The ligand-mediated down-regulation of the growth factor receptors is preceded by the involvement of various other factors. In particular, a ubiquitin ligase, Cbl, plays a central role in this event. Several candidates that have potential effects on the negative control of the epidermal growth factor (EGF) receptor have now been identified by our recent studies in phospho-proteomics. Among these molecules, we focus on characterizing a novel protein, Ymer, which is a tyrosine-phosphorylated and ubiquitinated protein. Ymer is found to be phosphorylated at tyrosine 145 and 146 upon EGF stimulation, and lysine 129 of Ymer has been identified as a ubiquitination site. Ymer has two motifs interacting with the ubiquitin (MIU) domains that might function as a binding site for the ubiquitinated EGF receptor. Although Ymer and EGF receptors are associated in an EGF-dependent manner, their interaction is required not only for MIU domains but also for the tyrosine phosphorylation of Ymer. Phosphorylated Ymer is mainly located at the plasma membrane with EGF receptor and functions in its endocytosis and degradation. Furthermore, EGF-mediated secondary modifications of an activated-EGF receptor are inhibited by overexpressing Ymer in COS7 cells. Therefore, Ymer may have competitive effects on the activation of the EGF receptor. Our findings suggest that Ymer functions as a novel inhibitor for the down-regulation of the EGF receptor and plays a crucial role for regulating the amount of the EGF receptor on the cell surface membrane.

Because the EGF receptor is well known for being involved not only in general cell growth but also in the generation or development of various human cancers (1), many research groups have applied a proteomic technique using mass spectrometry (MS) to identify novel molecules and posttranslational modifications such as phosphorylation and ubiquitination involved in this EGF signaling pathway (2). Recently, numerous proteins, including both well known and previously unknown proteins in growth factor receptor-mediated signal pathways, have been identified by proteomic studies carried out by several research groups including ours (3–7). To obtain a complete picture of EGF receptor-mediated signaling pathways by using variable experimental resources derived from proteomics, each identified molecule must be validated and characterized to ensure that it is involved in this pathway; this is an important step in the post-proteomics stage.

In the EGF receptor-mediated signaling pathway, receptor autophosphorylation after ligand binding is the first of the various intracellular events induced by EGF stimulation. After this step, protein-protein interactions of the assembled molecules with the activated receptor complex occurs for transducing the signal to several downstream kinase cascades (8). In general, these processes resulting from EGF receptor activation are likely to positively regulate this signaling pathway. On the other hand, ligand-mediated ubiquitination of the EGF receptor plays a role in its internalization to the endosomal pathway, which determines the fate of the intracellular EGF receptor; that is, its recycling or degradation (9, 10). Therefore, this modification is considered to be a negative regulator of the EGF receptor. Based on the results of phospho-proteomics that explores the downstream molecules of the EGF receptor, it is suggested that many proteins related to its internalization are involved in the interactome of Cbl, an E3 ubiquitin ligase that plays a central role in the growth factor receptor down-regulation (9). However, a newly identified molecule from the proteomics study that does not have any functional domain needs to have its functions in the EGF receptor-mediated signaling pathway deciphered using appropriate biochemical and cell biological experiments.

Ymer, which was originally named by Blagoev et al. (6), has been identified as a tyrosine-phosphorylated protein, and one of its phosphorylation sites (tyrosine 145) has also been detected (4, 5) by phospho-proteomic experiments performed by several research groups. Although Ymer has been hypothesized to function in the EGF signaling pathway, there is no biological evidence to this effect. Moreover, a recent report has
described that one of the newly identified ubiquitin binding motifs, MIU (motif interacting with ubiquitin) is contained within Ymer (11), which indicates that Ymer may be ubiquitinated and may function in the negative control of receptor signaling. In this study, we focus on Ymer that has been identified by our original phospho-proteomic experiments using EGF-stimulated A431 cells, and we demonstrate that the functional analysis in the EGF receptor down-regulation events is dependent upon the multiposttranslational modifications of Ymer.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Transfection, and Reagents—**COS7, HeLa, human embryonic kidney (HEK) 293T, and A431 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin. Transfection of a plasmid into COS7, HeLa, or HEK293T cells was carried out by electroporation using Gene Pulser (Bio-Rad). Before EGF stimulation, cells cultured for 48 h after transfection were serum-starved for 16 h, and 50 ng/ml EGF (Sigma) or 100 ng/ml Alexa-fluor 488-conjugated EGF (Molecular Probe) dissolved in serum-free medium was added. MG132 was purchased from Sigma.

**cDNA Cloning and Vector Constructions—**For cloning of the long and short forms of the Ymer cDNA, the A431 cDNA library were screened by PCR with two primers (EcoRI, 5′-ATGGCT-GAAGTCAGCATCGAC-3′, and BamHI, 3′, 5′-TTAATGGTT-TGAAATGAAA-3′) that were designed based on the sequence (gi 40675504) in the NCBI data base. The amplified products were first subcloned into a TOPO™ TA cloning vector, pCR 2.1-TOPO (Invitrogen), and then sequenced. The nucleotide sequences were determined using an ABI prism dye terminator cycle sequencing kit (PerkinElmer Life Sciences) and an ABI Prism 3100-Avant genetic analyzer. Ymer cDNAs were subcloned into a pCMV-FLAG6a (Sigma) expression vector in-frame and expressed as FLAG-tagged protein with the tag at the N terminus. The human HA-tagged ubiquitin construct was provided by Dr. S. Hatakeyama (Hokkaido University, Japan). The DNA fragments of Ymer for the construction of deletion mutants were prepared by using PCR or suitable restriction enzyme digests. After examining their DNA sequences, the constructs were subcloned into a pCMV-FLAG6a (Sigma) expression vector in-frame and expressed as FLAG-tagged protein with the tag at the N terminus. The human HA-tagged ubiquitin construct was provided by Dr. J. E. van Leeuwen (University of Nijmegen, The Netherlands).

**Antibodies—**Two polyclonal anti-Ymer antibodies were used in this study. Anti-Ymer(His) antibody and anti-Ymer(pep) were raised against a Ymer fragment containing residues 77–183 and 166–270, which were bacterially produced as His-tagged protein using PET vector (Novagen) and as glutathione S-transferase fusion protein using pGEX4T vector, respectively. These antibodies were purified using HiTrap N-hydroxy succinimide-activated Sepharose columns (Amersham Biosciences) coupled with each immunizing antigen. Phosphorylation site-specific antisera was raised against the phosphopeptides having an additional cysteine residue at the N-terminal end of the Ymer amino acid sequence, namely, CAYADSYpYYEDGGGM (amino acids 139–151; pY indicates phosphotyrosine). The phosphopeptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits. The phosphorylation site-specific antibodies were purified from antisera by successive column chromatography with the use of affinity resins coupled with each phosphopeptide and nonphosphopeptide. The Ymer antibodies used in this study were tested for their specificities for use in immunoprecipitation (anti-Ymer(pep)), immunoblotting (anti-Ymer(His) and (pep)), and immunofluorescence studies (anti-Ymer(pep)). Other antibodies that were obtained commercially from various suppliers included anti-FLAG M2 antibody (Sigma), anti-phosphotyrosine antibody (4G10; Upstate Biotech Inc.), anti-EGF receptor antibody (Santa Cruz Biotechnology, SC-03), anti-HA antibody (12CA5; Roche Applied Science), and anti-ubiquitin (P4D1, Santa Cruz).

**Semi-quantitative Reverse Transcriptase-PCR—**To assess the relative expression levels of Ymer transcripts, a reverse transcriptase-PCR assay was performed on each panel of eight different human culture cell lines and tissue cDNAs (human tissue and cell line MTC panel, Clontech) using the primers 5′-AAA-AGACCCTTTGAAACAACAA-3′ and 5′-TTATTTCTTCAGCATTAGAAG-3′. The normalized cDNA was amplified under the following conditions: denaturation at 94 °C for 1 min; 30 or 50 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and extension at 72 °C for 5 min. PCR products were electrophoresed in 1.5% agarose gels.

**Small Interference RNA (siRNA)—**The mammalian expression vector pSUPER-Retro-puro (Oligoengine) was used for expression of siRNA in HeLa cells. The targeted sequence of human Ymer was 5′-AAGATGGAGGAATGAAGCCAA-3′ (nucleotides 440–460), and a resulting plasmid for the knockdown of Ymer was named pSuper-Ymer. The empty pSuper vector was used as a control. HeLa cells were stably infected with an amphotropic receptor. The packaging cells, AmphotopoCMV293 cell line (Clontech), were transfected with the appropriate retroviral RNA-mediated interference construct by electroporation. Culture supernatants were collected after 48 h post-transfection and centrifuged. HeLa cells were infected with the viral supernatants in the presence of 8 μg/ml Polybrene (Sigma) for 12 h, after which the medium was replaced with fresh medium. After infection, cells were selected using 2 μg/ml puromycin.

**Immunoprecipitation and Immunoblot Analysis—**The following procedures were carried out at 0–4 °C. Cells were lysed in lysis buffer containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM dithiothreitol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM Na3VO4, and complete protease inhibitors mixture (Roche Applied Science). The lysate was used as a whole cell lysate. For immunoprecipitation experiments, the whole cell lysate was centrifuged, and the supernatant was incubated for 2 h with an appropriate antibody. Protein G-Sepharose (Amersham Biosciences) was added. Then the resulting mixture was rotated at 4 °C for additional hour. The beads were then washed three times with lysis buffer. The samples
FIGURE 1. Ymer is tyrosine-phosphorylated upon EGF stimulation. A, detection of Ymer by immunoblotting using a Ymer-specific antibody. Shown are whole cell lysates of COS7 cells transfected with empty vector (lane 1), the expression vector for FLAG-Ymer-S (lane 2), and FLAG-Ymer-L (lane 3). Immunoblot analysis was carried out using anti-Ymer antibody. The positions of each molecule are indicated by arrows. WCL, whole cell lysate. B, Ymer is included in the total pool of tyrosine-phosphorylated proteins in EGF-stimulated A431 cells. Tyrosine-phosphorylated proteins in A431 cells treated with (right) or without (left) EGF (50 ng/ml, 10 min) were immunoprecipitated (IP) using anti-phosphotyrosine antibody (α-pY). Immunoblot analysis was carried out using anti-EGF receptor antibody (upper panel) or anti-Ymer antibody (lower panel). Positions of each molecule are indicated by arrows. α-EGFR, anti-EGF receptor; α-Ymer, anti-Ymer; endo, endogenous. C, EGF receptor-mediated tyrosine phosphorylation of Ymer in A431 cells. Ymer was immunoprecipitated from the lysates of A431 cells with (center) or without (right) EGF stimulation for 10 min. A control experiment was carried out using control IgG. Immunoblot analysis was carried out using anti-Ymer antibody (upper panel) or anti-phosphotyrosine antibody (lower panel). D, schematic representation of the primary structure of Ymer isoforms. The wild-type long isoform of Ymer is composed of 12 exons, and the truncated form, Ymer-S, lacks the region from Asp-150 to Ala-325 encoded by exon 6. The numbers of amino acid residues of the boundaries are indicated. The positions of the common primers for reverse transcriptase-PCR to detect the amounts of expression level of each Ymer variant are indicated by thin lines. The amplified DNA fragments of the expected sizes derived from Ymer cDNAs are indicated by bold lines. E, expression of Ymer mRNA in various human cultured cells. Quantitative-PCR was carried out using specific internal oligonucleotide primers as indicated by arrows in the upper panel. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a loading control. The PCR cycle number for all samples was 30.

were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to an Immobilon P membrane (Millipore). Immunoblot analysis was carried out using primary antibodies as described in the figure legends. Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG and ECL reagent (Amersham Biosciences).

Purification of FLAG-Ymer and Its Binding Protein—293T cells (1 × 10⁶ cells) transiently expressing FLAG-tagged Ymer were lysed with lysis buffer. The lysate was centrifuged, and the supernatant was incubated with FLAG affinity gel (50-μl bed volume) (Sigma) for 2 h. The gel was applied to an empty mini column (Bio-Rad) and washed with lysis buffer several times. FLAG-Ymer was eluted with 45 μl of 100 mM glycine-HCl (pH 3.0). The eluate was neutralized with 5 μl of 1 M Tris-HCl (pH 9.0) and resolved by SDS-PAGE. All bands that were visible after Coomassie Brilliant Blue staining were subjected to MS/MS analysis after in-gel digestion with trypsin.

In-gel Digestion withTrypsin—Each gel slice was cut into small pieces and put into a new tube. The gel pieces were destained by rinsing in 50% acetonitrile containing 25 mM NH₄HCO₃. Disulfide bonds were reduced by incubating with 10 mM dithiothreitol in 25 mM NH₄HCO₃ at 56 °C for 1 h and alkylated with 55 mM iodoacetamide in 25 mM NH₄HCO₃ at room temperature for 45 min in the dark. Gel pieces were washed with 50% acetonitrile and dried in a vacuum concentrator (SpeedVac, Thermo Savant). Gel pieces were rehydrated with 15 μl of trypsin solution (10 ng/μl trypsin in 50 mM NH₄HCO₃ containing 5 mM CaCl₂). In-gel digestion was performed overnight at 37 °C. The resulting peptides were extracted with 50% acetonitrile and dried in a vacuum concentrator (SpeedVac) and redissolved in 0.1% (v/v) formic acid. The peptide mixtures obtained were subjected to LC/MS and data-dependent LC/MS/MS analyses.

Mass Spectrometric Analysis—LC/MS/MS analysis was carried out in a quadrupole-time-of-flight-type hybrid mass spectrometer (Micromass, UK) interfaced on-line with a capillary high performance liquid chromatography (Waters-Micromass modular CapLC, Micromass, UK) (12, 13). The linear gradient of acetonitrile produced was split at 1:10 ratio, and a slow flow of ~100 nl/min was injected into a nano-LC column (PepMap C18, 75 μm × 150 mm, LC Packings). The column eluate was directly injected into a self-constructed nanospray ion source with a tapered stainless capillary. The LC/MS/MS analysis was carried out in a data-dependent mode; peptide peaks above a certain threshold were subjected auto-
Fluorescence Microscopic Analysis—COS7, HeLa, and A431 cells with or without transfection were fixed with 5% formaldehyde in PBS for 10 min, washed with PBS, permeabilized with 0.1% Triton in PBS for 10 min, and washed with PBS again. After a blocking step with 3% bovine serum albumin in PBS for 30 min, the primary antibodies, as described in the figures, were applied for 1 h. After washing with PBS, cells were incubated with appropriate secondary antibodies conjugated with Alexa fluorescent dyes (Molecular Probes) for 45 min. If required, the cells were treated with rhodamine phalloidin (Molecular Probes). The cell nuclei were stained with 2 μM Hoechst 33342 (Molecular Probes). Finally, cells were rinsed 3 times with PBS and mounted onto microscope slides with ProLong Antifade reagents (Molecular Probes). The images taken were those observed under a Zeiss Axioskop 200 fluorescence microscope with a 40 × 1.0 numerical aperture PlanApo objective (Zeiss Axioskop, Carl Zeiss Inc.). Figures were prepared using Adobe Photoshop.

RESULTS

Ymer Is a Tyrosine-phosphorylated Protein upon EGF Stimulation—Ymer has been identified as a tyrosine-phosphorylated protein through the phospho-proteomic studies performed by three independent research groups (4–6). In addition, by using a similar strategy, we have also independently identified more than 150 proteins containing Ymer from the lysate of EGF-stimulated A431 cells.3 However, the function of Ymer in the EGF signaling pathway is yet to be elucidated. Although the study on the cDNA cloning of Ymer has revealed that two splice variants of Ymer transcriptants are expressed ubiquitously in mammalian tissues and that the short variant is dominantly expressed (14), little evidence for expression of this protein has been shown in cultured cells. Therefore, biochemical analysis on Ymer was carried out. It has been reported that the long form of Ymer (Ymer-L) is transcribed as a 1449-bp mRNA comprising a total of 12 exons, which corresponds to a 482-amino acid polypeptide. A splice variant lacking exon 6 encodes the short form of Ymer, which corresponds to a 306-amino acid polypeptide (Fig. 1D). We have produced a specific
antibody against Ymer, which recognizes the recombinant Ymer proteins with considerable accuracy (Fig. 1A, lane 2 and 3). By using this antibody, endogenous Ymer was also detected in the cell lysates from COS7 (Fig. 1A, lane 1). This band was not detected using preimmune serum or by incubating with immunizing antigen (data not shown). Furthermore, the immunoprecipitants with phosphotyrosine antibody from EGF-stimulated A431 cells included Ymer (Fig. 1B, right), and immunoprecipitated endogenous Ymer from EGF-stimulated cells was tyrosine-phosphorylated (Fig. 1C). Therefore, Ymer might be tyrosine-phosphorylated in an EGF-dependent manner. The size of this Ymer was apparently similar to the expected size of the short form, and the band of Ymer-L was very faint in the whole cell lysate (WCL; Fig. 1A). Therefore, Ymer-S is expressed dominantly in all examined cells with the exception of a breast cancer cell line, MCF-7 (Fig. 1F). The Ymer protein was not detected in the lysate from these cells (data not shown).

Tyrosine 145 and 146 of Ymer Are Phosphorylated upon EGF Stimulation—Two research groups have described that tyrosine 145 of Ymer is phosphorylated by growth factor stimulation (4, 5), and we have obtained similar results from a deletion study (data not shown). To confirm the phosphorylation of Tyr-145 of Ymer, point mutants replacing the tyrosines around this phosphorylation site with phenylalanine were constructed. Unexpectedly, the Y145F mutant of Ymer continued to be phosphorylated upon EGF stimulation, and a double mutant replacing both Tyr-145 and 146 with Phe abolished the EGF-dependent tyrosine phosphorylation (Fig. 2B). Therefore, in addition to Tyr-145, 146 of Ymer is also phosphorylated upon EGF stimulation. For further analysis on the physiological function of Ymer, we have developed a Tyr-145 phosphorylation-specific antibody. This antibody recognizes the phosphorylation of both Ymer isoforms with considerable accuracy (Fig. 2, B and C), and we demonstrated this in our study on the subcellular localization of phosphorylated Ymer using this antibody as described below.

Ymer Is a Ubiquitinated Protein—To gain insights into the function of Ymer, we analyzed proteins coprecipitated with Ymer by immunoaffinity purification. HEK293T cells transfected with the expression plasmid for FLAG-tagged Ymer-S were lysed with the buffer containing 1% Triton X-100, and immunoprecipitation was carried out using an anti-FLAG-affinity gel. Proteins eluted with glycerine buffer were analyzed with SDS-PAGE. In addition to Ymer, a protein ~45 kDa was detected only in the immune complex from the transfected cells (Fig. 3A). These bands were cut out from the gel and in-gel-digested with trypsin. The resulting extracted peptides were analyzed to identify their amino acid sequences using tandem MS. Interestingly, this band was Ymer itself, and several peptides derived from ubiquitin were also detected (data not shown). We carefully analyzed the MS/MS profile of the Ymer peptide containing the ubiquitin sequence; lysine 129 was identified as a ubiquitination site of Ymer (Fig. 3B). To confirm the data from MS/MS, an immunoprecipitation study was performed with co-expression of HA-tagged ubiquitin. We found that Ymer-S was ubiquitinated in COS7 cells, and the amount of the high molecular weight band increased after EGF treatment (Fig. 3C). This result suggests that Ymer might be polyubiquitinated in EGF-stimulated cells. On the other hand, several bands conjugated with HA-tagged ubiquitin might be mono- or multiubiquitinated Ymer, and the intensity of these bands also increased slightly upon EGF stimulation (Fig. 3C). In contrast to Ymer-S, which was strongly ubiquitinated in the cells, only a band of likely monoubiquitinated Ymer-L was detected (Fig. 3C). Although the mutant Ymer replacing Lys-129 with arginine was also strongly mono- or mult ubiquitinated, the polyubiquitinated Ymer levels did not increase upon EGF stimulation in COS7 cells (Fig. 3D). Because there are many Lys residues that could be putative ubiquitinated sites in the Ymer molecule, further studies must be carried out to identify the total ubiquitination sites. On the other hand, our previously identified protein, CFBP, which could play a role as the positive effector in the down-regulation of EGF receptor, was not ubiquitinated (Fig. 3D).

Next, we focused on the MIU region, which has been most recently identified as a new ubiquitin-binding site (11, 15, 16). Although the precise position of the monoubiquitination site of Ymer has not yet been identified, the double mutant of alanine 179 and 206 that plays crucial roles for the structures of two MIU domains replaced with glycine diminished the amounts of...
monoubiquitinated Ymer (Fig. 3E). Therefore, two MIU domains in Ymer play a role in its ubiquitination. However, the ubiquitination of exogenous Ymer normally occurred without EGF treatment in COS7 cells, and it was enhanced by pretreatment with a proteasomal inhibitor, MG132 (Fig. 3F). This result suggests that ligand-induced polyubiquitination and degradation of Ymer may be dependent on the proteasomal pathway. Ymer Binds to the EGF Receptor in an EGF Stimulation-dependent Manner—From our results, we found that multi-posttranslational modifications occurred in the Ymer molecule in an EGF-dependent manner. According to the results of quantitative phosphorylation proteomics, the EGF-induced tyrosine phosphorylation of Ymer was ~2.5-fold higher than its phosphorylation upon platelet-derived growth factor stimulation (7). Therefore, Ymer might be a suitable substrate for the EGF receptor. Likewise, the substrate of the EGF receptor, Eps15, is well known as a ubiquitinated and EGF receptor-binding protein, which functions in the process of EGF receptor down-regulation. Hence, we focused on the physical relationship between Ymer and the EGF receptor. Endogenous Ymer was found to be co-immunoprecipitated with the EGF receptor in the lysate from EGF-stimulated A431 cells (Fig. 4A). Next, we proceeded to identify the EGF receptor binding site in Ymer by using recombinant proteins in COS7 cells. Although it was impossible to detect endogenous EGF receptors bound to FLAG-Ymer-S, the complex of both proteins appeared due to the overexpression of EGF receptor in COS7 cells. Furthermore, the Ymer-S region of amino acids from 78 to 216 was found to be a binding site for the EGF receptor, which contained its ubiquitination and tyrosine phosphorylation sites and two MIU regions (Fig. 4, B and C). Therefore, we tested the effects of posttranslational modifications in Ymer on the interaction between Ymer and the EGF receptor. Because the Y145F/Y146F mutant of Ymer did not bind to the EGF receptor, the phosphorylation of these sites might be necessary for their binding. On the other hand, Ymer-L also had a low affinity for binding to the EGF receptor (Fig. 4D). This result suggests that the internal sequence between posttranslational modification sites and MIU regions, which are encoded by exon 6, may have negative effects on the binding to the EGF receptor. Next, we focused on the MIU region that has been most recently identified as a new ubiquitin-binding site. Because the mutant replacing Ala-179 with Gly diminished the binding activity to the EGF receptor as compared with the wild type and another mutant, the N-terminal MIU domain might be necessary for binding to the EGF receptor (Fig. 4E). To clarify the meaning of the interaction between EGF receptor and Ymer, we focused on the effect of Ymer on the posttranslational modification of the EGF receptor. Hence, the EGF-dependent ubiquitination of the EGF receptor was inhibited by the overexpression of Ymer. Moreover, tyrosine phosphorylation of EGF receptor was also diminished (Fig. 4F). These results suggest that Ymer might be localized with the EGF receptor in the cells and have negative effects on the secondary modifications of the EGF receptor for its activation.

Ymer Inhibits EGF Receptor Down-regulation—Because several structures of the EGF receptor have been reported to play an important role in the down-regulation of growth factor receptors (17), we investigated the role of Ymer through its association with the EGF receptor. First, a time course experiment to observe the amount of EGF receptor in the cells overexpressing Ymer or its tyrosine phosphorylation mutant was performed. Significantly, in contrast to control cells or the mutant cells, the amount of EGF receptor in cells overexpressing Ymer remaining at 60 min after stimulation was clearly high (Fig. 5A). To confirm this result by a cell biology experiment, we used fluorescent-labeled EGF. The EGF receptor was bound to labeled EGF at the plasma membrane of COS7 cells 5 min after EGF addition (Fig. 5B, a and f). Then, in the cells expressing Ymer mutant, EGF-EGF receptor complex was internalized into the cytosolic fraction within the endosomal vesicle 30 min after EGF addition (Fig. 5Bh). However, large amounts of labeled EGF remained at the plasma membrane of the cells expressing wild-type Ymer.

These data suggest that Ymer has a negative effect on the EGF receptor down-regulation. If the overexpression of Ymer inhibits the down-regulation of EGF receptor, knockdown of the protein should show a reversal effect. For this purpose, we have established Ymer knockdown HeLa cell line using a retrovirus gene transfer system. The effect of Ymer knockdown was clearly seen in the ligand-induced down-regulation of the EGF receptor.

After 30 min of EGF addition, a substantial amount of the receptor remained in the vector-transfected cells (Fig. 6B). Therefore, ligand-induced down-regulation of the EGF recep-
tor is promoted by knockdown of Ymer. Furthermore, internalization of the EGF receptor visualized by the immunofluorescent study was also promoted in the Ymer knockdown cells (Fig. 5D).

Ymer Co-localizes with the EGF Receptor at the Plasma Membrane and Sustains the Internalization of the EGF Receptor—We have found that one of the functions of Ymer may be to suppress the down-regulation of the EGF receptor through their mutual association. However, the molecular mechanism of this phenomenon is still unclear. Thus, the subcellular localization of Ymer and the EGF receptor were studied by fluorescence microscopy.

When the phospho-specific antibody of Ymer that did not cross-react with the Y145F/Y146F mutant (Fig. 6A, e and f) was used, phosphorylated Ymer was found to be concentrated with actin filaments on lamellipodia in EGF-stimulated COS7 cells (Fig. 6A, c and d). From our biochemical observations in Figs. 1 and 2, Ymer was found to be phosphorylated and bound to the EGF receptor in an EGF-dependent manner. The tyrosine-phosphorylated Ymer and EGF receptor complex was localized at the plasma membrane. In general, the EGF receptor has been known to be translocated into the early endosomal vesicles by clathrin-mediated endocytosis upon EGF stimulation, and during these steps the EGF receptor is ubiquitinated and degraded by the proteasomal pathway. However, Ymer was not recruited to those vesicles from the plasma membrane after EGF treatment (Fig. 6B, d and h). Interestingly, the internalization of the EGF receptor by endocytosis after EGF stimulation was delayed in the cells overexpressing Ymer-S but not in its phosphorylation mutant or Ymer-L (Fig. 6, B and C). These results were supported by our biochemical observations that the short isoform of Ymer, which was dominantly expressed in a wide variety of human cells and tissues,

**FIGURE 5.** Ymer suppresses the down-regulation of the EGF receptor. A, effect of the expression of Ymer in response to EGF stimulation. Comparison of the amount of each protein was monitored by immunoblotting with specific antibodies. Twenty micrograms of protein of each whole cell lysate prepared from COS7 cells transfected with the mock vector, the expression plasmid for FLAG-Ymer-S, or the Ymer-S(Y145F/Y146F) mutant stimulated with EGF was applied for the indicated times. B, fluorescence staining showed that comparison of the EGF receptor remained on the plasma membrane in COS7 cells expressing FLAG-Ymer-S (left panel set) or its phosphorylation mutant (right panel set). Cells were treated with Alexa-fluora 488-conjugated EGF (100 ng/ml) for 5 (each left panels) or 30 min. Cells were processed for immunofluorescence staining using anti-FLAG (red) antibody. The scale bars represent 10 μm. C, effect of siRNA knockdown of Ymer on the response to EGF stimulation as detected by immunoblotting. Comparison of the amount of the EGF receptor (upper) and Ymer (lower) were monitored by immunoblotting with each specific antibody. Twenty micrograms of protein of each whole cell lysate prepared from vector-infected (left six lanes) and siRNA construct-infected (right six lanes) HeLa cells stimulated with EGF for the indicated times were applied in each lane. D, effect of siRNA knockdown of Ymer on the response to EGF stimulation detected by immunofluorescence study. HeLa cells infected with empty vector (left panel set) or Ymer siRNA construct (right panel set) were processed for immunofluorescence staining using anti-Ymer (green) and anti-EGF receptor (red). Cells were treated with EGF for 15 min. The scale bars represent 10 μm. RNAi, RNA-mediated interference.
DISCUSSION

In this report we have demonstrated that the Ymer undergoes tyrosine phosphorylation and ubiquitination in response to EGF signaling, and it is involved in the negative regulation of the EGF receptor. Although the precise molecular mechanism is still unclear, Ymer modified with Tyr-145 and -146 phosphorylation and multiubiquitination binds to the activated EGF receptor. In our subcellular localization study using FLAG or green fluorescent protein-tagged proteins, Ymer-S was localized on the plasma membrane and not translocated into the endosomal pathway upon EGF stimulation. Therefore, the EGF receptor bound to Ymer-S might partially inhibit its tyrosine phosphorylation and polyubiquitination on the plasma membrane, and the degradation of the EGF receptor might be suppressed without its ligand-mediated internalization.

On the other hand, the protein of Ymer-L was expressed at extremely low levels in the examined cells (Fig. 1). Although the subcellular localization of both FLAG-tagged Ymer-S and -L were almost identical (data not shown), the level of posttranslational modifications of Ymer-L was lower as compared with Ymer-S (Figs. 2 and 3). Therefore, Ymer-L was not bound to the EGF receptor (Fig. 4) and had no effect on the down-regulation of the EGF receptor (Fig. 6). Further studies are required to identify the distinct roles of Ymer isoforms.

Ubiquitination is one of the most important secondary modifications in proteins, and it determines the fate of every protein in the cells (18). In contrast to polyubiquitination, which is known as a signal for degradation of the protein, mono- or multiubiquitination plays a role as the translocation signal for the protein to be targeted to specific locations or organelles (19, 20). The binding region of Ymer to activated-EGF receptor contains the MIU

![Image of the figure showing co-localization of phosphorylated Ymer with EGF receptor upon EGF stimulation.](image)

**FIGURE 6.** Co-localization of phosphorylated Ymer with EGF receptor upon EGF stimulation. A, confirmation of the specificity of phospho-specific Ymer antibody using a Ymer mutant. Subcellular localization of Tyr-145-phosphorylated Ymer and EGF receptor with the EGF treatment. COS7 cells expressing FLAG-Ymer-S (a–d) or FLAG-Y145F/Y146F Ymer-S (e and f) with (c–f) without (a and b) EGF treatment were processed for immunofluorescence staining using anti-FLAG (a, c, and e) and anti-phospho-Tyr-145 (α-pY) Ymer antibody (b, d, and f) followed by Alexa-floura 488-conjugated goat anti-rabbit IgG (Ymer) and Alexa-floura 592-conjugated goat anti-mouse IgG (EGFR) as the secondary antibodies, respectively. Selected areas of accumulation of Tyr-145-phosphorylated Ymer are shown with arrows. The scale bars represent 10 μm. B, comparison of the internalization of EGF receptor by immunofluorescence staining in COS7 cells with expressing FLAG-Ymer-S (a–d) or its phosphorylation mutant (e–h). Cells were treated with (c, d, g, and h) or without (a, b, e, and f) EGF for 15 min. Cells were processed for co-immunofluorescence staining using anti-FLAG (green) and anti-EGF receptor (red) antibodies. The nuclei were visualized with Hoechst 33342 dye (blue). Cells expressing FLAG-Ymer-S(Y145F/Y146F) (lower) or FLAG-Ymer-S (upper) were indicated by arrows. The scale bars represent 10 μm. C, graph showing the frequency of inhibition the internalization of the EGF receptor in cells that express FLAG-tagged Ymer-L, Ymer-S, and Ymer-S(Y145F/Y146F). This value was calculated by counting the number of the cells that showed delayed the internalization of EGF receptor in FLAG-positive cells. FLAG-positive cells were detected by immunofluorescence staining of COS-7 cells that express the indicated proteins, and cells were fixed after treatment with EGF for 15 min as shown in (B). Data were obtained from 50 randomly selected FLAG-positive cells in one experiment, and three independent sets of experiments were carried out. Error bars show the S.D.
domain, which is the most recently identified ubiquitin binding domain. Penengo et al. (11) has described that Rabex-5 has UBD (ubiquitin binding domains) containing RUZ (Rabex-5 ubiquitin binding zinc-finger) and MIU domains that can be recognized by the ubiquitinated EGF receptor (11). Our results indicate that not only the MIU domain in Ymer but also its tyrosine phosphorylations are required for binding to activated EGF receptor. Moreover, the internal region encoded by exon 6 in Ymer-L, which is divided into its phosphorylation sites and the MIU domain, has a negative effect on the binding to activated EGF receptor (Fig. 4D). The role for these secondary modifications of Ymer in terms of the effects on its structural alterations or its binding to activated EGF receptor still remains unknown, and further studies will have to be carried out to gain a better understanding.

Many molecules related to the down-regulation of growth factor receptors have been identified. The Sts family has been known to stabilize the EGF receptor by inhibiting its negative regulation similar to Ymer (21). In contrast to Ymer, Sts is an endosomal protein like Eps15 (22) and Hrs (23), containing a ubiquitin-associated domain that can recognize monoubiquitinated proteins. However, the SH3 domain in Sts binds directly to Cbl, and Sts inhibits the ubiquitination of the EGF receptor and its endocytosis. Therefore, this mechanism of stabilizing the EGF receptor may be different from that used by Ymer, which is localized on the plasma membrane. Although we have attempted to find a partner of Ymer by using an immunopurification method, only actin was found as a trigger factor for fixing Ymer onto the plasma membrane (data not shown). Ymer has low sequence homology to the ERM (ezrin, radixin, and moesin) family (24), and phosphorylated Ymer co-localizes with the family at the tight junction of cell-cell contacts in A431 cells (data not shown). It is possible that Ymer may have a function similar to the cytoskeletal protein that cooperates with actin at the plasma membrane.

Recently, the precise monoubiquitination sites in the EGF receptor have been identified (8), and the function of each ubiquitination site as well as its tyrosine phosphorylation sites have been described (25). The exact ubiquitination site recognized by the MIU domain still remains unknown, and further analysis is needed for the detection to gain a more precise mechanistic understanding of the binding.

In addition, we speculate that the complicated secondary modifications on Ymer induced by EGF stimulation may simply have complex effects on the inhibition of excess phosphorylation and ubiquitination of the EGF receptor. This is supported by our observation that the secondary modification of the EGF receptor is inhibited by overexpression of Ymer in COS7 cells (Fig. 3F).

Because Ymer may play a role for maintaining the amounts of growth factor receptor linked to various cancers, Ymer may have a potential to link to cancer pathology. If Ymer is overexpressed or strongly phosphorylated in some cells, these cells would be tumorigenic because of lack of normal receptor regulation. Although the cDNA of Ymer has been originally cloned in a screen for the gene directly linked to hereditary spastic paraplegia on chromosome 6, the genomic DNA encoding Ymer is not mutated or deleted (14). Interestingly, the mRNA of Ymer is not expressed in the breast cancer cell line MCF7, which is known to have a low expression level of the EGF receptor (26). The function of Ymer at physiological levels in normal cells or at abnormal levels in various tumor cells remains to be elucidated. We are currently analyzing the amounts of the Ymer protein and the level of its Tyr-145/146 phosphorylation in various carcinoma cells to understand the relationship between Ymer expression and tumorigenesis. Furthermore, screening of other interacting molecules in other cell lines will help achieve a detailed understanding of its physiological functions.

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