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A novel method, using ubiquitin-trapping with a UIM peptide and immunoprecipitation, for identification in non-denaturing conditions of molecules associated with ubiquitinated proteins

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Objective: To demonstrate a novel method, using ubiquitin (Ub)-trapping with a ubiquitin-interacting motif (UIM) peptide and immunoprecipitation, for purification of Ub-proteins and isolation of proteins that associate with epidermal growth factor (EGF) receptors.
Methods: HeLa cells were cultured with or without EGF. Cell lysates were subjected to Ub-trapping, immunoprecipitation with an anti-EGFR antibody, and then liquid chromatography (LC)-tandem mass spectrometry (MS/MS) (LC-MS/MS) or immunoblotting with antibodies to proteins identified by LC-MS/MS.
Results: Ub-trapping isolated Ub-proteins, including Ub-EGFR, but not non-Ub-EGFR. The combination of Ub-trapping and immunoprecipitation with an anti-EGFR antibody isolated pure Ub-EGFR. Cbl-c and c-Cbl were detected by LC-MS/MS in EGF-stimulated cell lysates. Some Cbl-b was shown to be associated with Ub-EGFR, unlike c-Cbl. Although HUWE1 was also detected by LC-MS/MS, HUWE1 was shown to interact with Ub-proteins other than Ub-EGFR.
Conclusions: The method described, a combination of Ub-trapping, immunoprecipitation and immunoblotting, is effective to obtain information regarding specific interactions of various proteins with a target Ub-protein. It should prove useful in future investigations involving ubiquitination mechanisms.

Key words: ubiquitin, epidermal growth factor receptor, UIM (ubiquitin-interacting motif), Cbl, HUWE1

Abbreviations: Ub, ubiquitin; Ub-, ubiquitinated; ASB2, ankyrin repeat-containing protein with a suppressor of cytokine signaling box 2; UIM, ubiquitin-interacting motif; EGF, epidermal growth factor; Cbl, Casitas B lymphoma protein; Ab, antibody; 2-ME, 2-mercaptoethanol; IP, immunoprecipitation; IB, immunoblotting; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; FT, flow-through fraction

Introduction

Ubiquitin (Ub) is a highly conserved 76-amino acid protein and is so named because it is distributed ubiquitously in eukaryotic cells. Protein conjugation with Ub, also known as ubiquitination, is involved in multiple cellular events, including proteasomal degradation, protein trafficking, endocytosis, and signal transduction.[1,2] In addition to single Ub conjugation (monoubiquitination), successive attachment of Ub to previously conjugated Ub (polyubiquitination) occurs in many kinds of proteins. The ubiquitination is caused by the sequential catalytic actions of Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3).

There are a large number of E3 Ub ligases, which are classified into three families-RING, HECT and U box-based on differences in their functional domains.[3] Since E3 Ub ligase determines specificity for ubiquitination

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and degradation, it is a key molecule in the regulation of Ub-dependent proteolysis. ASB2 (ankyrin repeat-containing protein with a suppressor of cytokine signaling box 2) is an E3 ligase that was recently found to ubiquitinate filamins.[4] A splice variant, ASB2β, shares with ASB2α the amino acid sequence at position 69-635. A previous study[5] revealed that there is a ubiquitin-interacting motif (UIM) in the N-terminal region of ASB2β. UIM is a well characterized Ub-binding domain (UBD) that has a highly conserved sequence, XeeeXϕXXAXXXSXXe, where e is a negatively charged residue, ϕ is a hydrophobic residue, and X is any amino acid.[3] Chemically synthesized UIM peptides were found to bind to Ub-proteins (both Lys48 and Lys63 polyUbs) in cell lysates.[5]

We have developed the "Ub-trapping" method to efficiently isolate Ub-proteins by immobilizing UIM peptides on sepharose beads via disulfide bonds. The Ub-proteins that bind to this UIM resin are released by addition of a low concentration of a reducing agent such as 2-mercaptoethanol (2-ME). In this study, Ub-trapping was followed by immunoprecipitation (IP) with antibody (Ab) targeting the protein of interest (the "objective" protein), and then IP with Abs against candidates that possibly interact with that protein. For this study, we chose epidermal growth factor (EGF) receptor (EGFR), an interesting target molecule, because: first, it is involved in epithelial tumors; second, Cbl-c monoubiquitinates EGFR; and third, the mechanism of polyubiquitination of EGFR remains to be elucidated.[6]

In the present study, HeLa cells were stimulated with EGF and then cell lysates were analyzed by the combination of Ub-trapping, IP with an anti-EGFR Ab, and liquid chromatography (LC)-tandem mass spectrometry (MS/MS (LC-MS/MS) or immunoblotting (IB) with Abs to molecules possibly interacting with Ub-EGFR.

Materials and Methods

Reagents and antibodies

Reagents and antibodies were purchased from the following companies: proteasome inhibitor MG132 from Calbiochem, Darmstadt, Germany; protease inhibitor cocktail from Sigma-Aldrich, Saint Louis, MO, USA; N-ethylmaleimide (NEM) and human recombinant EGF from Wako Pure Chemical Industries, Osaka; mouse monoclonal Ab to Ub (FK2) from Nippon Biotest Laboratories, Tokyo; mouse monoclonal Abs to Ub (2C5) and EGFR (6F1) from Medical & Biological Laboratories, Nagoya; mouse monoclonal Abs to EGFR (528), Cbl-b (sc-1704) and c-Cbl (sc-170) from Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit polyclonal Abs to HUWE1 (ab70161) from Abcam plc, Cambridge, UK; horseradish peroxidase (HRP)-conjugated goat Abs to mouse Igs from Biosource International, Camarillo, CA, USA; and HRP-conjugated goat Abs to rabbit IgG from Rockland Immunochemicals, PA, USA.

Cell culture and treatment

HeLa cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containing 10% heat inactivated fetal bovine serum, 1,000 U/ml penicillin, and 1 mg/ml streptomycin (Sigma-Aldrich) at 37℃ in humidified air with 5% CO_2. After 24 hours, HeLa cells were serum-starved overnight and then treated with 20 μM MG132 for 3 hours and subsequently stimulated with or without 50 ng/ml EGF for 10 minutes at 37℃ in 5% CO_2.

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated in 0.5 ml of a lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, 0.5% NP40, 10 mM NaF, 1 mM Na_3VO_4, 10 mM NEM and 1% PIC on ice for 30 minutes. The cell extracts were centrifuged at 13,200 rpm for 30 minutes at 4℃, and the resulting cell lysates (supernatants) were used for experiments.

Ub-trapping

To prepare the resin for Ub-trapping, 12.5 μl of 1 nmol/μl ASB2β (12-41) oligopeptide N-CTIGQEEYSLYSSLSEDELVQMAIEQSLAD-C in lysis buffer was incubated with 10 μl of Activated Thiol Sepharose 4B (GE Healthcare Bio-Sciences, Uppsala, Sweden) for 30 minutes at room temperature with gentle rotation. The resin was washed three times with lysis buffer, and then stored at -80℃ before use, and was designated "UIM resin." Forty microliters of UIM resin was washed twice with 0.2 ml Buffer C (PBS + 1% NP40) by centrifugation at 13,200 rpm at 4℃ for 3 minutes. Two mg of protein from the HeLa cell lysates were incubated in lysis buffer with gentle rotation for 2 hours at 4℃. Suspensions were centrifuged at 13,200 rpm for 3 minutes at 4℃. The resulting supernatants (UIM-flow through; UIM-FL) were mixed with the same volume of 2× SDS sample buffer containing 50 mM dithiothreitol and heated at 95℃ for 1 minutes. Precipitates were washed twice with Buffer C and then reduced with 20 mM 2-mercaptoethanol (2ME) at 37℃ for 30 minutes. The supernatants were used as the UIM sample and were treated with 2× SDS sample buffer. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

stained by immunoblotting and silver staining (Wako Pure Chemical Industries) using standard methods previously described.

Liquid chromatography (LC)-tandem mass spectrometry (MS/MS) and database search

The silver-stained protein bands were excised from the SDS-PAGE gels and subjected to Cys S-alkylation and tryptic digestion, extracted from the gel matrix and dried under vacuum using standard methods described previously, and then analyzed with a fully automated LC-MS/MS system,[7] with modifications. Briefly, reversed-phase peptide separation was performed with an acetonitrile gradient in 0.1% formic acid solution on a C_{18} capillary column (Michrom Bioresources, Auburn, CA, USA). The RPLC (reversed phase liquid chromatography) effluent was interfaced with an electrospray ionization source in positive ion mode on a Finnigan LTQ linear ion trap quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).[8] The electrospray ionization spray emitter (FortisTip, AMR [Akamon Management Review], Tokyo) was directly connected with the outlet of the capillary column. Protonated peptides were analyzed sequentially for MS/MS in a data-dependent scanning mode, consisting of a full-range scan at the m/z range of 450 to 1,800. For protein identification, a database search was performed using Mascot software (Matrix Science, London, UK). Peptide identifications with a Mascot matching score greater than 40 were considered significant.

Immunoprecipitation

Sample solutions (0.1 ml) or cell lysates containing 2 mg protein were allowed to react with 1 μg of an anti-EGFR Ab (528) overnight with gentle rotation at 4℃. Next, they were mixed with protein A-Sepharose and incubated for 1 hour with gentle rotation at 4℃. Suspensions were centrifuged at 13,200 rpm for 3 minutes at 4℃, and the supernatants were used as immunoprecipitation flow-throw fraction (IPFT), which was allowed to treat with Ub-trapping (UIM/IPFT). The precipitates were washed twice with buffer C and mixed with the same volume of 2× sample buffer containing 50 mM DTT (dithiothreitol) and then analyzed by SDS-PAGE as IP and UIM/IP.

Immunoblotting

Proteins were separated by SDS-PAGE using 4% to 12% Bis-Tris Gels (NuPAGE, Life Technologies, CA, USA) for EGFR, Cbl-b and c-Cbl and 3% to 8% Tris-Acetate Gels (NuPAGE, Life Technologies) for HUWE1. Gels were subjected to immunoblotting as described previously.[9] After SDS-PAGE, EGFR, Cbl-b, c-Cbl and HUWE1 were detected with combinations of individual Abs and HRP-conjugated goat Abs targeting mouse Igs. Membranes used for immunoblotting were stripped and re-probed with an anti-Ub Ab (FK2 or 2C5) and HRP-conjugated goat Abs targeting mouse Igs.

Results

Ubiquitination of EGFR on EGF stimulation

HeLa cells were stimulated with different concentrations of human EGF for different periods of time. The cell lysates were treated as described above. As shown in Figures 1A and B, IP efficiently concentrated EGFR. Ub-EGFR rapidly increased when cells were stimulated with 10 ng/ml EGF, and the amount of Ub-EGFR peaked after 5 minutes (Figure 1A). In the next experiments, the cells were stimulated with different concentrations of EGF for 10 minutes. As shown in Figure 1B, Ub-EGFR increased with increasing EGF concentration and non-

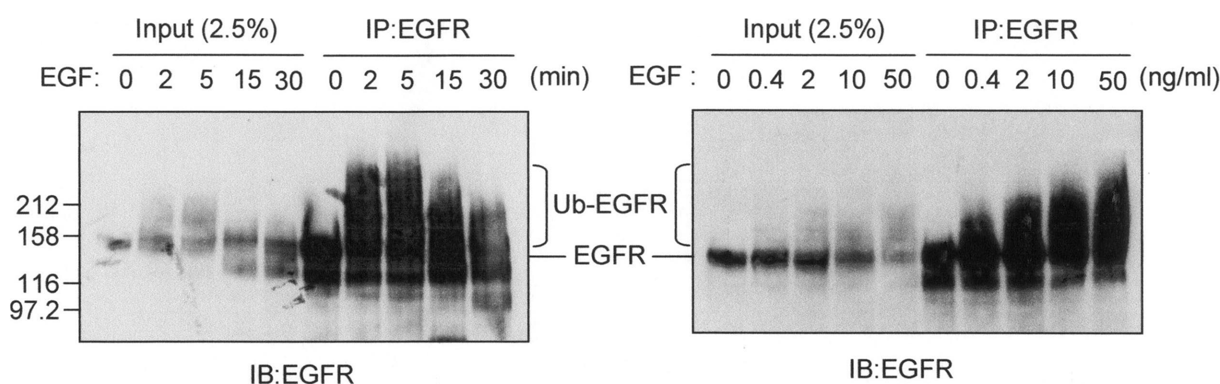

Figure 1. Ubiquitination of EGFR resulting from EGF stimulation. (**A**) HeLa cells stimulated with 10 ng/ml EGF for various times and cell lysates subjected to IP and then IB with an anti-EGFR Ab. A total of 2.5% of cell lysates were directly subjected to IB without IP (Input). (**B**) HeLa cells stimulated with various EGF concentrations for 10 minutes.

Ub-EGFR decreased simultaneously. Based on these results, in the next experiments, cells were stimulated with 50 ng/ml EGF for 10 minutes.

Search for proteins possibly interacting with EGFR using mass spectroscopy

To identify functional proteins associated with Ub-EGFR, cell lysates from HeLa cells treated with or without 50 ng/ml of EGF for 10 minutes were subjected to Ub-trapping followed by IP with an anti-EGFR Ab. The immunoprecipitates were subjected to SDS-PAGE, then proteins were detected by silver staining. Several well-defined bands, in addition to a smear of Ub-EGFR, were observed in EGF-stimulated cells, but not in unstimulated cells (Figure 2). LC-MS/MS and database search unambiguously identified HUWE1 in the region larger than 400 kDa with the unique 7 tryptic peptide fragments. The MS/MS-based analysis also identified Cbl-b (8 peptides) and c-Cbl (4 peptides) at the region from 100 kDa to 120 kDa (data not shown). Since these molecules are known to be E3 Ub ligases, it is likely that they would interact with Ub-EGFR. Therefore, their possible interactions with Ub-EGFR were studied by the combination of Ub-trapping, IP with an anti-EGFR Ab and IB with Abs to those molecules (Figure 3).

Interaction of Cbl-b and c-Cbl with EGFR

Cbl-b and c-Cbl were found in LC-MS/MS of immunoprecipitates obtained from cell lysates of EGF-treated cells by the combination of Ub-trapping and IP with an anti-EGFR Ab (Figure 2). Both Cbl-b and c-Cbl have been reported to bind to and to function as E3 Ub ligases for EGFR, although this remains a matter of some debate.[10,11] They were analyzed by a combination of Ub-trapping, IP and then IB with an anti-EGFR Ab (Figure 4).

As shown in Figures 4A and 4B, the Ub-trapped fraction (UIM, lanes 7 and 8), but not the non-trapped fraction (UIMFT, lanes 13 and 14), resulted in smears of Ub-proteins. Also, the Ub-trapped and IP flow-through fractions (UIM/IPFT, lanes 11 and 12), but not the non-trapped and IP flow-through fractions (UIMFT/IPFT), contained Ub-proteins. Thus, Ub-trapping efficiently isolated Ub-proteins in cell lysates. Comparing IP vs. IPFT and UIMFT/IP vs. UIMFT/IPFT, IP most efficiently collected most of the EGFR. The combination of Ub-trapping and IP isolated Ub-EGFR with very faint Ub-protein smears but did not isolate non-Ub-EGFR.

It is notable that the UIM/IP fraction of EGF-treated cells (lane 10) contained not only Ub-EGFR but also Cbl-b; whereas, EGF-untreated cells (lane 9) contained

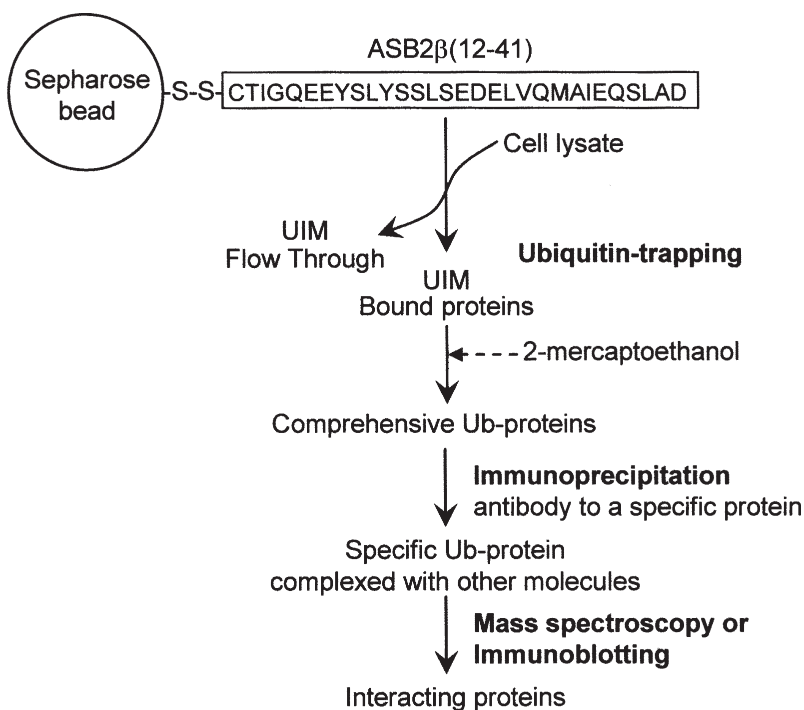

Figure 2. Silver-stained proteins on SDS-PAGE and analysis by LC-MS/MS. HeLa cells were cultured with or without 10 ng/ml EGF for 5 minutes, and then cell lysates were subjected to Ub-trapping and IP with an anti-EGFR Ab. Proteins on gels were S-alkylated and digested with trypsin. Peptide mixtures were analyzed by LC-MS/MS.

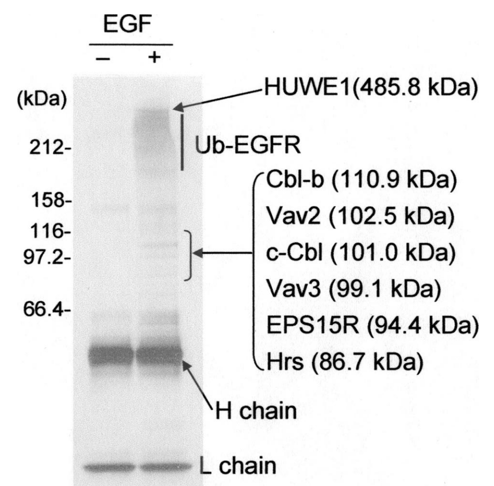

Figure 3. Schematic illustration of the combination of Ub-trapping, IP and subsequent IB using an Ab targeting a specific protein of interest ("objective" protein). UIM peptides or ASB2β (12-41) were fixed onto sepharose beads via disulfide bonds.

none of these (Figure 4A). These results indicate that some Cbl-b was associated with Ub-EGFR when cells were treated with EGF, while the rest of the Cbl-b seem to have been associated with non-Ub-EGFR (lanes 3, 4, 15, and 16).

As shown in Figure 4B, unlike Cbl-b, c-Cbl did not precipitate along with Ub-EGFR (UIM/IP, lane 10) even in cells treated with EGF. c-Cbl does not seem to be associated with either Ub-EGFR or non-Ub-EGFR, as seen by comparing lanes 3 and 4 vs. lanes 5 and 6, and lanes 15 and 16 vs. lanes 17 and 18. In addition, c-Cbl did not bind to UIM peptides, irrespective of EGF treatment (lanes 7-12). Therefore, these results suggest that c-Cbl does not bind to either UIM peptides or EGFR.

Binding of HUWE1 to UIM peptide

An apparent band of about 500 kDa was found in the silver-stained gel of EGF-treated cells. It was identified by LC-MS/MS to be HUWE1, which is known to be an E3 Ub ligase.[12] HUWE1 was also analyzed by the

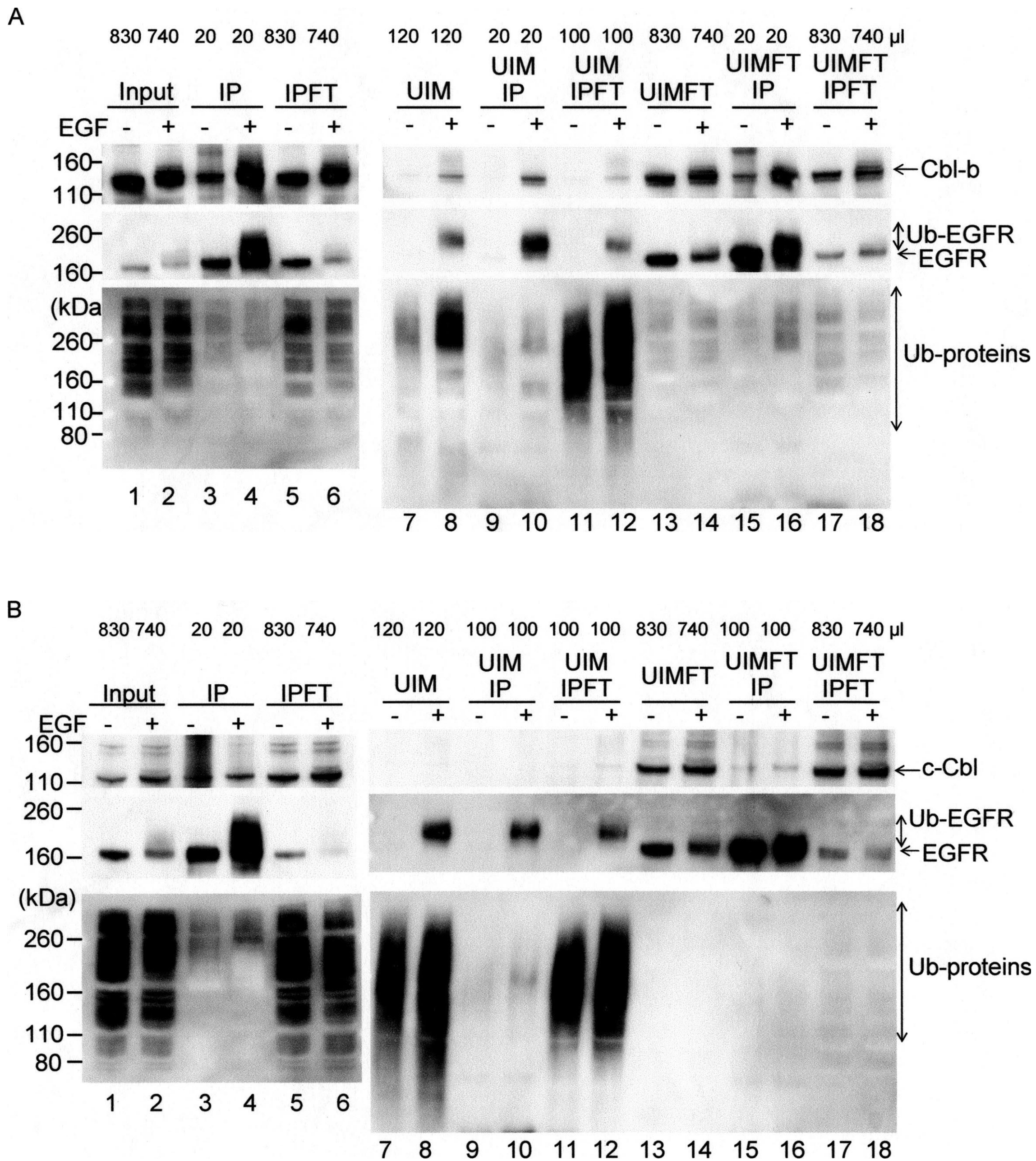

Figure 4. Analyses of interactions of (**A**) Cbl-b and (**B**) c-Cbl with EGFR. HeLa cells were stimulated with 50 ng/ml EGF for 10 minutes, and then the cell lysates were subjected to Ub-trapping, IP with an anti-EGFR Ab and then IB with Abs to EGFR, Cbl-b or c-Cbl, and Ub. Numbers at the top: fraction volumes. UIM, Ub-trapped fraction; UIMFT, flow-through fraction after Ub-trapping; IP, fraction immunoprecipitated with an anti-EGFR Ab; IPFT, non-precipitated flow-through fraction of IP.

combination of Ub-trapping, IP with an anti-EGFR Ab and IB with Abs to EGFR, HUWE1, and Ub (Figure 5).

HUWE1 appears to bind to UIM peptides: the Ub-trapped fraction (UIM, lanes 9 and 10) contained HUWE1 to the same extent as the UIMFT fraction (lanes 15 and 16), and the UIM/IPFT fraction (lanes 13 and 14) had almost the same amount of HUWE1 as did the UIMFT/IPFT fraction (lanes 7 and 8). IP with an anti-EGFR Ab rarely precipitated HUWE1, as compared with IP (lanes 3 and 4) vs. IPFT (lanes 5 and 6), and UIMFT/IP (lanes 17 and 18) vs. UIMFT/IPFT, suggesting that HUWE1 was partially independent of EGFR. These results show that half of HUWE1 bound to UIM peptides and was independent of EGFR, irrespective of EGF treatment.

Discussion

There are two splice variants of ASB2, ASB2α, and ASB2β. The amino acid sequence of ASB2β (Arg69-Gln635) is identical to that of ASB2α (Arg21-Gln587). We previously identified a Ub-interacting motif (UIM) in the N-terminal region (Cys12-Asp41) of ASB2β.[5] By making use of this sequence, a novel affinity purification method that enables efficient isolation of Ub-proteins from cell lysates, in the form of complexes with their functionally associated molecules, was established; we designated this "Ub-trapping." Ub-trapping followed by IP with an anti-EGFR Ab afforded isolation of not only Ub-EGFR, but also Cbl-b, which was previously reported to be associated with EGFR,[10,11] and another Ub ligase, HUWE1,[12] not previously known to interact with EGFR.

The Ub-trapped protein fractions, UIM and UIM/IPFT, contained very dark smears of Ub-proteins, whereas the non-Ub-trapped protein fractions, UIMFT and UIMFT/IPFT, had faint or no smears (Figures 4A, B, and 5), suggesting that most Ub-proteins were isolated by Ub-trapping. Furthermore, the combination of Ub-trapping and IP with an anti-EGFR Ab isolated Ub-EGFR without either non-Ub-EGFR or other Ub-proteins. Current proteomic approaches for investigating cellular Ub-proteins are based on affinity purification with anti-Ub antibodies[13,14] using a GST (Glutathione S-transferase) fusion protein with S5a having two UIM motifs.[15] In these approaches, the bound proteins are eluted by protein denaturation and then separated with SDS-PAGE. These proteomic methods have contributed to comprehensive identification of cellular Ub-proteins. However, since those methods include a protein denaturation process, one cannot tell which Ub ligase acts on which protein substrate, or what kinds of proteins are associated with the Ub-protein. In the method presented here (Ub-trapping), UIM peptide immobilized through a disulfide bond efficiently captured both K48- and K63-linked Ub-proteins, and 2-ME reduced the disulfide bonds to release the Ub-proteins, which remain complexed with

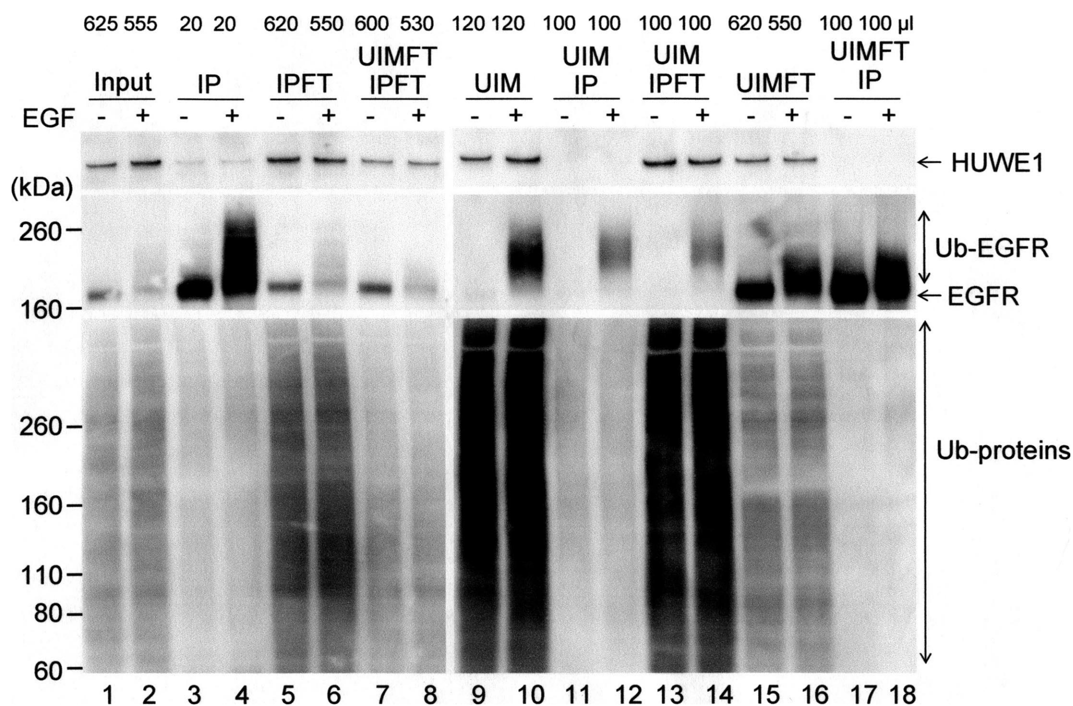

Figure 5. Analysis of the interaction between HUWE1 and EGFR. HeLa cells were treated in the same manner as described in the Figure 4 legend, using an anti-HUWE1 Ab for IP and IB.

functionally associated proteins. Unlike previously reported methods, because the peptide involved is as short as 30 amino acids. Theoretically, there should be very few nonspecifically bound proteins among those eluted; and, therefore, Ub-trapping should identify proteins associated with Ub-proteins better.

Cbls are E3 Ub ligases, which mediate the ubiquitination of activated tyrosine kinases, including EGFR.[16] Although Cbl-b and c-Cbl seem to be involved in the ubiquitination of EGFR, in the present study, Cbl-b associated with EGFR but c-Cbl did not.[10] The Ub-associated domain of Cbl-b had a higher affinity than that of c-Cbl.[10] In the present study, some Cbl-b was found to be associated with Ub-EGFR, but not non-Ub-EGFR, and c-Cbl did not bind to either Ub-EGFR or non-Ub-EGFR fractions (Figure 4A). These results are consistent with those from previously reported studies.

HUWE1 identified by LC-MS/MS in the cell lysates of EGF-treated cells is also an E3 Ub ligase[12] involved in the regulation of cell proliferation, apoptosis, DNA damage responses, and base excision repair.[17] LC-MS/MS revealed that HUWE1 precipitated with Ub-EGFR in EGF-treated cells, leading us to anticipate that HUWE1 might be the E3 Ub ligase involved in EGFR polyubiquitination. However, analysis by the combination of Ub-trapping, IP and IB revealed that there was no HUWE1 in the Ub-EGFR fraction (Figure 5, lane 12) and that some portion of HUWE1 was in the UIM fraction, suggesting some interaction of HUWE1 with the UIM resin. HUWE1 has tandemly repeated Ub-binding domains[12] and, therefore, may bind to Ub-proteins.

Ubiquitination is involved in many kinds of diseases such as cancer[18] and Alzheimer's disease, and therapeutics targeting it are actively being investigated.[19,20] The method described here should prove useful in future studies designed to further elucidate the ubiquitination system and pathogenic mechanisms involved in diseases possibly resulting from this process.

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