Proteomic Survey of Ubiquitin-Linked Nuclear Proteins in Interferon-Stimulated Macrophages

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Ubiquitin modification plays a critical role in immune responses. Some cytoplasmic factors require ubiquitination to execute proper signaling upon pathogen and cytokine stimulation. However, ubiquitin modification and its functional significance have not been fully studied for many nuclear proteins. We report here that stimulation of RAW macrophages with interferon- γ and toll-like receptor ligands that activates innate immune responses triggers a global increase in ubiquitinated proteins in the nucleus, pointing to the role for ubiquitin modification in regulating nuclear events during innate immune responses. By immunopurification and mass-spectrometry analyses, we found that more than 200 proteins are directly or indirectly associated with ubiquitin in stimulated RAW cells. These proteins included proteins in the ubiquitin pathways, those involved in DNA metabolism, chromatin and transcriptional regulation, and mRNA processing. The largest group of proteins found in our list was ribosomal proteins important for protein translation. Other proteins found here were heat shock proteins and stress-response factors, suggesting a link between macrophage activation and stress response. In conclusion, upon macrophage activation, a large number of nuclear proteins become associated with ubiquitin modification, presumably leading to a global shift in the genome activity, important for proper execution of innate immune responses.

Introduction

UBIQUITIN CONJUGATION, deconjugation, and proteasome-mediated degradation are an important mechanism of regulation of innate and adaptive immunity. Various proteins are ubiquitinated in response to signaling, which activates the toll like receptor (TLR) pathway and the retinoic acid inducible gene-I (RIG-I) pathway, leading to induction of a series of cytokines in macrophages and dendritic cells (Gack and others 2007; Bhoj and Chen 2009; Wang and others 2009). Recent evidence indicates that ubiquitin can even serve as part of ligands for the RIG-I pathway to activate interferon (IFN) induction (Zeng and others 2010). Underscoring the role of ubiquitin modification in innate immunity, TRIM family proteins, many of which carry an E3 ubiquitin ligase activity, are induced by both type I and type II IFNs and regulate cytokine induction (Ozato and others 2008).

The levels of free ubiquitin and those of ubiquitinated proteins are maintained in homeostasis under unstimulated conditions. However, cellular stress such as heat shock, radiation induced stress, and DNA damage sets off ubiquitin modification events (Bergink and others 2007; Kimura and

Tanaka 2010). Ubiquitin is conjugated to substrates by 3-step enzymatic reactions, producing mono- or polyubiquitinated proteins (Hershko and Ciechanover 1998). Polyubiquitinated proteins, particularly those modified through lysine (K) 48 of ubiquitin, are often driven to 26S proteasome-dependent degradation, whereas monoubiquitination is more frequently associated with other types of regulation (Gallastegui and Groll 2010). Ubiquitination is a reversible process: the ubiquitin moiety can be removed from the substrates by deubiquitinating enzymes, such as CYLD and A20, which also affects innate and adaptive immune responses (Sun 2008). Despite accumulating reports attesting the role for ubiquitin modification in immune stimulation, little information is available as to ubiquitination events in the nucleus during macrophage activation, where large changes occur in chromatin and gene expression patterns.

Proteomic approaches have been used to identify and characterize ubiquitinated proteins in the yeast as well as mammalian cells (Kirkpatrick and others 2005; Matsumoto and others 2005; Jeon and others 2007; Ota and others 2008; Jeram and others 2009). The majority of these studies analyzed proteins bound to an exogenously expressed, tagged ubiquitin followed by immunopurification and

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mass-spectrometry (MS) analysis. Recently, a new method to profile ubiquitinated proteins has been reported that utilizes monoclonal antibody for the adduct of ubiquitination (Xu and others 2010). These studies collectively indicate that many more proteins are associated with ubiquitin than those studied individually so far, thus expanding the range of potential substrates conjugated to ubiquitin and those modified or interacting with ubiquitin.

We show here that IFN- γ /TLR stimulation triggers a large increase in ubiquitinated proteins in the nucleus of RAW macrophages. Some of the increase observed here was not directly tied to proteasome-mediated degradation, since ubiquitination was increased even in the absence of a proteasome inhibitor. These data raised the possibility that ubiquitin modification mediates changes in nuclear events necessary for macrophage activation. To gain a clue as to the range of nuclear proteins modified by ubiquitin or directly or indirectly interacting with ubiquitin, we employed a proteomic approach and identified, by MS analyses, more than 200 candidate proteins associated with Flag-tagged ubiquitin expressed in RAW cells. Our analyses revealed that nuclear proteins of diverse functions are covalently or noncovalently, or directly or indirectly associated with Flag-ubiquitin in stimulated macrophages. Other proteins identified here may interact with ubiquitin indirectly. These proteins include factors involved in DNA metabolism, chromatin regulation, transcription, mRNA processing, and protein translation, as well as stress responses. Many of these proteins have not previously been implicated for ubiquitin association. These results indicate that ubiquitin modification has a pivotal role in globally shifting nuclear events in macrophages after IFN- γ /TLR stimulation.

Materials and Methods

Cell lines and reagents

RAW 264.7 (RAW) macrophages and 293T cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. RAW cells were stimulated with 150 U/mL of IFN- γ (PeproTech) overnight or restimulated with 150 ng/mL of CpG or indicated TLR ligands (Kong and others 2007). Antibodies for ubiquitin, ARF-BP1, DDX17, UBC13, SIN3a, and TFIIB were purchased from Santa Cruz, Novus Biologicals, and Zymed laboratories, respectively. Anti-HA antibody was from Roche. Anti-Flag antibody and anti-FlagM2 agarose, MG132, and LPS were from Sigma.

Plasmids, retroviral vectors, and transduction

The expression vector for Flag-tagged ubiquitin (Flag-Ub) was constructed by amplification of Ub cDNA from pcDNA-HA-Ub using the 5' primer, ccggaattcaatgcagatcttgaag, and the 3' primer, cgcggatctcagccacctctgaggcgaa. Resultant PCR fragments were cloned into p3 $\times$ Flag-CMV. A retroviral vector for Flag-Ub was constructed by inserting the Flag-Ub cDNA above into pMSCV-CD8. RAW cells were transduced with viral supernatants containing pMSCV-Flag Ub-CD8 as described (Kim and Ozato 2009). Mouse Sqstm1 (p62) was amplified from the Image clones (ID, 3487289) and cloned into pcDNA-HA. Mouse Ddx17 cDNA was amplified from cDNAs from RAW cells and cloned into pCMV-Flag 2. All constructs were sequenced to confirm correct cloning.

Immunopurification of Flag-Ub-associated proteins

RAW cells ($\sim 6 \times 10^9$) were transduced with control pMSCV-CD8 vector or pMSCV-Flag-Ub-CD8 vector, and nuclear extracts were prepared as described (Kong and others 2007). Nuclear extracts were precipitated on anti-Flag M2 agarose, and bound materials were eluted with 3 $\times$ Flag peptides (100 μ g/mL; Sigma). The eluted materials ($\sim 150 \mu$ g) were further separated on the ubiquitin enrichment column containing the polyubiquitin affinity resin, according to the manufacturer's protocol (Thermo Scientific-Pierce). The flow through was regarded as a fraction enriched with monoubiquitin associated proteins, whereas the eluted fraction was considered enriched with polyubiquitin associated proteins. These fractions were acetone-precipitated and pellets were dissolved in 2 $\times$ sample buffer, heated at 70°C for 10 min, and separated on a Nu-PAGE gel (Invitrogen). Proteins were observed by colloidal blue staining.

In gel digestion and high-performance liquid chromatography-coupled tandem MS

Bands that appeared to be unique in the lane from the pull-down sample as well as corresponding areas of a control lane were excised (~ 26 and 12 bands from gels of the flow-through and eluate fractions, respectively). Remaining blank areas of the gels and corresponding areas of the control lane were also excised. Gel pieces were destained as described by the stain vendor and next processed through an automated version of reduction/alkylation and in-gel trypsin digestion, essentially as described by Shevchenko and others (2006), and then analyzed by liquid chromatography (LC)/MS/MS with the set of control samples run first. Peak lists of the data were used to search against an *in silico* database of all known proteins (Perkins and others 1999) using the Mascot program (Matrix Sciences).

Transient transfection and in vivo ubiquitination assay

To test ubiquitination of Sqstm1 (p62), 293T cells were transfected with pcDNA-HA-Sqstm1(p62) and pCMV-Flag-Ub using Superfect (Qiagen). For Ddx17, cells were transfected with pCMV-Flag-Ddx17 and pcDNA-HA-Ub. Twenty-four hours after transfection, cells were lysed in buffer containing 25 mM HEPES, 150 mM NaCl, 0.5 mM MgCl₂, 1 mM EDTA, 1% sodium dodecyl sulfate, 0.1% NP40, 10 mM NaF, 1 mM Na₂MoO₄, 1 mM Na₃VO₄, and protease inhibitors (Roche). Lysates in 10-fold diluted buffer without 1% sodium dodecyl sulfate were precipitated with anti-Flag or anti-HA antibody at 4°C for overnight. Immune precipitates were washed 3 times, eluted in 2 $\times$ sample buffer, and heated at 70°C for 10 min. Eluted materials were resolved on a Nu-PAGE gel, transferred onto PVDF membrane (Millipore), and immunoblotted with indicated antibody. Antibody binding was detected by the ECL kit (SuperSignal West Dura extended Duration Substrate, Thermo Scientific-Pierce).

Results

IFN- γ /TLR stimulation triggers a global increase in ubiquitinated proteins in macrophage nuclei

Many ubiquitin E3 ligase belonging to the TRIM family are induced in macrophages upon IFN- γ /TLR stimulation

(Kong and others 2007; Ozato and others 2008; Rajsbaum and others 2008). However, relatively few substrates ubiquitinated under this stimulation have been identified. Particularly, little is known about substrates in the nucleus. To examine whether IFN- γ /TLR signaling alters the pattern of ubiquitinated nuclear proteins, immunoblot analysis was performed for nuclear extracts from RAW macrophages with anti-ubiquitin antibody. As shown in Fig. 1A, IFN- γ stimulation led to a large, time-dependent increase in ubiquitin-conjugated proteins that were mostly >50 kDa in size. Data in Fig. 1B show that while treatment with CpG alone did not increase ubiquitin-conjugated proteins, a combined treatment with IFN- γ plus CpG increased ubiquitin conjugated proteins over those by IFN- γ alone: the increase was clearer at 4 h than 1 h of CpG treatment. Similarly, combined treatment with IFN- γ and LPS led to an increase in ubiquitinated proteins at 4 h. These results suggest that IFN- γ treatment was a prerequisite for the increased nuclear protein ubiquitination, whereas TLR signals further augment the effects. The experiments in Fig. 1A and B were performed in the absence of a proteasome inhibitor, indicating that ubiquitin-conjugated proteins were not immediately processed for degradation under these conditions. We next tested the effect of proteasome inhibitor, MG132. In Fig. 1C, IFN- γ pretreated RAW cells were restimulated with LPS, CpG, as well as poly IC and R848 in the presence of MG132. All 4 TLR ligands led to a noticeable increase in the levels of ubiquitin conjugated proteins over those by IFN- γ alone and the extent of increase was greater in the presence of MG132 than in the absence, indicating that some proteins ubiquitinated after TLR stimulation were degraded during the 4 h incubation period. It is of note that our nuclear fractions were largely devoid of cytoplasmic proteins (see Fig. 2C). These results indicate that stimulation of macrophages with IFN- γ /TLR leads to a large-scale ubiquitin modification of nuclear proteins, some of which are not immediately degraded, and may be involved in regulating innate immunity.

Isolation of Flag-ubiquitin immune complexes

To detect ubiquitin-conjugated and/or ubiquitin-associated proteins in IFN- γ /TLR-stimulated macrophages, we opted for a strategy outlined in Fig. 2A. First, RAW cells were transduced with a retroviral vector expressing Flag-tagged ubiquitin (Flag-Ub), and selected by the CD8 marker, expanded, and stimulated with IFN- γ /CpG in the absence of MG132. Nuclear extracts from these cells were immunoprecipitated with anti-Flag antibody and bound proteins were eluted by Flag peptides. Eluted materials were further separated on the poly-ubiquitin enrichment column into the flow-through and eluted fractions. Proteins in these samples were separated on gel electrophoresis and analyzed by LC/MS/MS. Data from Fig. 2B through Fig. 2E confirm that these steps were correctly executed. Flow cytometry data in Fig. 2B showed a uniform CD8+ population in Flag-Ub- and Mock-transduced cells (empty pMSCV-CD8 vector), but not in untransduced RAW cells. In Fig. 2C, immunoblot analysis of nuclear extracts with anti-ubiquitin and anti-Flag antibody showed extensive modification of nuclear proteins with ubiquitin and Flag-Ub. Nuclear extracts used here were relatively free of cytoplasmic proteins as evidenced by the absence of Cbl and the presence of YY1, a representative of cytoplasmic versus nuclear proteins, respectively. Data in Fig. 2D (see first step, left) showed that anti-Flag immune precipitates contained proteins conjugated to Flag-Ub. In the second step, shown on the right, both the flow-through and eluates recovered from the poly-ubiquitin enrichment column abundantly contained Flag-Ub conjugated proteins. The molecular mass of the proteins in the flow-through fraction ranged from 60 kDa to over 200 kDa, whereas proteins from the eluted fraction were largely greater than 100 kDa. The flow-through was regarded as a fraction enriched with monoubiquitin-associated proteins bound to monoubiquitin. Whereas, the fraction eluted from this column may be enriched with polyubiquitin-associated proteins. However, we consider it unlikely that this step would

FIG. 1. IFN- γ /TLR stimulation increases ubiquitin-conjugated nuclear proteins in macrophages. (A) Nuclear extracts from RAW cells stimulated with 150 U/mL of IFN- γ for indicated times were immunoblotted with anti-ubiquitin antibody. Immunoblot for TFIIB represents a loading control. (B) *Left:* RAW cells treated with CpG for 4 h or IFN- γ overnight and immunoblot was performed as above. *Right:* RAW cells pretreated with IFN- γ overnight were stimulated by CpG or LPS for 1 h, or 4 h. (C) RAW cells pretreated with IFN- γ were stimulated with indicated TLR ligands for 4 h in the presence of 10 μ M of MG132. IFN- γ , interferon- γ ; TLR, toll like receptor.

FIG. 2. Strategy and isolation of Flag-Ub immune complexes from stimulated RAW cells (**A**) A schematic diagram of procedures used for identifying ubiquitin-conjugated/associated nuclear proteins in macrophages. (**B**) RAW cells transduced with empty MSCV vector (Mock-CD8) or with Flag-Ub (Flag-Ub-CD8) or without transduction (RAW) were stained with anti-CD8 antibody, and CD8-positive cells were detected by flow cytometry. (**C**) Nuclear extracts from RAW cells transduced with empty vector (Mock, M) or Flag-Ub (U) vector were resolved on Nu-PAGE electrophoresis and immunoblotted with antibody for ubiquitin (Ub), Flag (Flag), or TFIIB. Cbl and YY1 were immunoblotted to confirm relative purity of the cytoplasmic (C) and nuclear (N) fractions. (**D**) The first step: Immune complexes from mock (M) or Flag-Ub (U)-transduced cells were eluted with 3×Flag-peptides and immunoblotted with anti-Flag antibody. The second step: the above materials were separated on the polyubiquitin enrichment column, and the flow-through and eluted fractions were tested. M and U indicate mock and Flag-Ub-transduced samples. (**E**) The above fractions were stained with Colloidal blue and gel slices were subjected to liquid chromatography/MS/MS analysis. MS, mass-spectrometry.

completely separate monoubiquitin-linked proteins from polyubiquitin-associated counterparts. In Fig. 2E, proteins from the flow-through and eluted fractions were resolved on the Nu-PAGE gels and stained with colloidal blue. The flow-through fraction showed a broad smear of stained proteins, whereas the eluates exhibited proteins of larger sizes, some appearing as discrete bands. It should be noted here that because Flag-Ub complexes were immunopurified under conditions expected to preserve protein-protein interactions, where it is possible that some of the proteins identified in our analyses were not covalently linked to ubiquitin, but associated with ubiquitinated proteins directly or indirectly through protein-protein interactions.

MS/MS analysis reveals nuclear protein of wide-ranging functions associate with ubiquitin after macrophage stimulation

MS/MS analyses were performed for the flow-through and eluates from the polyubiquitin enrichment column for 3 independent preparations of nuclear extracts. We found a total of 228 proteins that consistently showed MS scores higher than 100. We adopted this as an arbitrary cut off to focus on proteins, which we feel are most confidently identified. Tables 1 and 2 each lists 27 proteins that were detected in the flow-through and the eluates, respectively. Supplementary Table S1 (Supplementary Data are available online at www.liebertonline.com/jir) compiles the rest of proteins with >100 MS scores.

Data in these tables indicate that nuclear proteins of diverse functions are bound to ubiquitin. Ubiquitin modification or ubiquitin association has not been reported for many of these proteins. In Fig. 3, proteins with known functions were classified into 12 groups. As would have been expected, enzymes in the ubiquitination pathways were abundantly found in both fractions, including E1, E2, and E3 ligases, deubiquitination enzymes (eg, UBC 13, BIRC6, MCL1-1, and HUWE1) (Ren and others 2005; Zhong and others 2005; Plans and others 2006; Liu and others 2007). Also, proteins with the Ub binding domain, such as SQSTM1 (p62) and BAT1A were found in the Flag-Ub immune complex (Dikic and others 2009). Furthermore, 19 proteasome subunit components are found in our analysis. Together, proteins belonging to the ubiquitin/proteasome pathway comprised 15% of the total proteins detected in these analyses. The abundance of this class of proteins found in our study validates the procedures employed, and lends credence to the idea that proteins identified in our work properly reflect ubiquitination events in stimulated RAW macrophages.

About 5% of the proteins found here are involved in DNA metabolism, regulation of chromatin, and transcription. The relatively large representation of proteins in this group is likely attributed the fact that nuclear extracts, as opposed to total lysates, were analyzed in the present study, although the latter have been more commonly used in proteomic analyses of ubiquitinated proteins (Matsumoto and others 2005; Jeon and others 2007; Jeram and others 2009; Xu and others 2010). Proteins in this group include ATP-dependent SWI/SNF chromatin remodeling factors of the SMARCA subfamily (better known as BRG1/BAF complex). Ubiquitin binding states of these proteins have not been studied in detail. Given that the BRG1/BAF complex plays a role in IFN-stimulated transcription, ubiquitin association indicated

TABLE 1. PROTEINS IDENTIFIED FROM THE FLOW-THROUGH FRACTION

No.	Protein name	Mass-spectrometry score	DB source accession	Category	Gene symbol
1	Hectm uba and vwv domain containing 1 (Mcl-1 ubiquitin ligase)	2447	NM_021523.4	1	<i>Huwe1</i>
2	Talin 1	959	NM_011602.5		<i>Tln1</i>
3	DNA methyltransferase (cytosine-5) 1	830	NP_034196.3	4	<i>Dnmt1</i>
4	Ubiquitin-activating enzyme E1	615	BC058630.1	1	<i>Uba1</i>
5	Ribosomal protein S3	594	AY043296.2	9	<i>Rps3</i>
6	Dynein, cytoplasmic, heavy chain 1	566	NM_030238.2	11	<i>Dync1h1</i>
7	Myb binding protein (p160) 1	556	AL662812.12	5	<i>Mybbp1a</i>
8	Valosin containing protein	537	BC049114.1	3	<i>Vcp</i>
9	Actin, beta	507	BC138611.1	11	<i>Actb</i>
10	Phosphoglycerate kinase 1	463	NM_008828.2		<i>Pgk1</i>
11	ATP-dependent RNA helicase A (nuclear DNA helicase II, DEAH box protein 9, DDX9)	452	NM_007842.2	7	<i>Dhx9</i>
12	Heat shock protein 1, beta	445	NM_008302.3	12	<i>Hsp90ab1</i>
13	Ribosomal protein S8	423	X73829.1	9	<i>Rps8</i>
14	Ribosomal protein S4, X-linked X isoform	422	M73436.1	9	<i>Rps4x</i>
15	Dedicator of cytokinesis protein 2 (Protein Hch)	414	NM_033374.2		<i>Dock2</i>
16	Swi/snf related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	412	BC079560.1		<i>Smarca4</i>
17	Eukaryotic translation elongation factor 1, alpha 1	402	BC108391.1	10	<i>Eef1a2</i>
18	Valyl-tRNA synthetase	402	NM_011690.3	8	<i>Vars</i>
19	Gamma actin-like protein	399	AF195094.1	11	<i>Actl</i>
20	Heterogeneous nuclear ribonucleoprotein K	384	NM_025279.2	6	<i>Hnrnpk</i>
21	Tropomodulin 3	383	NM_016963.2	11	<i>Tmod3</i>
22	Alpha-tubulin isotype M-alpha-2	383	M13446.1	11	<i>Tuba1b</i>
23	Leucine-rich ppr-motif containing (leucine rich protein mLRP130)	372	Q6PB66		<i>Lrp130</i>
24	Poly(rc) binding protein 1	371	NM_011865.3	8	<i>Pcbp1</i>
25	U5 snRNP-specific protein, 200kDa	368	NM_177214.4	8	<i>Snrnp200</i>
26	Fatty acid synthase	366	NM_007988.3		<i>Fasn</i>
27	Midasin homolog	362	NM_001081392.1		<i>Mdn1</i>

DB, Data Box.

in this study may pose an implication on the function of the complex (Cui and others 2004). Twelve percent of the proteins listed here fell into the group representing RNA metabolism, including heterogeneous ribonucleoproteins, proteins of mRNA processing, and 8 of the Dead Box proteins. Dead box proteins, having an RNA helicase motif, are involved in various aspects of RNA metabolism (Fuller-Pace and Ali 2008). Ubiquitin modification and its functional consequence are largely unknown for this group of proteins, although DDX5 (p68), a Dead Box protein, is shown to be polyubiquitinated in some cancer cells (Causevic and others 2001). A surprisingly large number of ribosomal proteins, as many as 33, were found in our list, comprising 14.5% of total proteins. Together with eukaryotic translation initiation/elongation factors identified here, as much as 17% of the proteins identified here were classified as proteins involved in protein translation. Ribosomes are the primary site of translation: composed of a small (40S) and large (60S) subunits, each ribosomal subunit contains RNA, and ~33–49 proteins that coordinately execute protein biosynthesis (Korostelev and others 2008). Neither the event of ubiquitin conjugation nor the functional meaning of ubiquitin association has been studied in detail for most, if not all ribosomal proteins. The last group of proteins (~9%) found in this work were actin, myosin, tubulin, and associated proteins, involved in adhesion, migration, and microtubule dynamics. Ubiquitination has been documented for some of these proteins. For example, tubulin α , β , and γ are shown to be

ubiquitinated for timely degradation (Sankaran and others 2005; Bheda and others 2010). Actin is present in both the cytoplasm and nucleus, and some fractions are ubiquitinated by Trim 32 (Kudryashova and others 2005; Hofmann and others 2009; Louvet and Percipalle 2009). Ubiquitination of the actin- and integrin-binding protein talin is also reported that affects cell adhesion and migration (Huang and others 2009). Together, our MS analyses reveal ubiquitin association of nuclear proteins vital to basic cellular functions, indicating that ubiquitination is part of a global shift in basic cellular activities in macrophages that follows after IFN- γ /TLR stimulation. However, it should be born in mind that an immunopurification strategy such as this could pick up interactions that may not be relevant to macrophage stimulation.

Spot validation of MS/MS analyses

To begin validating our MS/MS data, we asked whether some proteins with higher than 100 MS scores were indeed linked to ubiquitin. Nuclear extracts from Mock or Flag-Ub-transduced cells were immunoprecipitated with anti-Flag antibody and blotted against indicated proteins. Data in Fig. 4A show that HUWE1, UBC13, DDX17, SQSTM1 (p62), and SIN3A found in our list of proteins with significant MS score were all associated with Flag-ubiquitin. These proteins represent an ubiquitin ligase, E2 enzyme, Dead Box protein, ubiquitin binding protein, and transcription factor, respectively, and were tested because antibodies suitable for

TABLE 2. PROTEINS IDENTIFIED FROM THE ELUTED FRACTION

No.	Protein name	Mass-spectrometry score	DB source accession	Category	Gene symbol
1	Ubiquitin B	714	BC100341.1	1	<i>Ubb</i>
2	PREDICTED:similar to fusion protein:ubiquitin (base 43_513); Ribosomal protein S27a (bases 217_532)	536	NM_024277.2	9	<i>Rps27a</i>
3	Heat shock protein 70 cognate	313	X54401.1	12	<i>hsc70</i>
4	Heat shock protein 8	287	BC066191.1	12	<i>Hspa8</i>
5	Proliferating cell nuclear antigen	230	NM_011045.2	4	<i>Pcna</i>
6	Heat shock protein 1, beta	206	NM_008302.3	12	<i>Hspb1</i>
7	PREDICTED: similar to ubiquitin A-52 residue ribosomalprotein fusion product 1	198	BC014772.1		<i>Uba52</i>
8	PREDICTED: Heterogeneous nuclear ribonucleoprotein A/B isoform 2	188	NM_010448.3	6	<i>Hnrnpab</i>
9	84 kda heat shock protein	172	M18186.1	12	<i>Hsp90ab1</i>
10	Eukaryotic translation initiation factor 2, subunit 1 alpha	158	BC016497.1	10	<i>Eif2s1</i>
11	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin c2 isoform 3	138	BC075641.1	5	<i>Smarca2</i>
12	Chloride intracellular channel 1	129	BC004658.1		<i>Clic1</i>
13	Ubiquitin c	127	D50527.1	1	<i>Ubc</i>
14	HLA-B-associated transcript 3 (Bat 3)	124	NM_057171.1	3	<i>Bat3</i>
15	Ribosomal protein S14	122	Y08307.1	9	<i>Rps14</i>
16	Ribosomal protein S17	121	D25213.1	9	<i>Rps17</i>
17	Ribosomal protein L12	121	L04280.1	9	<i>Rpl12</i>
18	Ribosomal protein, large P2	121	BC012413.1	9	<i>Rplp2</i>
19	Deubiquitinating enzyme (Ubiquitin specific peptidase 14)	119	Q9JMA1	1	<i>Usp14</i>
20	Proteasome subunit alpha type-2 (Proteasome component C3)	118	P49722	2	<i>Psm2</i>
22	Heterogeneous nuclear ribonucleoprotein U	113	NM_016805.2	6	<i>Hnrnpu</i>
23	Ubiquitin specific protease 5 (isopeptidase T)	113	NM_013700.1	1	<i>Usp5</i>
24	PREDICTED: similar to ubiquitin A-52 residue ribosomal protein fusion product a	106	P62984		<i>Uba52</i>
25	PREDICTED: similar to Ribosomal protein S27a	106	BC096392.1	9	<i>Rps27a</i>
26	78 kDa glucose-regulated protein (Heat shock 70kD protein 5)	101	NM_001163434.1	12	<i>Hspa5</i>
27	Tropomyosin alpha 3 chain nonmuscle tropomyosin	101	P21107 1615126A	11	<i>Tpm3</i>

DB, Data Box.

immunoblot were available. Since the size of precipitated proteins closely matched with that found in total input proteins, these proteins are likely to be modified by or directly bound to ubiquitin. We next examined ubiquitination of SQSTM1 and DDX17 *in vivo*. In Fig. 4D, 293T cells were transfected with HA-tagged SQSTM1 (p62) and Flag-Ub, and anti-HA precipitates were tested for Flag-Ub in immunoblot. Similarly, in Fig. 4C, cells expressing Flag-tagged DDX17 and HA-tagged ubiquitin were precipitated with anti-Flag antibody and tested for HA. In both cases, precipitated proteins were associated with Ub. Multiple slow mobility bands detected with SQSTM1 suggest that this protein was associated with both mono- and polyubiquitin chains under these conditions. Finally, we found that SQSTM1 was induced in response to IFN- γ /CpG stimulation in RAW cells coinciding with global ubiquitin conjugation of nuclear proteins (Fig. 4D). To verify ubiquitin conjugation of endogenous SQSTM1, extracts from untreated or IFN- γ /CpG treated RAW cells were precipitated with anti-SQSTM1 antibody and blotted against ubiquitin. As shown in Fig. 4E, SQSTM1 from IFN- γ /CpG-treated cells was conjugated to ubiquitin. Ubiquitin conjugation was not detected in untreated cells

expressing a low level of SQSTM1. These results support the view that many of the proteins identified in this study indeed represent those directly modified by ubiquitin or indirectly associated with ubiquitin upon IFN- γ /CpG stimulation.

Discussion

We found that IFN- γ /TLR stimulation results in a striking increase in ubiquitin-conjugated nuclear proteins in RAW macrophages. IFN- γ alone led to a noticeable increase in ubiquitinated proteins. Additional stimulation with TLR ligands further increased the levels of ubiquitinated proteins, although TLR ligands alone did not have a significant effect. The requirement of IFN- γ for augmenting the effect of TLR signaling is reminiscent of synergistic activation of cytokines by IFN- γ and TLR ligands in macrophages (Kong and others 2007; Kim and Ozato 2009). Increased ubiquitination was detected in the absence of proteasome inhibitor, suggesting that this increase partly changes regulatory activities not directly linked to proteolytic degradation. To our knowledge, this work is the first to document the induction of large-scale ubiquitination of nuclear proteins triggered by

FIG. 3. Classification of ubiquitin-associated nuclear proteins in stimulated macrophages. Proteins showing >100 in the MS scores were classified according to functions/biological activities. The percentages of proteins in each category were a summary of data in Tables 1 and 2, and Supplementary Table S1. The numbers of proteins in each category are shown in the *bottom table*. EIF, eukaryotic translation initiation factor; ELF, eukaryotic translation elongation factor.

macrophage activation. Our results indicate that many nuclear proteins are targeted for ubiquitination after IFN- γ /TLR stimulation, likely causing extensive alterations of nuclear activities required for efficient progression of innate immune responses.

Our MS/MS analysis identified a total of 228 candidate proteins likely associated with ubiquitin either directly or indirectly. We presume that this number is a considerable underestimate, since only those showing higher than 100 MS score in all 3 analyses were included. Ubiquitin-associated proteins detected in this work are of diverse functions, vital to fundamental cellular metabolism. Besides proteins involved in ubiquitin modifications and proteasome activities, proteins regulating DNA metabolism, transcription, and chromatin and those involved in RNA metabolism comprised prominent groups, amounting to ~18% of total proteins. A striking observation is the high representation of ribosomal proteins in our list. As many as 33 of ribosomal proteins were found directly or indirectly associated with ubiquitin. Since each of the 2 ribosomal subunits contain ~33 and ~49 peptides, respectively, it seems that a large

fraction of ribosomal proteins are ubiquitin modified in IFN- γ /TLR-stimulated RAW cells. Given that ribosomes are the protein translation machinery, these results indicate that ubiquitination may play an important role in regulating protein synthesis in stimulated macrophages. However, ubiquitin modification and its impact on ribosomal function have not been extensively documented so far. Nevertheless, Matsumoto and others (2005), through a proteomic analysis of ubiquitin bound proteins in 293T cells, reported extensive ubiquitin binding of ribosomal proteins. It is of note here that although a bulk of ribosomes resides in the cytoplasm, some are in the nucleus. Because our analyses are based on nuclear extracts, it is possible that the ubiquitination status of ribosomal proteins differ between the nucleus and cytoplasm.

Some proteins classified in the DNA metabolism and the transcription/chromatin regulation in Fig. 3 function as stress response factors and are known to be ubiquitin-conjugated in response to stress. For example, DNA methyltransferase I (DNMT1) is ubiquitinated and brought to proteasome-mediated degradation upon drug treatment

FIG. 4. Partial validation of ubiquitin association for identified proteins. **(A)** Nuclear extracts (*left*) and anti-Flag immune complexes (*right*) from mock or Flag-Ub transduced cells were blotted with the indicated antibodies. **(B)** *In vivo* ubiquitination of Sqstm1(p62). 293T cells were transfected with expression vectors for HA-Sqstm1 and Flag-Ub. Lysates were precipitated with anti-HA antibody and blotted with anti-Flag antibody. In the lower panels total lysates were immunoblotted for HA and Flag to confirm the expression of transfected proteins. **(C)** *In vivo* ubiquitination of Ddx17. 293T cells were transfected with vectors for Flag-Ddx17 and HA-Ub. Lysates were precipitated with anti-Flag antibody blotted with anti-HA antibody. **(D)** Nuclear extracts from RAW cells pretreated with IFN- γ and stimulated with CpG for indicated times were blotted against Sqstm1, TFIIB, or Ub. **(E)** Ubiquitination of endogenous Sqstm1. Extracts from untreated RAW cells (-) or treated with IFN- γ /CpG were immunoprecipitated with anti-Sqstm1 antibody and blotted against Ub. In the IgG lane, extracts were precipitated with normal IgG. IgG, immunoglobulin G.

(Zhou and others 2008). Downregulation of DNMT1 is linked to activation of previously silent genes as well as some autoimmune diseases known to have aberrant activation of repressed genes (Chen and Li 2006; Pan and Sawalha 2009). In addition, MYBBP1 is a nuclear stress response protein and upon stress, processed into smaller peptides, presumably by ubiquitin conjugation. MYBBP1 interacts with nucleophosmin, and plays a role in ribosome biogenesis (Yamauchi and others 2008). Our MS/MS analyses found both MYBBP1 and nucleophosmin as ubiquitin-associated proteins. Also, we found ubiquitin association for PCNA, the proliferating cell nuclear antigen, a factor important for DNA replication. PCNA is ubiquitinated in response to DNA damage,

through which it contributes to RAD6-dependent DNA repair pathway (Ulrich 2009). These data suggest a possible link between stress response and IFN- γ /TLR-activated macrophages. Supporting this link, heat shock and related proteins were also found associated with ubiquitin in IFN- γ /TLR-stimulated RAW cells. These factors regulate folding, aggregation, processing, and clearance of many proteins in coordination with the ubiquitin-proteasome network (Powers and others 2009). A link between IFN- γ /TLR-stimulated ubiquitin modifications and stress response pathways suggested above may indicate that innate immune responses share some functional features with broader stress responses.

Only few proteins previously known to be involved in IFN- γ and TLR signaling were found in our list of ubiquitin-associated factors. Nevertheless, SQSTM1 (also known as p62) offers a good example of proteins involved in innate immunity. SQSTM1 carries an ubiquitin binding domain and is induced and ubiquitin conjugated after IFN- γ /TLR stimulation (Fig. 4D, E). Our subsequent study showed that SQSTM1 negatively regulates expression of proinflammatory cytokines, including interleukin-12p40, tumor necrosis factor α , and interleukin-1 β by regulating ubiquitin modification of several transcription factors (Kim and Ozato 2009). SQSTM1-mediated cytokine downregulation presumably contributes to inhibition of uncontrolled cytokine burst.

In summary, the extensive ubiquitin modification of macrophage nuclear proteins likely plays critical roles in redirecting the mode of gene expression, protein synthesis and turnover, and even cell motility, contributing to well-orchestrated elicitation of innate immune responses.

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Author Disclosure Statement

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