RESEARCH ARTICLE

Proteomic analysis of ubiquitinated proteins in normal hepatocyte cell line Chang liver cells

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Post-translational modification by ubiquitin (Ub) and Ub-like modifiers is one of the most important mechanisms regulating a wide range of cellular processes in eukaryotes. Through mediating 26S proteasome-dependent degradation of substrates, the covalent modification of proteins by multiple Ub (ubiquitination) can regulate many different cellular functions such as transcription, antigen processing, signal transduction and cell cycle. To better understand ubiquitination and its functions, proteomic approaches have been developed to purify and identify more protein substrates. The S5a subunit of the 26S proteasome binds to poly-Ub chains containing four or more Ub. In this study, immobilized GST-S5a fusion protein was used to affinity-purify ubiquitinated proteins from Chang liver cells. The purified proteins were then identified with multi-dimensional LC combined with MS/MS. Eighty-three potential ubiquitination substrates were identified. From these proteins, 19 potential ubiquitination sites on 17 potential substrates were determined. These potential ubiquitination substrates are mainly related to important cellular functions including metabolism, translation and transcription. Our results provide helpful information for further understanding of the relationship between ubiquitination machinery and different cell functions.

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Abbreviations: CDC2, cell division cycle 2; **HDAC3**, histone deacetylase 3; **SCX**, strong cation exchange; **THRA**, Thyroid hormone receptor α; **Ub**, ubiquitin

1 Introduction

PTM of proteins are essential processes modulating their properties and functions. Many of the most important PTM regulatory switches belong to the ubiquitin (Ub) protein family, including Ub, SUMO (small ubiquitin-like modifier),

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and numerous other Ub-like proteins. Ub is a 76-amino acid peptide that widely exists in eukaryotes. Ubiquitination is a process during which multiple Ub are covalently conjugated to the protein substrate [1]. This process is performed through the actions of Ub-activating (E1), Ub-conjugating (E2) and Ub-ligating (E3) enzymes. In this process, Ub is conjugated to the amino group of lysine side chain in the target protein through its most C-terminal glycine. Ub conjugation can be formed on multiple lysine residues of the substrate and Ub itself (K6, K11, K48 and K63). The poly-Ub chain formed through K48 of Ub with four or more Ub is a recognition signal for 26S proteosome, and therefore can mediate the degradation of substrates [2, 3]. In addition to multiple Ub modification by four or more Ub, ubiquitination can also be mono-Ub conjugation formed through other lysines (*e.g.* K63). Rather than causing degradation, this kind of ubiquitination usually changes the activities of substrate proteins [4]. Since ubiquitination substrates are widely distributed in cells, Ub modification, especially the Ub-proteasome pathway, is related to almost all cellular processes. Alterations of ubiquitination pathways contribute to the pathogenesis of diseases from cancer to neurodegenerative disorders to viral infections [3].

Since Ub and ubiquitination exist widely in cells and they are involved in many cell processes and related to many diseases, identification of ubiquitination substrates is essential to unveil some of the mechanisms corresponding to different cell functions and the pathogenesis of different diseases. Until now, several proteomic strategies, including different methods of affinity purification, trypsin digestion, and analysis of peptides with MS, have been applied to different species, tissues and cells to identify ubiquitinated proteins. In 2003, Peng *et al.* [5] first identified 1075 potential ubiquitination substrates from a yeast strain stably expressing 6x His-tagged Ub. The Ub-conjugated proteins were isolated with Ni-NTA resin, trypsin digested and identified with multi-dimensional LC followed by MS/MS (LC/LC-MS/MS). Excellent work was also performed by Hitchcock *et al.* [6] and Kirkpatrick *et al.* [7]. These studies greatly contributed to the understanding of the Ub modification profiles in large scale and integrated view.

Although it is quite direct and easy to affinity purify ubiquitinated substrate using stable cell lines overexpressing epitope-tagged Ub, there is some disadvantage for this strategy. First, since Ub is a small protein, fused tag may somehow affect its interaction with ubiquitination enzymes and conjugating with the substrate. Secondly, the exogenously overexpressed Ub may interfere with the original ubiquitination state in the cell and make it different from the normal physiological situations. Using Ub-specific antibody or Ub interaction reagent to affinity purify ubiquitination substrates can avoid the problems above. In 2005, Matsumoto *et al.* [8] purified ubiquitinated proteins from HEK293 cells using Ub antibody. Layfield *et al.* [9] and Weekes *et al.* [10] purified the ubiquitin-modified proteins from animal tissues using S5a-agarose affinity chromatography.

The S5a subunit of the 26S proteasome was originally identified as a protein capable of binding poly-Ub chains of four or more Ub in the absence of other 26S proteasome subunits [11, 12]. The observation that the *Saccharomyces cerevisiae* S5a homolog is not essential suggests that other poly-Ub 'receptor' elements of the 26S proteasome exist *in vivo* [13]. Mammalian S5a contains two independent poly-Ub-binding sites [14] and binds avidly to poly-Ub chains via hydrophobic interactions [15] recognizing chains linked through Lys6, Lys11 and Lys48 [16].

2. Materials and methods

2.1 Cloning and plasmids construction

S5a was cloned into GST-fusion protein expression vector pGEX-6P-1 (Pharmacia Biotech). The Ub and UbΔGG (Ub with C-terminal two glycine residues removed) were cloned into mammalian expression vector pEF with hemagglutinin tag (HA) fused at the N terminus. Thyroid hormone receptor α (THRA) and cell division cycle 2 (CDC2) were cloned into pEF vector with Flag tag fused at the C terminus. All cloning was performed by PCR amplification from a cDNA library in our laboratory. The cloning reagents were purchased from Takara Bio and New England Biolabs; pcDNA3.1-histone deacetylase 3 (HDAC3)-Flag is a kind gift from Prof. Boqu Huang of Northeast Normal University, Changchun, China.

2.2 Purification of proteins and preparation of GST and GST-S5a affinity columns

GST or GST-S5a was purified from *Escherichia coli* strain BL-21 (DE3) transformed with pGEX-6P-1 or pGEX-6P-1-S5a using glutathione Sepharose affinity column. The purified proteins were then coupled to CNBr-activated Sepharose-4B beads (Amersham Biosciences) according to the manufacturer's guide. The final amounts of coupled proteins were approximately 5 mg for GST-S5a and 2 mg for GST (with similar molar amount to GST-S5a) per mL of beads.

2.3 Cell culture and affinity purification of ubiquitinated proteins

The Chang liver cell line was a kind gift from Prof. Yinkun Liu of Fudan University, Shanghai. Chang cells were cultured in DMEM (PAA) containing 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2. At about 90% confluence, cells were collected with trypsin-EDTA, counted, washed once with PBS and stored at −80°C. Approximately 1×10^9 cells were collected for one batch of purification.

For affinity purification, cells were first lysed in 20 mL of lysis buffer containing 100 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 mM N-ethylmaleimide and 50 μM MG132. After

centrifuging at 14 000 rpm for 15 min, the supernatant was collected and pre-cleared with GST-Sepharose beads twice. The resulting pre-cleared lysate was then separated into two 50-mL centrifuge tubes with approximately 10 mLcell lysate (200 mg total proteins) in each tube. The two tubes of cell lysate were firstly mixed with 0.5 ml GST-Sepharose 4B beads or GST-S5a-Sepharose 4B beads, respectively. The mixtures were then put on a roller incubator and shaked gently at 4°C for 4 h. After incubation, the beads were put into mini-columns, washed with ten bed volumes of wash buffer (100 mM Tris-HCl, pH 7.0, 500 mM NaCl), and finally eluted with eluting buffer (7 M urea, 100 mM Tris-HCl, 150 mM NaCl pH 7.0).

2.4 Trypsin digestion and strong cation exchange chromatography

To digest the protein mixture in-solution, the eluant from S5a affinity purification was first reduced by adding DTT to 5 mM, mixed gently and incubated in 37°C for 2.5 h. Alkylation was performed by adding 25 mM iodoacetamide to the samples and incubating in the dark at 37°C for 1 h. The samples were then dialyzed against trypsin digestion buffer (1 M urea, 100 mM NH_4HCO_3). Trypsin was then added into the protein mixture. For 50 mg total protein, 1 mg trypsin was used. The samples were mixed gently and the digestion was processed at 37°C for 16 h.

The peptides in the trypsin digested samples were first separated in the first dimension by strong cation exchange (SCX) chromatography run in a ProteomeLab™ PF 2D Protein Fractionation System from Beckman. A SCX column purchased from the Great Eur-Asia Sci&Technology Development (Product No.: GEA0412016) with the length of 15 cm and id of 4.6 mm was used for the separation. The column was filled with Hypersil SCX packing material with particle diameter of 5 μm and pore diameter of 100 Å. For each sample, peptides were fractionated in a 40-min gradient of 1~100% solvent B (solvent A: 5 mM $NH_4H_2PO_4$, 40% ACN, pH 2.7; solvent B: solvent A with 350 mM NH_4Cl) with flow rate of 0.7 mL/min. The effluents were monitored at 214 nm, and fractions were collected every 1 min with an automated Gillson 215 liquid handler (Gillson, Middleton, WI). The system was controlled by 32 Kraft software for SCX and Uniprot software (Beckman coulter) for collector. Forty fractions were collected for each sample.

2.5 RPLC-MS/MS

All collected fractions were then subjected through a RPLC running on a capillary LC system from Waters. The RPLC column used was a C18 column with 15 cm length and 100-μm id. The fractions were eluted in two-step gradient (A phase: 5% ACN, 0.1% FA; B phase: 95% ACN, 0.1% FA. 0~5 min: 100% A; 5~30 min: 100%A to 50% A; 30~40 min: 50%A to 0%A) with flow rate of 200 nL/min. The RPLC was automatically connected through an electro-spray ionization interface to a LCQ-IT

MS system from ThermoFinnigan (LCQ DECA XP PLUS). The spray voltage was set as 1.8 kV. The temperature of the ion transfer capillary was set as 180°C. Peptide ions were detected in a full scan followed by three data-dependant MS/MS scans on the three most intense ions. The isolation width was 3 Da and normalized collision energy was 35%.

2.6 Data processing

All MS/MS spectra were searched against the composite database containing the normal human IPI protein database (Ver. 3.07) and its reversed database using the SEQUEST algorithm with trypsin constraints and a mass increase of +114.1 Da on lysine residue to indicate the ubiquitination sites. It was required that peptide matches must (i) be fully tryptic, (ii) have cross-correlation (XCorr) values of ≥2.0, 2.2, and 3.75 for 1+, 2+, and 3+ charge-state peptides, respectively, and (iii) have a delta correlation (dCn) score ≥0.1. For all identified peptides, the false positive rate was less than 5%. Then, AMASS version 1.16 (available at www.proteomicscams.com) was used to filter the SEQUEST results with three parameters: MatchPct ≥60, Cont ≥40, and Rscore <2.6.

2.7 Cell transfection, co-immunoprecipitation and Western blotting

To validate the ubiquitination of candidate proteins, co-immunoprecipitation was performed in 293T cells co-transfected with potential substrate and Ub. The 293T cells were cultured in DMEM containing 10% fetal bovine serum at 37°C with 5% CO_2. The cells were transfected using calcium phosphate method. Briefly, 3×10^6 cells were plated in 10-cm dishes 1 day before transfection. For transfection, 12~20 ng of plasmid DNA was first mixed with 50 μL 2.5 M $CaCl_2$ in a 1.5-mL centrifuge tube. The volume of the mixture was adjusted to 500 μL with 0.1x TE buffer (1 mM Tris-Cl, 1 mM EDTA, pH 7.6). Then 500 μL of 2xHEPES (50 mM HEPES, pH 7.0, 140 mM NaCl, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$) was added into the tube. The tube was tapped quickly to mix immediately after the 2x HEPES buffer was added and sat at room temperature for 1 to 5 min. The DNA mixture (1 mL total) was then added drop by drop onto 293T cells plated 1 day before. The culture medium was replaced with fresh medium in 3~6 h. Twenty-four hours post-transfection, cells were washed in cold PBS and lysed in 100 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1 % NP-40, 1 mM DTT, 1 mM PMSF, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 10 mM N-ethylmaleimide, 50 μM MG132 for 30 min on ice. Normally, cells from one 10-cm dish were lysed with 0.5 mL lysis buffer. After centrifugation (12 000 × g, 15 min, 4°C), the cell extracts were incubated with 3 μg of indicated antibody for 1 h at 4°C. Protein G-Sepharose beads (30 μL) were added and the incubation was continued for another 3 h. The precipitates were washed with 100 mM Tris-HCl, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM

PMSF three times, PBS twice and finally re-suspended in SDS-PAGE loading buffer. Western blot analysis was performed using standard procedures.

3   Results

3.1   Preparation of affinity column with recombinant GST-S5a or GST

Recombinant GST or GST-S5a were expressed in *E. coli* and purified by glutathione-Sepharose affinity chromatography. GST and GST-S5a proteins ran as almost single bands of approximately 27 and 77 kDa, respectively, on SDS-PAGE followed by CBB staining (Fig. 1A). Purified recombinant GST-S5a (5 mg) and GST protein (2 mg) were conjugated to cyanogens bromide-activated Sepharose 4B beads with similar molar amount. SDS-PAGE using the coupled beads boiled with SDS loading buffer showed the bands with the same molecular mass (Fig. 1B), indicating that the conjugation was successful.

3.2   Purification of ubiquitinated protein from Chang liver cells

Cell lysate from 1×10^9 Chang cells were prepared for one batch of purification. The N-ethylmaleimide and MG132 were added to the lysis buffer to prevent de-ubiquitination and proteosome degradation of ubiquitinated proteins. For coupling proteins to GST or GST-S5a affinity beads, normal immunoprecipitation conditions and 100 mg total proteins for each were used. The coupled potential ubiquitinated

proteins were eluted from the beads with denaturing condition including 7 M urea under which all the non-covalent bindings should dissociate. Nearly 256 μg protein was obtained from GST-S5a affinity purification whereas GST affinity purification yielded 222 μg protein.
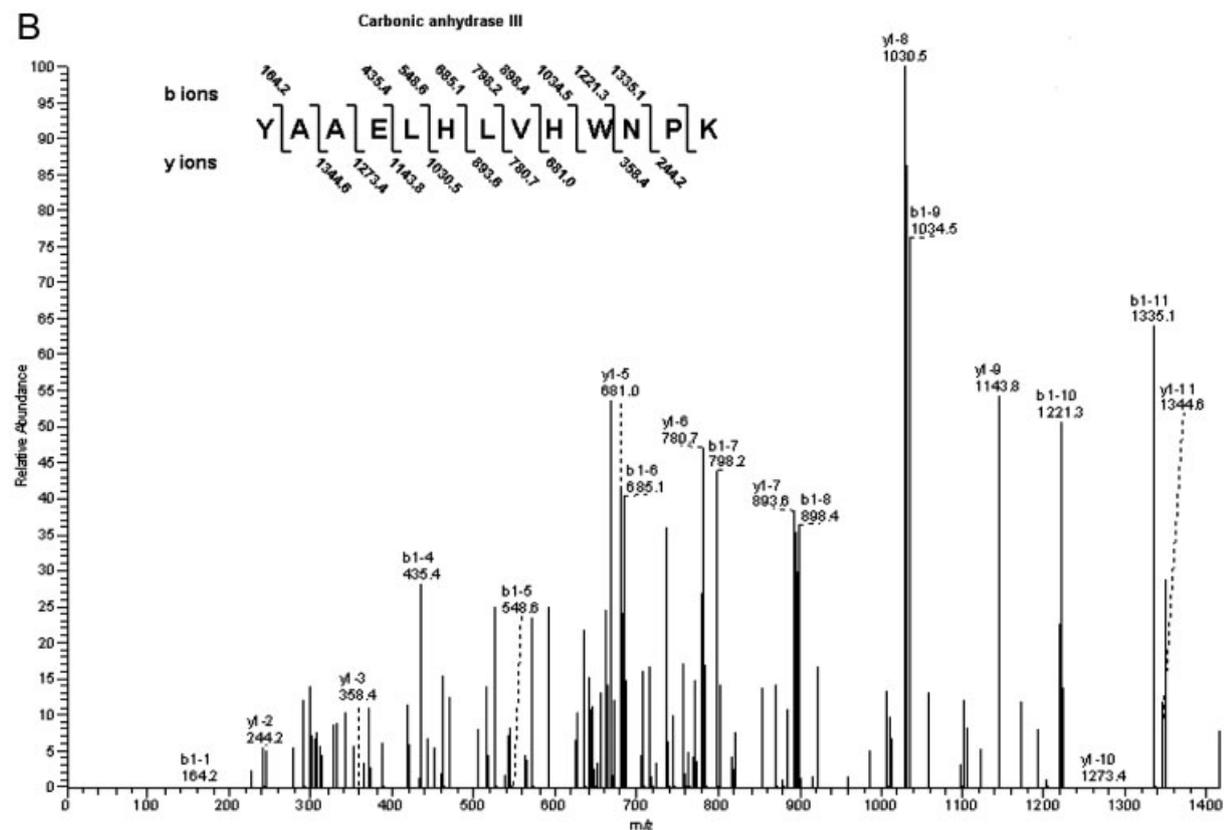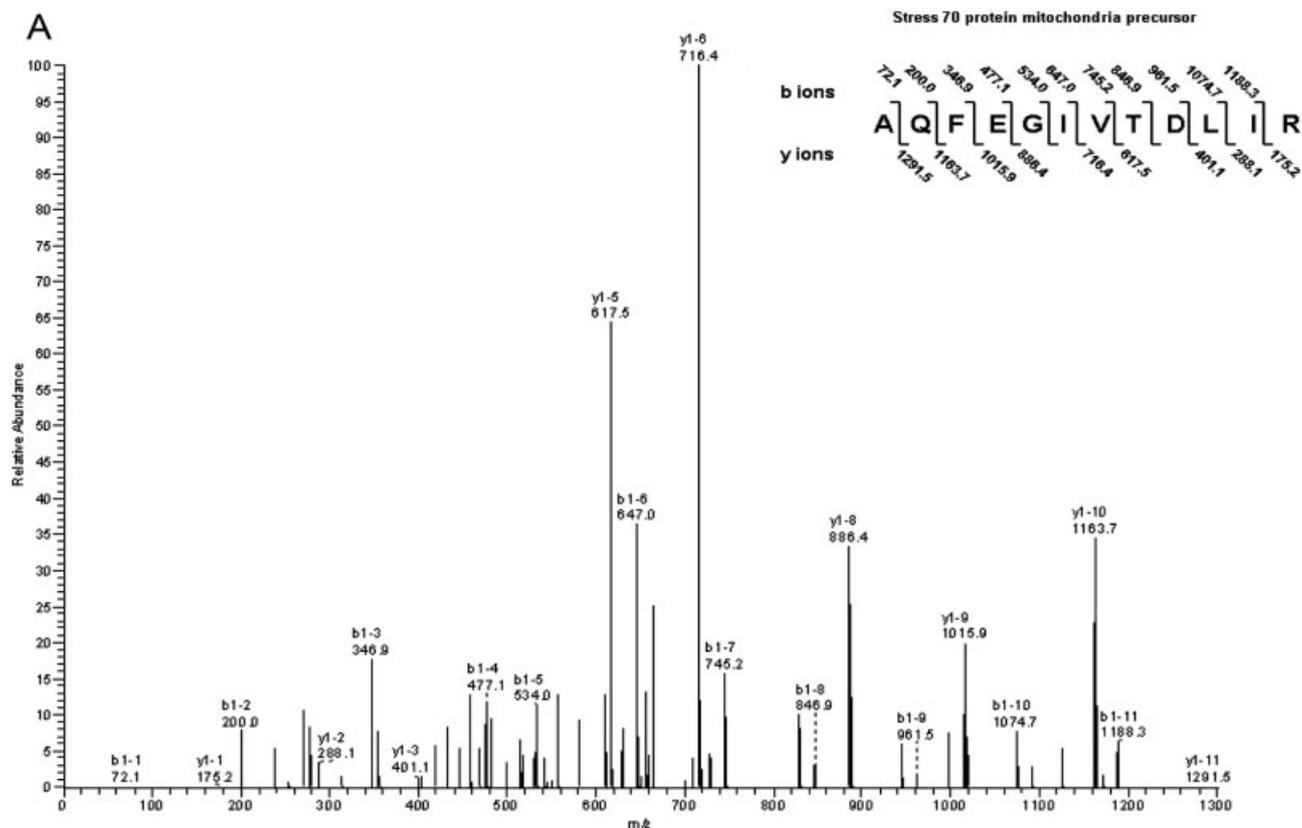
SDS-PAGE analysis showed that the predominant product eluted from GST affinity purification is GST itself while the products eluted from GST-S5a affinity purification contain many different proteins other than GST-S5a. These proteins were clearly visible with CBB staining. Western blotting of purified material with Ub antibody (Sigma) showed a high-molecular mass Ub-positive smear in the GST-S5a column eluant (Fig. 1C) that is characteristic of Ub conjugates. Lower molecular mass Ub-positive species under 45 kDa were almost unable to be detected in the GST-S5a eluant. No binding of ubiquitinated proteins to the GST (control) column was detected, confirming that the interactions were S5a dependent.

3.3   Characterization of S5a affinity purified proteins

Although 1-D- or 2-DE was used by many researchers to effectively separate the proteins before MS analysis, these techniques are not appropriate for ubiquitinated proteins. As we know, substrates can be modified by Ub at different positions and gain poly-Ub chains with various lengths. One substrate can appear as several bands or dots on 1-D- or 2-D gels. Therefore, it is hard to determine whether the bands or dots on the gels belong to the same substrate or not. In this study, we used the shotgun sequencing approach, the similar characterization strategy that many people used for identifying proteins in a complex [17] or ubiquitinated protein mixtures [5, 7].
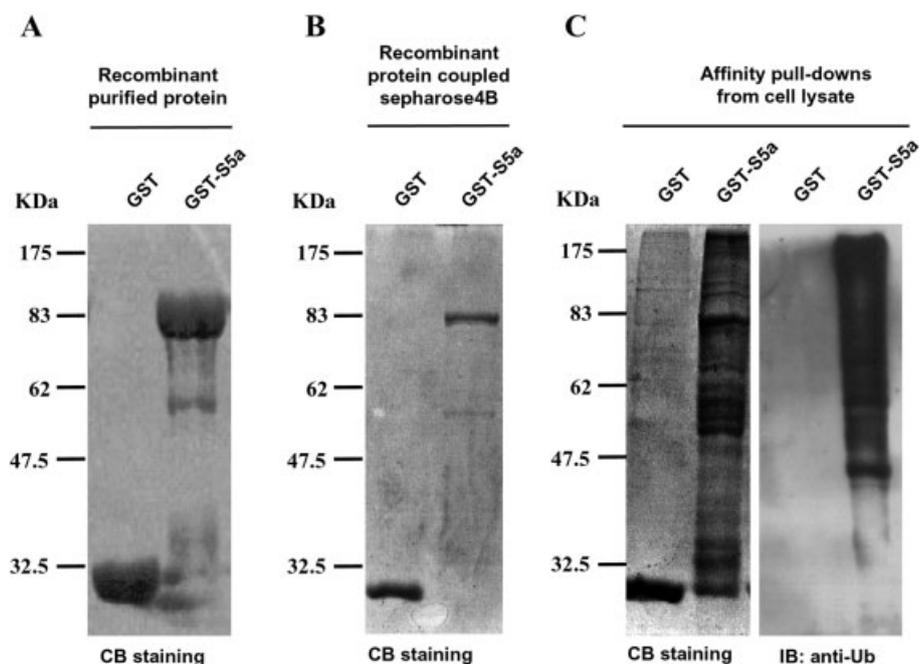
Figure 1. Purification of ubiquitinated proteins from Chang liver cells. (A) Purification of GST and GST-S5a. GST and GST-S5a were expressed in *E. coli* and purified by glutathione-Sepharose column. Protein purity was demonstrated by CBB staining. (B). Coupling of GST and GST-S5a to Sepharose 4B. The coupled beads were treated with SDS loading buffer and analyzed with SDS-PAGE. (C) Ubiquitinated proteins were purified by S5a-affinity chromatography from Chang liver cells. Products of affinity chromatography were separated by SDS-PAGE, demonstrated by CBB staining and immuno-blotted with anti-ubiquitin antibody.

A

B

Figure 2. MS/MS spectra of the representative peptides from four proteins (stress 70 protein mitochondria precursor; carbonic anhydrase III; 40S ribosomal protein S7 and SEC-14 like protein 2) with the biggest differences between the GST-S5a and GST purified samples.

Both GST-S5a and GST affinity purified samples were adjusted to the same protein concentration level and digested independently with trypsin. The digested peptide mixtures were then fractionated through SCX chromatography. The peptide fractions were subjected to RPLC that was directly connected to an MS/MS spectra system. Among those proteins with peptides detected with MS/MS in GST and GST-S5a purified samples, we considered those with a minimum of two valid peptides detected in GST-S5a purified sample but not in GST purified sample as potential ubiquitinated proteins or proteins specifically bound to S5a. Those proteins with more peptides detected in GST-S5a purified sample than in GST purified sample (more than threefold) were also considered as ubiquitinated proteins or S5a-related proteins, as they might have much higher affinity to GST-S5a than GST. According to these criteria, 83 proteins were obtained. The MS/MS spectra representing the peptides from four proteins with the biggest differences between the GST-S5a and GST purified samples are shown in Fig. 2. Additionally, 73 proteins were identified with a single peptide detected in GST-S5a purified sample but not in GST purified sample. Further biochemical experiments are needed to confirm whether these 73 proteins are real candidates for ubiquitination.

3.4 Identification of ubiquitination sites

Like other protein modifications, ubiquitination can give special signal in the spectra of the substrate. After trypsin digestion, the ubiquitinated protein can yield a signature peptide with the C-terminal glycine residues remaining on the modified lysine. Since trypsin digestion cannot occur at the modified lysine, this signature peptide gives a mass shift of 114.1 Da at lysine residue and a missed proteolytic cleavage [5]. According to this, the detection of ubiquitination sites was allowed by approximately adding 114.1 Da to the mass shift of lysine during database search. Through analyzing the spectrum data from GST-S5a purified samples, 19 peptides with ubiquitination sites belonging to 17 proteins were identified (Table 1). Three of the Ub-conjugating sites were detected on ubiquitin itself (Table 1 and Fig. 3). Although a few of these 17 proteins did not have much more peptides identified in GST-S5a purified sample than the sample pulled down by GST (*e.g.* DNA ligase I and CDC2), they were considered to be potential substrates because we think the existence of potential ubiquitination sites is quite convincing for characterizing a substrate. Taken together, 83 proteins that could be potential ubiquitination substrate or proteins tightly bound to S5a were obtained.

### 3.5 Confirmation of ubiquitination modification on some of the potential substrates

To confirm whether these proteins are ubiquitinated, some of the potential substrates were selected to examine their ubiquitination by *in vivo* ubiquitination assay. The assays were performed simply by co-transfecting the substrate with Ub, immunoprecipitation and Western blot. Ubiquitin with the two C-terminal glycines removed (UbΔGG) was used as the negative control since it is unable to modify proteins. As shown in Fig. 4, strong ubiquitination on HDAC3, THRA and CDC2 was observed.

Table 1. List of the proteins with ubiquitination sites identified with MS/MS

Protein	p*I*	WM	Accession number	Ubiquitinated sites	Charge	XCorr
Ubiquitin K (48)	7.72	147335.1	NP_002945	LIFAGK*a)QLEDGR	2	4.15
Ubiquitin K (11)				TLTGK*TITLEVEPSDTIENVK	2	4.71
Ubiquitin K (29)				AK*IQDKEGIPPDQQR	2	3.45
4 kDa protein	9.52	4299.9	ENSEMBL:ENSP 00000299455	SSLK*LVKENNIR	2	2.85
Similar to ribosomal protein S23	10.1	13643.8	XP_373033.1	MLGSSIVTEK*TRSGVINTK	2	2.75
Sal-like protein 2	5.92	105398.9	NP_005398.1	K*ACEVCGQAFPSQAALEEHQK	2	2.65
Cell division cycle and apoptosis regulator protein 1	8.59	132821.2	NP_060707	LDPK*TMKVNDLR	2	3.05
Hypothetical protein FLJ43983	6.38	75690.9	XP_113947	MK*VDMSGLQAKNEILSEK	2	2.84
Potassium voltage-gated channel subfamily H member 5	4.56	111907.1	NP_647479.2	DAMELK*PNGGADQK	2	2.51
Probable G-protein coupled receptor 158 precursor	8.57	135489.2	NP_065803.2	EDK*EGADHGTAKGTALIR	2	2.57
KLF4 protein	8.66	51076.6	NP_004226.2	EK*TLRQAGAPNNR	2	2.6
Thyroid hormone receptor alpha	6.42	54815.7	NP_955366	K*FLPDDIGQSPIVSMPDGDK	2	2.28
Histone deacetylase 3	9.2	48847.5	NP_003874	K*MIVFKPYQASQHDMCR	2	2.22
High-mobility group box 1	6.69	24218.2	NP_002119	PK*IKGEHPGLSIGDVAK	2	2.49
RNA binding motif protein 26	9.37	72129.4	XP_291128	K*AISSTEAVLNNR	2	3.72
Heterogeneous nuclear ribonucleoprotein K	5.39	50976.4	NP_002131.2	HESGASIK*IDEPLEGSEDR	2	2.41
Transcription elongation factor A protein 2	9.32	33600.8	NP_942016	K*QSSDEEVIALAK	2	2.73
DNA ligase I	5.49	101736.5	NP_000225.1	DIARLTGSASTAK*K	1	1.91
Cell division cycle 2 protein isoform 1	5.96	34095.3	NP_001777	K*YLDSIPPGQYM DSSLVK	2	2.29

a) *The marked Ks are the lysine residues predicted to be ubiquitin modified.

A

B
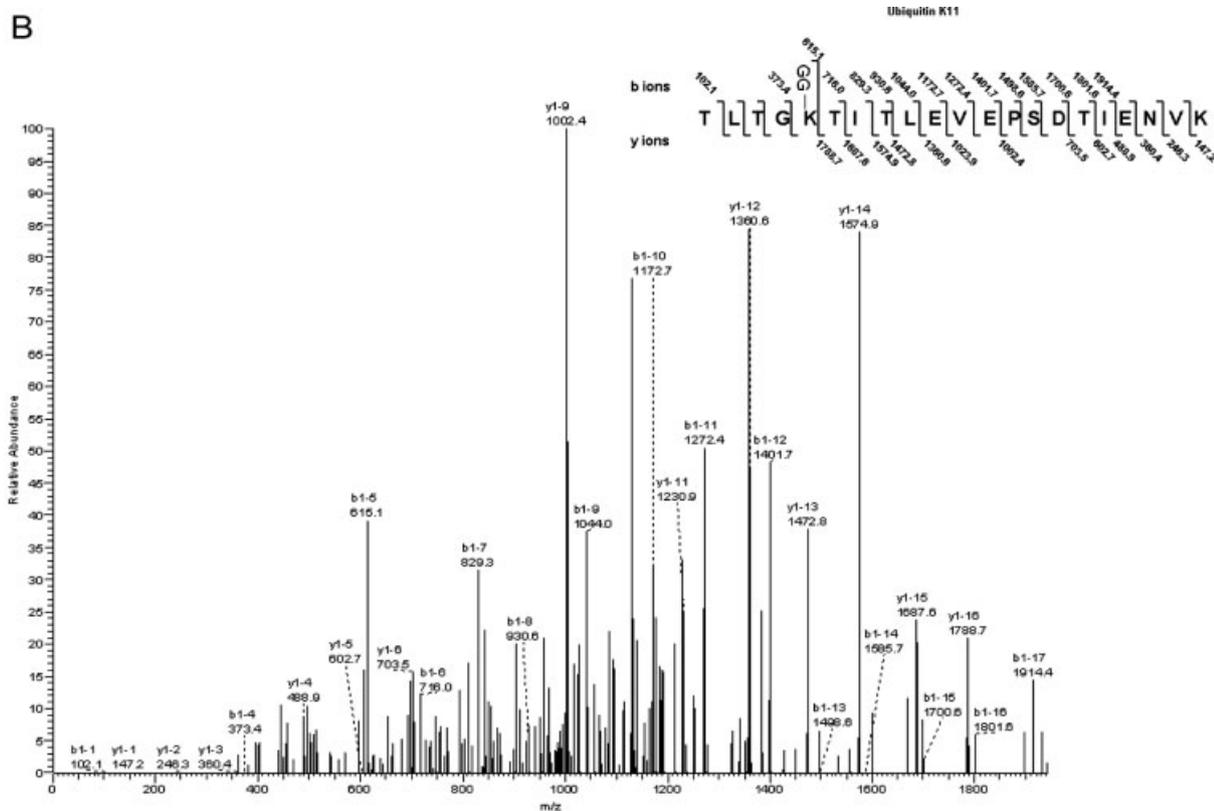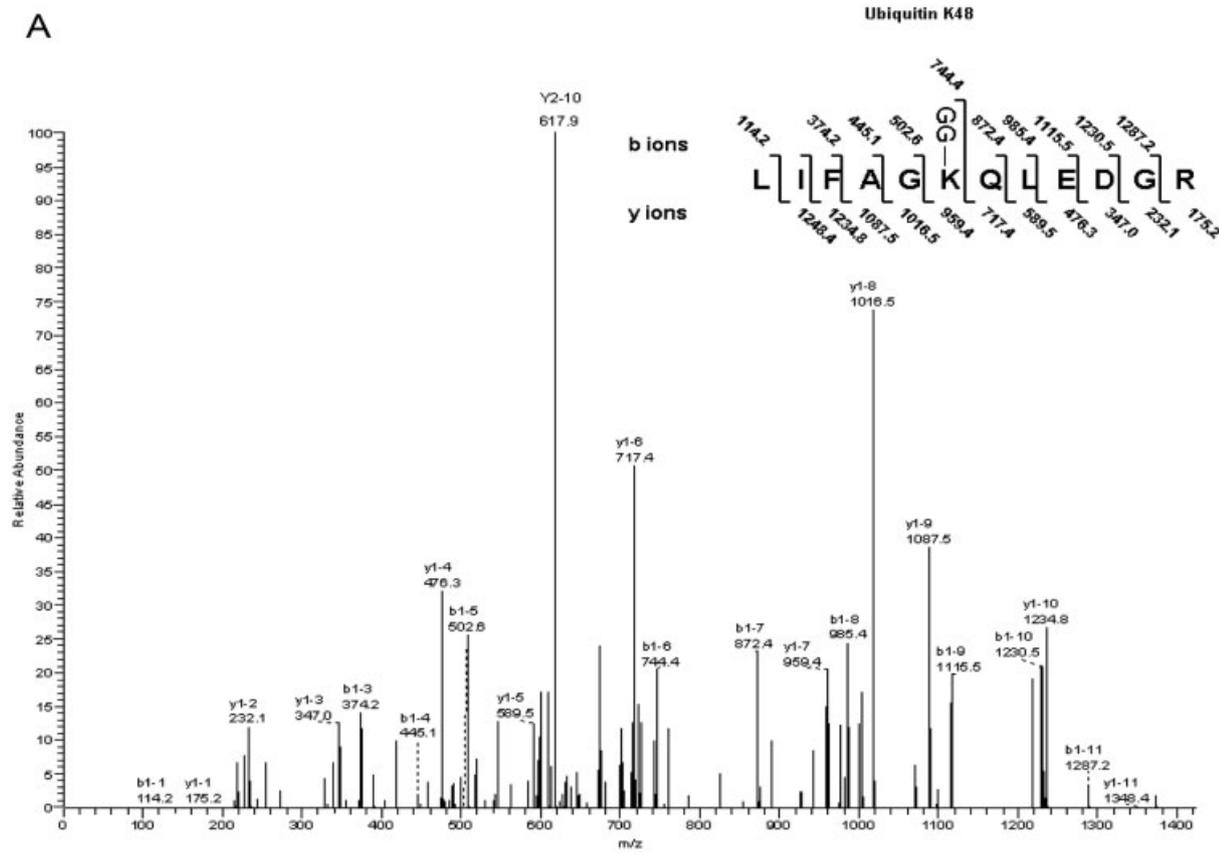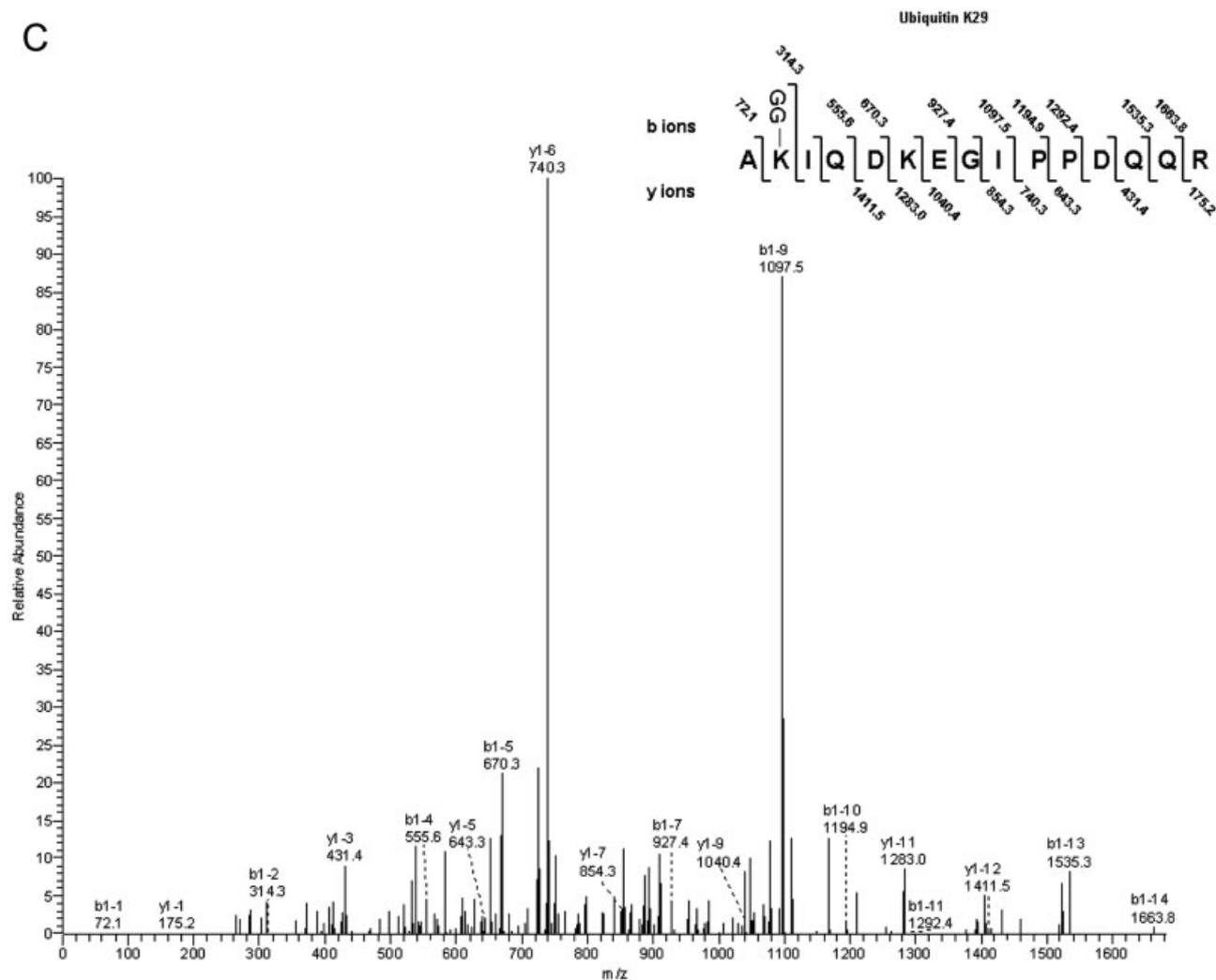
Figure 3. MS/MS spectra of the peptides of ubiquitin showing the identification of ubiquitination sites. The ubiquitination sites were identified by adding 114.1 Da to the molecular mass of lysine. Three ubiquitin chain conjugating sites on ubiquitin (K48, K11 and K29) were identified and shown.

4 Discussion

Chang liver cell is a normal hepatocyte cell line derived from non-malignant tissue. The cells were first grown as HeLa cell contaminant but were later characterized by its expression of hepatocyte markers like α-fetoprotein and albumin [18, 19]. In the past years, Chang liver cell line was widely used as human normal hepatocyte model in the studies of cell signaling [20], gene expression profiling [21], apoptosis [22], and oxidative stress [23].

In this study, the potential ubiquitinated proteins in Chang liver cells were analyzed with S5a protein-based affinity purification followed by proteomic approach as shown in Fig. 5. S5a, a member of the 19S modulation subunit of 26S proteosome, can bind ubiquitinated proteins through its two independent Ub-interacting motifs (UIM) and modulate

their degradation by the 20S core subunit. Using GST-S5a-conjugated Sepharose-4B affinity column, Ub-modified proteins were efficiently pulled out. A smear could be detected with Western blotting when the GST-S5a purified sample was blotted with polyclonal Ub antibody. This smear typically represents Ub-modified proteins because many substrates are modified by multiple Ub molecules.

The purified substrates were identified using shotgun sequencing, a strategy first established by the Yates lab [17]. Combined with trypsin digestion of the protein mixture and LC/LC-MS/MS, this strategy was first designed to analyze protein complexes. This strategy is also appropriate for analyzing ubiquitinated protein mixture because one protein is usually modified by multiple Ub molecules and therefore exists as multiple forms in the mixture. The trypsin digestion step can make it easier to analyze the mixture than running
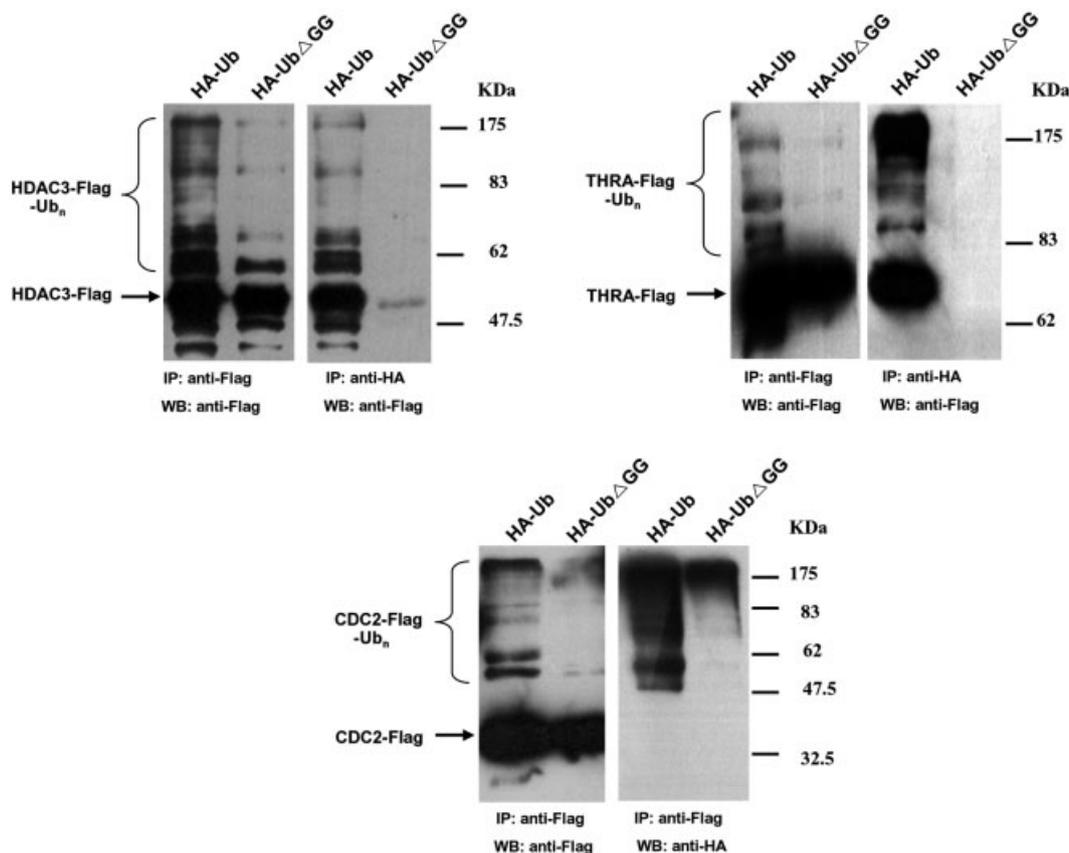
Figure 4. Confirmation of ubiquitination modification on specific substrates. Three potential ubiquitination substrates identified from Chang liver cells, HDAC3, THRA and CDC2, were subjected to this ubiquitination assay. Either HDAC3, THRA or CDC2 were constructed with C-terminal Flag-tag and overexpressed in 293T cells with HA-tagged ubiquitin or UbΔGG (with C-terminal two glycines removed). The cell lysates were then immunoprecipitated with Flag antibody and blotted with HA antibody or immunoprecipitated with HA-antibody and blotted with Flag antibody.
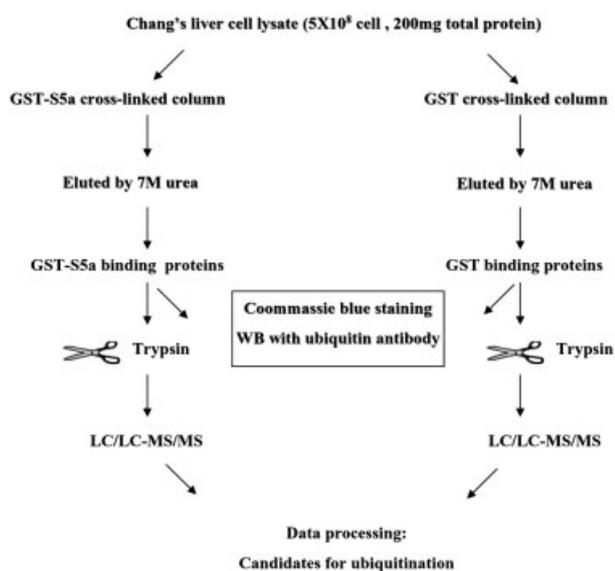
Figure 5. Flow chart representation of S5a-affinity chromatography.

1-D or 2-D gels. Using shotgun sequencing, we identified 83 potential ubiquitination substrates or S5a-related proteins because they have high affinity for S5a and/or have ubiquitination sites identified.

Taking a brief view of the function of these 83 proteins according to the literature (Fig. 6), 10% of the proteins belong to the Ub-proteasome system, including Ub-binding protein VCP (valosin-containing protein), NPLOC4 (nuclear protein localization 4), p62 and some other modulating subunits in 26S proteosome interacting with S5a. These are thought to be proteins non-covalently bound to S5a or Ub chains and are not real ubiquitination substrates. Among the remaining 90% of the proteins that are potential ubiquitination substrates, the most abundant ones are catalytic enzymes related to cellular metabolism (24%). This is not surprising because the liver is the core for metabolism in the body and ubiquitination might regulate the most important functions of this metabolism core. In addition to these metabolic enzymes, more than 60% are the proteins playing essential roles in transcription, translation, protein transport, DNA replication, DNA repair, and chromatin main-
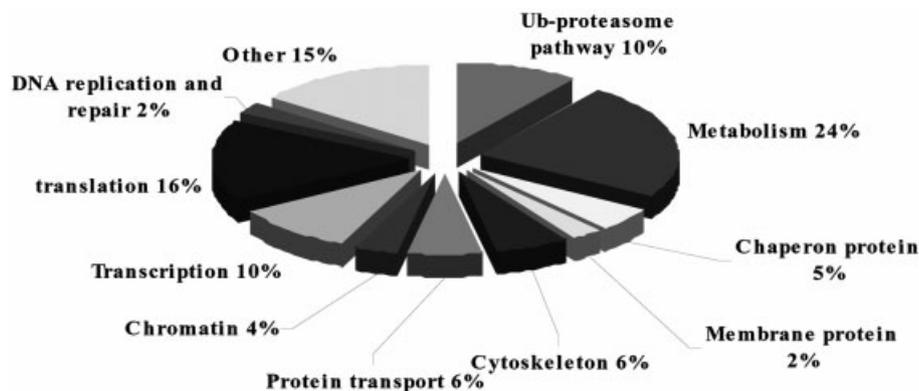
Figure 6. Categorical analysis of poly-ubiquitinated proteins or S5a-related proteins purified by S5a-affinity chromatography.

tenance etc. It is well known that ubiquitination is a very important PTM type regulating protein levels and protein functions related to many diseases. Although the ubiquitination and related function of these proteins need to be further validated, these results provide some information of the status of ubiquitin modification and its related functions in Chang liver cells.

From these potential ubiquitination substrates revealed in Chang liver cells, some of them have already been reported to be possibly regulated by Ub modification in other cells. The zinc finger transcription factor Krüppel-like factor 4 (KLF4), a negative regulator of cell proliferation, was shown to be ubiquitinated when the cells are either treated with proteosome inhibitor or transfected with exogenous Ub [24]. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is rapidly induced by DNA damage. The up-regulation of hnRNP K is carried out through the inhibition of its Ub-dependent proteasomal degradation mediated by the Ub E3 ligase HDM2/MDM2 [25]. Prohibitin is one of the ubiquitination substrates in the sperm mitochondria, which might make the sperm mitochondria recognizable by the egg's Ub-proteasome-dependent proteolytic machinery after fertilization and most likely facilitates the marking of the defective spermatozoa in the epedidymis for degradation [26]. Whether and how these proteins and the other potential substrates are regulated by ubiquitination in the liver need to be further studied.

Through analyzing the MS data from GST-S5a purified samples, we identified 19 peptides with ubiquitination sites belonging to 17 proteins. Among these ubiquitination sites, three of them are located on Ub (K48, K29 and K11). These three ubiquitination sites identified on Ub itself comprise the three well-known residues to which other Ubs conjugate. The identification of ubiquitination sites other than K48 on Ub also suggested that the chains of four or more Ub targeting the substrates for S5a binding and proteolysis are not limited to K48 [3]. It is also very interesting that the ubiquitination site we identified on another potential substrate, heterogeneous nuclear ribonucleoprotein K, is also located in the consensus sumoylation sequence and shares the same lysine for potential sumoylation site [27]. This might be one

more case that sumoylation and ubiquitination compete on the same residue and lead to different consequences. Although these potential ubiquitination substrates and ubiquitination sites need to be validated, they do provide some information about the ubiquitination profile and the roles, which ubiquitination may play in the liver.

In conclusion, using GST-S5a affinity purification, we purified the ubiquitination proteins from normal heptocyte Chang liver cells. We identified 83 potential ubiquitination related proteins or substrates and 19 ubiquitination sites in 17 proteins. These findings provide novel insight into the profile of ubiquitination in the liver and its possible functions, while further studies are necessary to characterize the detailed mechanism and function of the ubiquitination of these proteins.

5 References

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