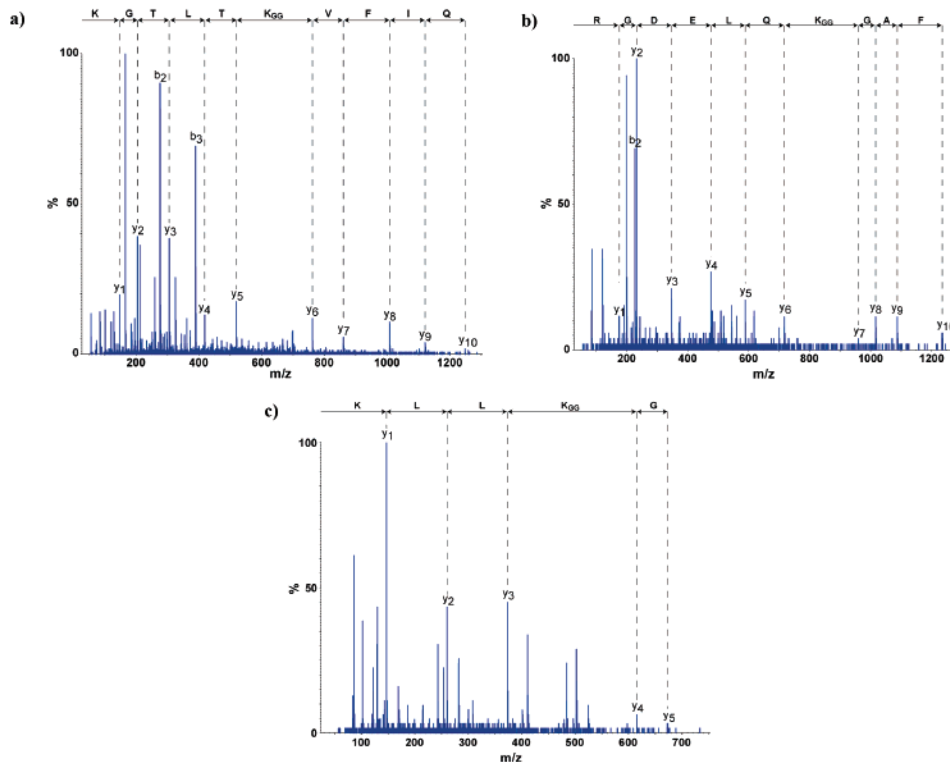

Figure 5. Representative MS/MS spectra of signature peptides. MS/MS spectra of doubly charged $[M + 2H]^+$ peptide ions at *m/z* 698.3 (a), 730.9 (b), and 401.2 (c). The peptide sequences MQIFVK_{GG}TLTGK, LIFAGK_{GG}QLEDGR, and EGK_{GG}LLK all contain an internal lysine with a di-glycine remnant that was derived from Ub.

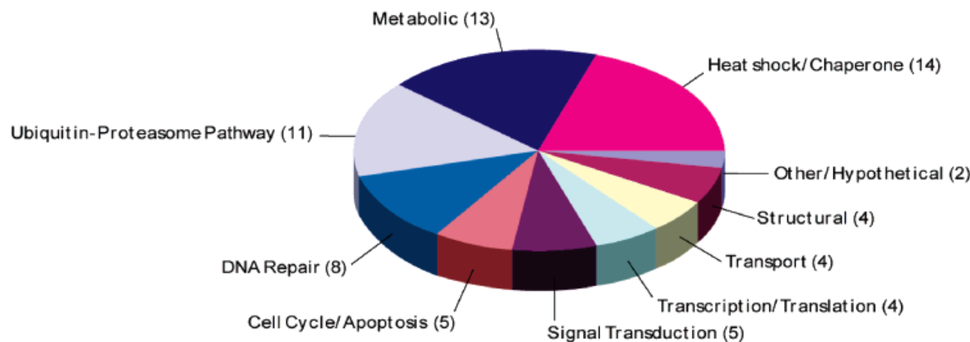

Figure 6. Pie-chart distribution of ubiquitinated proteins into functional categories. Ubiquitinated proteins identified from MG132-treated MCF-7 cells were grouped into 10 functional categories.

untreated MCF-7 cells. Instead, it was our goal to develop a method for the analysis of ubiquitinated proteins and to validate it using a biologically relevant sample. An important conclusion that can be drawn from a qualitative/visual inspection of the results shown in Figure 4a,b is that application of our immunoaffinity purification protocol will result in the isolation of additional target proteins when there is an increase in the level of ubiquitination in a given sample (e.g., after MG132 treatment). Currently, we are developing a gel-free protocol for the differential analysis of multiple samples using LC−MS/MS, and a commercially available mass spectrometry software package. In this way, we will be able to quantitatively monitor the expression levels of ubiquitinated proteins before and after drug treatment.

Thirty bands were excised from the MG132-treated lane shown in Figure 4a and were subjected to in-gel tryptic digestion. Peptides were extracted, separated by nanoflow LC, and then introduced into a quadrupole-TOF mass spectrometer. MS/MS analysis and database searching resulted in the identification of 70 proteins, each matching with 2 or more unique peptide sequences. Identification of signature peptides (see Figure 1b) was made possible by selecting a ubiquitinated lysine residue with a monoisotopic mass of 242.13788 Da in the Mascot variable modification list prior to database searching. This mass corresponds to a lysine residue with an attached di-glycine moiety derived from Ub (128.09496 Da + 114.04292 Da).[32] A complete list of the ubiquitinated proteins identified with their corresponding Mascot scores is found in Table 1.

Representative MS/MS spectra of three signature peptides are shown Figure 5a−c. Doubly charged $[M + 2H]^+$ peptide ions at *m/z* 698.3 (a), 730.9 (b), and 401.2 (c) were fragmented via collision-induced dissociation. The masses of the fragments were measured and the peptide sequences MQIFVK_{GG}TLTGK, LIFAGK_{GG}QLEDGR, and EGK_{GG}LLK, all of which contain an internal lysine with a di-glycine remnant that is derived from Ub, were obtained. Subsequent database searching matched

these peptide sequences to proteins found in bands 5, 12, and 24. In all cases, signature peptides were manually verified to confirm protein ubiquitination.

The two bands present in the IgG control lane in Figure 4a, with molecular weights of approximately 50 kDa and 40 kDa, were also subjected to LC−MS/MS analysis and database searching. This resulted in the identification of Tubulin and Actin isoforms as co-purifying background proteins (see Table 2). As a result, they were removed from consideration as ubiquitinated proteins. Tubulin and Actin have also been reported as commonly observed contaminants with other affinity purifications using agarose and sepharose-based resins.[33,34] Recently, isoforms of each were reported as nonspecific proteins when Ni−NTA-agarose beads were used for the isolation His-tagged GFP−Ub conjugates from HEK293 cells.[17]

Among the proteins listed in Table 1 are the E3 and EDD ubiquitin protein ligases, both of which were identified from band 2 with Mascot scores of 769 and 201, respectively (the statistically significant threshold score was 38). These enzymes are attached to Ub within cells and act as readily accessible storage pools of Ub. Also identified were Poly-Ub and a protein containing multiple Ub-like sequences (FLJ32377). Interestingly, both proteins have theoretical molecular weights well below the apparent molecular weight of band 2. Therefore, it is likely that they comigrated with either of the two Ub-ligating enzymes and were still covalently attached due to incomplete tryptic digestion. A similar explanation is likely for all of the bands that were found to contain Poly-Ub and/or Ub.

Numerous proteins belonging to the 26S proteasome complex are listed in Table 1, including 6 ATPase and 3 non-ATPase regulatory subunits. Each subunit is known to interact with a number of related regulatory proteins as well as a distinct subset of cytosolic, nuclear, and/or membrane proteins that are known to be ubiquitinated. For example, the 26S proteasome non-ATPase regulatory subunit 2, identified from Band 9 with a Mascot score of 707, is known to directly bind to ATPase regulatory subunits 1 and 2, non-ATPase regulatory subunit 5, the E3 ubiquitin protein ligase, and the intracellular domain of the Tumor Necrosis Factor (TNF)-type 1 receptor.[35−37]

Eleven members of the heat-shock protein family and three members of chaperonin protein family were identified from bands 11, 13, and 15−19. This was not surprising as the heat shock response is known to be directly regulated through the Ub-proteasome pathway. By tagging and degrading unfolded or damaged proteins, the Ub-proteasome pathway helps to prevent the accumulation of heat shock proteins under normal conditions.[13] However, when this degradative process is blocked by a proteasome inhibitor such as MG132, there is an induction of heat shock proteins and molecular chaperones (e.g., BiP and glucose-regulated stress proteins) which will in turn bind to Ub and Ub-tagged proteins.[38,39]

All other ubiquitinated proteins that were identified are listed in Table 1. This includes thirteen metabolic enzymes, eight DNA repair proteins, five cell cycle and apoptosis-related proteins, five proteins involved in signal transduction, four transcription and translation elongation factors, four transport proteins, four structural proteins, and two hypothetical proteins with currently unknown functions. A pie-chart distribution of these proteins into different functional categories is shown in Figure 6. As expected, the diversity of proteins identified in this study confirms that the important role that ubiquitination plays in the regulation of many key cellular functions in MCF-7 cells.

Conclusion

Taken together, our findings represent the first proteomic analysis of native ubiquitinated proteins from a genetically unmodified/untransfected mammalian cell line. Our method was shown to enable the profiling of ubiquitinated proteins from human MCF-7 cells under normal physiological conditions and/or after drug treatment. Further work is underway to improve the sensitivity of the method in order to permit the identification of very low-abundance ubiquitinated proteins which may serve as biomarkers of disease progression or as targets of novel therapeutics that interfere with the Ub-proteasome pathway. We expect our method to soon be adapted for the study of ubiquitinated proteins in other complex biological material, including human serum and plasma samples.

Acknowledgment. Funding for this study was provided by MDS Inc., MDS-Sciex, MDS-Pharma Services, Protana Inc., and the Ontario Genomics Institute.

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PR050265I