CFBP Is a Novel Tyrosine-phosphorylated Protein That Might Function as a Regulator of CIN85/CD2AP*

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To decipher the global network of the epidermal growth factor (EGF) receptor-mediated signaling pathway, a large scale proteomic analysis of tyrosine-phosphorylated proteins was conducted. Here, we focus on characterizing a novel protein, CFBP (CIN85/CD2AP family binding protein), identified in the study. CFBP was found to be phosphorylated at tyrosine 204 upon EGF stimulation, and the CIN85/CD2AP family was identified as a binding partner. A proline-rich motif of CFBP is recognized by one of the three Src-homology 3 domains of CIN85/CD2AP, and the affinity of the interaction is regulated by the tyrosine phosphorylation of CFBP. They co-localize in actin-enriched structures, and overexpression of CFBP induced morphological changes with actin reorganization. Furthermore, CFBP accelerated the EGF receptor’s down-regulation by facilitating the recruitment of Cbl to the CD2AP/CIN85 complex. Two spliced variants of CFBP lacking either exon 5 or 8 are also expressed, and the variant lacking exon 5 without the proline-rich motif lacks the ability to bind to the CIN85/CD2AP family. The CFBP protein seems to play a key role in the ligand-mediated internalization and down-regulation of the EGF receptor.

Proteomics is a powerful tool for discovering a whole range of protein molecules involved in specific biological phenomena. In the field of growth factor receptors, which are often directly linked to disease, many groups have applied the technique to identify novel molecules and posttranslational modifications, such as phosphorylation and ubiquitination, involved in the signaling pathway downstream of the receptors (1). To obtain a complete picture of the growth factor receptor-mediated signaling pathways, it is not only necessary to understand the cellular signal transduction networks, but it is also important to develop more effective and safer drugs against signaling-related disease. The EGF receptor is well known for being involved in the generation or development of various human cancers, and its antagonists have been used to develop effective drugs for cancer (2). The signaling network downstream of the EGF receptor has been studied extensively in the pre- and post-genomic era; several groups have applied the proteomic approach and have found many new connections to known proteins as well as identifying new molecules (3–5). The strategy of these proteomic studies features the same approach; anti-phosphotyrosine antibody was used to isolate tyrosine-phosphorylated proteins from EGF-stimulated cell lysates, and the isolated proteins separated and visualized by SDS-PAGE were identified by mass spectrometry combined with a data base search. The identified proteins can be considered to be either tyrosine-phosphorylated themselves or bound to the tyrosine-phosphorylated proteins. The site of phosphorylation can then be analyzed by tandem mass spectrometry (6). More recently, Rush et al. (4) have reported a new approach in which total protein is first digested with protease, and tyrosine-phosphorylated peptides rather than proteins are isolated by the anti-phosphotyrosine antibody. Although this approach enables the identification of a much larger number of tyrosine-phosphorylated sites, non-phosphorylated proteins forming complexes with tyrosine-phosphorylated proteins escape detection. Since not only protein phosphorylation but also protein–protein interactions play important roles in signaling (7), the conventional approach in which the signaling complexes are isolated should bring additional knowledge about the component proteins. In fact, adaptor proteins in growth factor receptor-mediated signaling play key roles, serving as scaffold proteins for recruiting essential partners to the appropriate complex (8), and such protein–protein interactions are often regulated by protein phosphorylation and other posttranslational modifications (9, 10). To obtain a more complete picture of the growth factor signaling, it will be of prime importance to analyze both the secondary modifications and the interacting proteins.

In the present study, we have reanalyzed the protein complex isolated by the anti-phosphotyrosine antibody. The main differences are the use of the combination of different antibodies, scale-up of the amount of samples analyzed, and application of the newest sensitive mass spectrometric analyses. This strategy has enabled us to detect not only the major component proteins but also the less abundant proteins that are not visible even in...
Silver-stained gels. We have currently identified over 150 polypeptides as downstream molecules of the EGF receptor, which include both well known and previously unknown proteins in this signaling pathway. Understanding the function of each novel protein will make it possible to better understand the remaining steps in the pathway. In this report, we have selected a novel protein containing no known functional domains. This protein composed of 273 amino acid residues is tyrosine-phosphorylated upon EGF stimulation. During the screening of binding partners to the protein, we identified CD2AP (CD2-associated protein), which contains several structural domains involved in protein-protein interactions. CD2AP has been known as a key regulator maintaining the integrity of kidney glomerular filtration and cytoskeletal polarization in T cells (11, 12). A protein structurally related to CD2AP, CIN85 (Cbl-interacting protein of 85 kDa), was identified as a scaffold protein that functions to down-regulate the growth factor receptor in cooperation with proto-oncogene product Cbl, an E3 ubiquitin ligase (13). We report here that CFBP interacts and co-localizes with CIN85/CD2AP family proteins and functions as a key component in EGF receptor internalization and down-regulation mediated by the CIN85-Cbl complex.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—COS7, HeLa, human embryonic kidney HEK293, HEK293T, and A431 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml streptomycin, and 100 units/ml penicillin. For maintaining the NIH3T3 cells, 10% calf serum was used in substitution of fetal bovine serum. Transfection of a plasmid into COS7, HeLa, or HEK293T cells was carried out by electroporation using a Gene-Pulser (Bio-Rad). For transfection of COS7 cells, 2 mgl/10 cm dish of DNA was used.

**cDNA Cloning and Vector Constructions**—For cloning of the CFBP cDNA, the HeLa cDNA library was screened by PCR with two primers (HindIII 5′-ATGGATCCCCTACCGGGGGGAGC-3′ and EcoRI 5′-CTATGACCGCTGCGGGGCC-3′), which were designed based on the sequence (gi/12408440) in the NCBI data base. Full-length human CD2AP and EGF receptor cDNAs were cloned from the HeLa cDNA library by PCR. The amplified products were first subcloned into a TOPO™ TA cloning vector, pCR®2.1-TOPO (Invitrogen) and 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. The EGF receptor cDNA was inserted into a pcDNA3 vector (Invitrogen). CFBP and CD2AP 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. Human FLAG-tagged CIN85 construct was provided by Dr. I. Dikic (University of Hospital of the Johann Wolfgang Goethe University, Germany) and Dr. S. Kajigaya (National Institutes of Health). The DNA fragments of CFBP, CD2AP, and CIN85 used for the construction of deletion mutants were made by using PCR or suitable restriction enzyme digests. After checking their DNA sequences, the constructs were subcloned into a pCMV-FLAG6 series vector to express as N-terminal FLAG-tagged proteins into a pGEX4T vector (Amersham Biosciences) or to express as gultathione S-transferase (GST) fusion proteins or subcloned into a pEGFP-C1 vector (Clontech) to express as N-terminal green fluorescent protein (GFP)-tagged proteins. Point mutations or deletions were introduced using the QuikChange kit (Stratagene) following the manufacturer’s protocol. The mutations were verified by DNA sequencing.

**Antibodies**—Three kinds of rabbit polyclonal anti-CFBP antibodies were used in this study. Anti-CFBP(His) antibody and anti-CFBP(GST) were raised against a CFBP fragment containing residues 102–213 and 135–273, which were bacterially produced as His-tagged protein using pET vector (Novagen) and GST fusion protein, respectively. Anti-CFBP(C-ter) was raised against a keyhole limpet hemocyanin-conjugated peptide, which corresponds to the carboxy-terminal 13 residues of CFBP. These antibodies were purified through HiTrap N-hydroxysuccinimide-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 amino-terminal end of the amino acid sequence of CFBP: CLRRNSpYEASSLY (amino acids 197–210; where pY indicates phosphotyrosine). The phosphopeptides were coupled to keyhole limpet hemocyanin and used to immunize rabbits. The phosphorylation site-specific antibodies were purified from antiserum by successive column chromatographies with the use of affinity resins coupled with each phosphopeptide and non-phosphopeptide. All CFBP antibodies used in this study were tested for their specificities of use in immunoprecipitation (anti-CFBP(C-ter)), immunoblotting (anti-CFBP(His), anti-CFBP(C-ter), and anti-CFBP(GST)), and immunofluorescence studies (anti-CFBP(His) and anti-CFBP(C-ter)). Other antibodies were obtained commercially from various suppliers: anti-FLAG M2 antibody (Sigma), anti-phosphotyrosine antibody (4G10; Upstate Biotechnology, Inc., Lake Placid, NY), anti-GFP antibody (Molecular Probes, Inc., Eugene, OR), anti-CD2AP antibody (SC-9137; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Cbl antibody (SC-1651; Santa Cruz Biotechnology), anti-CIN85 antibody (Calbiochem), anti-EGF receptor antibody (SC-03; Santa Cruz Biotechnology), anti-β-actin antibody (Sigma), and anti-HA antibody (12CA5; Roche Applied Science).

**Semiquantitative Reverse Transcription-PCR**—To assess the relative expression levels of CFBP transcripts, a reverse transcription-PCR assay was performed on each panel of eight different human culture cell and tissue cDNAs (human tissue and cell line MTC panel; Clontech) using the following primers: 5′-ATGGATCCCAGTACCGGG-3′ and 5′-CTATGACCGCTGCGGGGCC-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 or 7.5% polyacrylamide gels containing ethidium bromide.

Cloning of the A431 Stable Cell Line—Full-length CFBP cDNA was inserted into a pcDNA3-based N-terminal FLAG-tagged vector (gift from Dr. U. Kikkawa, Kobe University, Japan) in frame. A431 cells were transfected with the empty vector or the resulting plasmid. Cells resistant to G418 were isolated in the presence of 0.4 mg/ml G418 (WAKO) and cloned.

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 CFBP was 5’-AAGACAGTGCCTGGATACCTT-3’ (from nucleotide 385 to 405), and a resulting plasmid for knockdown of CFBP was named pSuper-CFBP. The empty pSuper vector was used as a control. Transfection of pEGFP and either pSuper or pSuper-CFBP into HeLa cells was carried out by electroporation. HeLa cells were stably infected with an amphotropic receptor. The packaging cells (AmphoPack™-293 cell line; Clontech), were transfected with the appropriate retroviral RNA 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 changed. Following infection, cells were selected with 2 µg/ml puromycin.

Immunoprecipitation, Immunoblot Analysis, and GST Pull-down Assay—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 inhibitor 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. For the in vitro binding assay by GST pulldown, bacterially expressed GST fusion protein was immobilized on glutathione-Sepharose (Amersham Biosciences). Then the resulting mixture was rotated at 4 °C for an additional 1 h. The beads were then washed three times with lysis buffer. The samples were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to an Immobilon P membrane (Millipore). Immunoblot analysis was carried out with 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-CFBP and Its Binding Protein—293T cells (1 × 10⁸ cells) transiently expressing FLAG-tagged CFBP 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-CFBP 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 visible bands with Coomassie Brilliant Blue staining were subjected to MS/MS analysis after in-gel digestion with trypsin.

In-gel Digestion with Trypsin—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 in 50 mM NH4HCO3 containing 5 mM CaCl2). In-gel digestion was performed at 37 °C overnight. Resulting peptides were extracted with 50% acetonitrile containing 5% trifluoroacetic acid. Extracts were dried in a vacuum concentrator and resuspended 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 Q-Tof-type hybrid mass spectrometer (Micromass) interfaced on-line with a capillary high pressure liquid chromatograph (Waters-Micromass modular CapLC, Micromass) (6, 14). The linear gradient of acetonitrile produced was split at a 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 automatically to collision-induced dissociation. Peak lists obtained from the MS/MS spectra were used to identify proteins using the Mascot search engine (Matrix science).

Fluorescence Microscopy Analysis—For expression of the GFP-fused CFBP in cultured cells, the full-length CFBP cDNA was inserted into a pEGFP-C3 vector (Clontech) in frame. HEK293T, 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. Following a blocking step with 3% bovine serum albumin in PBS for 30 min, the primary antibodies 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 necessary, cells were treated with rhodamine phalloidin (Molecular Probes), and the cell nuclei were stained with 2 μl Hoechst 33342 (Molecular Probes) simultaneously. Finally, cells were rinsed three times with PBS and mounted onto microscope slides with ProLong Antifade reagents (Molecular Probes). Images were taken on a Zeiss Axiovert 200 fluorescence microscope using a 40 × 1.0 numerical aperture PlanApo objective (Zeiss Axioskop; Carl Zeiss Inc.). Figures were prepared using Adobe Photoshop.

RESULTS

Identification of CFBP as a Novel Component of EGF Signaling—To identify proteins acting downstream of the EGF receptor, total protein was isolated using column chromatog-
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raphy packed with three different anti-phosphotyrosine antibodies from the lysate of EGF-stimulated A431 cells, which express large amounts of EGF receptor. The proteins were separated by SDS-PAGE, and the whole lane was cut out to pieces of equal size regardless of the presence of bands visible by silver staining (15). Even from pieces without stained bands, many proteins were identified with confidence. Consequently, over 150 proteins, including not only well studied proteins but also several previously unidentified ones, were detected. In the present study, we focus on the functional characterization of one of the newly identified molecules, named CFBP, in the EGF signaling pathway. Its cDNA has been cloned in the human full-length cDNA sequencing projects, and the nucleotide sequence is available in the public data base. Based on the sequence information, we cloned the CFBP cDNA from the HeLa cDNA library using PCR. In addition to the full-length cDNA, an alternatively spliced variant lacking exon 8 was also obtained. CFBP is composed of 273 amino acid residues, and no significant homology to other proteins of known functions or to known functional domains was detected (Fig. 1A). To assess the expression of two CFBP mRNA species, semiquantitative reverse transcription-PCR was carried out using the wide variety of cDNA libraries derived from human tissues and cultured cells. Both sizes of mRNA were expressed ubiquitously except in skeletal muscle (Fig. 1B). Interestingly, although CFBP was originally identified by mass spectrometric analysis from the lysates of A431 cells stimulated with EGF, the transcriptional level of the CFBP in A431 cells was unexpectedly low. This issue prompted us to conduct a more detailed analysis of the expression of CFBP in A431 cells. When extensive PCR using the A431 cDNA library as a template with 50 cycles was carried out, another small fragment of 400 bp in addition to the two splice variants was detected (Fig. 1C). This fragment was identified as another spliced variant lacking exon 5 by sequencing. Schematic structures of CFBP isoforms are shown (Fig. 1D).

CFBP Interacts with CD2AP/CIN85—To gain insights into the function of CFBP, we analyzed proteins co-purified with CFBP by immunoaffinity purification. HEK293T cells transfected with the expression plasmid for FLAG-tagged CFBP were lysed with the buffer containing 1% Triton X-100, and immunoprecipitation was carried out with an anti-FLAG affinity gel. Proteins eluted with glycine buffer were analyzed with SDS-PAGE. In addition to the full-length CFBP, a protein of ~80 kDa was detected only in the immune complex from the transfected cells (Fig. 2A). 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 mass spectrometry (LC/MS/MS). Five independent peptides derived from CD2AP were detected. The primary structure of CD2AP is shown in Fig. 2B together with the identified peptides. The association of CFBP with CD2AP in cells was confirmed further by an immunoprecipitation study using their specific antibodies. CD2AP was detected in the immune complex precipitated with the anti-CFBP(C-ter) antibody from the lysate of HEK293 cells, demonstrating that the endogenous CFBP and CD2AP are associated in cultured cells derived from human kidney (Fig. 2C). The degree of association was elevated upon EGF stimulation. Moreover, a protein structurally related to CD2AP, CIN85, was also bound to CFBP in HEK293 cells. Although CIN85 was not detected as a binding protein to CFBP by MS/MS analysis, the result of immunoblot analysis revealed that endogenous CIN85 could be bound to CFBP. These results were confirmed by reciprocal immunoprecipitation experiments using anti-CD2AP and anti-CIN85 antibodies (Fig. 2D).

A Proline-rich Motif in CFBP Is the Binding Site for the SH3 Domain of CD2AP—To analyze the binding mechanisms of CFBP and CD2AP in detail, several constructs were expressed in HEK293T cells, and the interaction was assessed by immunoprecipitation. Reexamination of the CFBP sequences suggested a proline-rich region (amino acid residues 155–160) as a likely candidate for the binding site for CD2AP. The PVPKPR sequence in CFBP shows a good match to the PX(P/A)XXR sequence that has been recently identified as the target of the SH3 domain of CD2AP/CIN85 (16, 37). Accordingly, several deletion mutants lacking the proline-rich region were constructed (Fig. 3A). HEK293T cells expressing FLAG-tagged CFBP cultured in the medium containing 10% fetal bovine serum were harvested, and immunoprecipitation was carried out with anti-CD2AP antibody. FLAG-CFBP was found in the immune complex obtained with anti-CD2AP antibody from the lysate of HEK293T cells expressing FLAG-CFBP, demonstrating that endogenous CD2AP associates with FLAG-tagged CFBP (Fig. 3B). Next, the reciprocal experiment was carried out with various deletion mutants expressed in HEK293T cells (Fig. 3C). Wild type (CFBP-L) and a spliced variant (CFBP-S) still bound to endogenous CD2AP and CIN85. On the other hand, another spliced variant lacking exon 5 (CFBP-L Δex5) and the deletion mutant lacking the proline-rich motif (CFBP-S ΔPro) lost the ability to bind to CD2AP and CIN85. We conclude that CFBP binds to the CD2AP/CIN85 family through the proline-rich motif containing a proline-arginine motif. Since the spliced variant lacking the proline-rich region (CFBP-L Δex5) in A431 cells did not bind to CD2AP, there seems to be a functional difference among the splicing variants. Interestingly, FLJ00022, a homologous protein of CFBP (34% identity) in which the tyrosine phosphorylation site is conserved but which lacks the PX(P/A)XXR sequence, failed to bind to CD2AP (Fig. 3C).

Reverse experiments were performed to identify the binding site for CFBP in CD2AP. The proline-rich sequence is a well known binding site for the SH3 domain, and the design of the constructs (Fig. 3D) was facilitated by recent studies on the binding proteins to CD2AP/CIN85 (4). HEK293T cells expressing the FLAG-tagged CD2AP derivatives and GFP-fused CFBP-S were lysed, and immunoprecipitation was carried out using the anti-FLAG antibody. It is clear that the deletion of the most N-terminal of the three SH3 domains of CD2AP (referred to as SH3A, with the second and third domains being referred to as B and C, respectively) abolished the binding (Fig. 3E). The SH3A domain might be involved in the binding of CFBP in HEK293 cells.

Next, to clarify whether the SH3 domains of CD2AP/CIN85 directory bind to CFBP, GST pull-down assays with the isolated SH3 domains of CD2AP/CIN85 were carried out. Although all

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A nucleotide and deduced amino acid sequence of CFBP. The full-length cDNA of CFBP encompasses 822 nucleotides and encodes a 273-amino acid polypeptide. In addition to the full-length cDNA, splicing variants were cloned as short forms lacking either exon 5 or 8, which result in 233 or 254 amino acid polypeptides, respectively. The regions of exons 5 and 8 are boxed in the nucleotide sequences, and the amino acid sequences encoded in exons 5 and 8 are indicated with **boldface italic type**.

Expression of CFBP mRNA in various human tissues and cultured cells. Quantitative PCR was carried out using specific internal oligonucleotide primers, as indicated by the underlines in A. The results of various human culture cells (top panels) and tissues (bottom panels) are shown. The amplified DNA fragments of the expected size (520 and 463 bp) derived from CFBP cDNAs are indicated by arrows in the upper panel. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as a loading control. The PCR cycle number of all samples is 30.

Identification of a second spliced variant in A431 cells. Extensive PCR (50 cycles) was carried out using the cDNA library derived from A431 cells. Resulting PCR fragments were separated by 7.5% PAGE. The presence of a smaller fragment (400 bp) lacking exon 5 was confirmed by sequencing.

Schematic representation of the primary structure of CFBP isoforms. Wild-type CFBP is composed of nine exons, and the truncated forms lack the region from Ala235 to Glu253 (ex8), or that from Asp139 to Ser178 (ex5). Numbers of amino acid residues of the boundaries are indicated.
SH3 domains of CD2AP/CIN85 were able to bind directly to CFBP to some extent, the SH3A domain of each molecule had the highest binding affinity to CFBP in vitro (Fig. 3F).

Identification of the Tyrosine Phosphorylation Site of CFBP That Is Related to the Binding Affinity to CD2AP/CIN85

Since CFBP was originally identified in the immune complex of the anti-phosphotyrosine antibody, it should be either tyrosine-phosphorylated itself or associated with another tyrosine-phosphorylated protein. The results shown above suggest that the binding of CFBP to the CD2AP/CIN85 family might be regulated by their tyrosine phosphorylation upon EGF stimulation. Because immunoprecipitated endogenous CFBP from EGF-stimulated HEK293 cells was blotted by anti-phosphotyrosine antibody, CFBP is a tyrosine-phosphorylated protein response to EGF stimulation (Fig. 4A). To identify the tyrosine phosphorylation sites, wild-type and mutant CFBPs lacking tyrosine residues were expressed in cells, and tyrosine phosphorylation was analyzed using the anti-phosphotyrosine antibody (Fig. 4B). When cells were stimulated with EGF, the wild-type CFBP was clearly tyrosine-phosphorylated upon EGF stimulation (Fig. 4C). Although the wild-type and
two C-terminal truncated mutants containing 1–255 and 1–209 amino acid residues were tyrosine-phosphorylated with EGF treatment, the shortest mutant containing the N-terminal 1–200 amino acid residues abolished detectable tyrosine phosphorylation under the same conditions (Fig. 4B). Consequently, a phenylalanine point mutant (Y204F) was made and tested for EGF-dependent tyrosine phosphorylation (Fig. 4C). The results established that Tyr204 in CFBP is the major phosphorylation site upon EGF stimulation. Next, we produced a specific antibody that is able to recognize the phosphorylation at Tyr204 in CFBP (α-pY204). This phosphorylation resulted from overexpressing EGF receptor in COS7 cells (Fig. 4D), which was more enhanced with EGF treatment and was sustained for up to 3 h after the EGF addition (Fig. 4E). The association of CFBP and CD2AP/CIN85 was correlated with the phosphorylation at Tyr204 in CFBP. Furthermore, CD2AP/CIN85 was not associated with the Y204F mutant of CFBP with or without EGF addition. The mutant with Tyr204 replaced by Asp, which mimics a constitutively phosphorylated form, gained the ability to bind CIN85/CD2AP without EGF treatment (Fig. 4F). These results indicate that the phosphorylation at Tyr204 in CFBP is a significant post-translational modification for binding to CD2AP/CIN85.

CFBP Co-localizes with the CD2AP/CIN85 Family and the EGF Receptor—Subcellular localization of the protein and that of CD2AP was then studied by fluorescence microscopy. First, the subcellular localization of endogenous CFBP detected with anti-CFBP antibody and exogenous protein with FLAG or GFP fusion protein was compared. They showed almost identical localization, with the only difference being the signal intensity caused by the expression level (data not shown). Thus, exogenous expression of FLAG-tagged or GFP-fused CFBP will not affect the localization of the protein in transfected cells. Next, FLAG-tagged or GFP-fused CFBP

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**FIGURE 3.** A proline-rich motif in CFBP is required for the binding to the SH3 domain of CD2AP/CIN85. A, schematic representation of the constructs of CFBP. The numbers indicate the length in amino acid residues. FLJ00022 is a protein homologous to CFBP, and the boxed region has a 34% amino acid sequence identity to CFBP. B, the binding of FLAG-tagged CFBP to endogenous CD2AP. Co-immunoprecipitation (IP) studies were carried out using the cell lysates from HEK293T cells transfected with the empty vector or the expression plasmid encoding FLAG-CFBP indicated above the upper panel. Endogenous CD2AP was immunoprecipitated by α-CD2AP. Immunoblot (IB) analysis was carried out using α-CD2AP (upper panel) or α-FLAG (lower panel). Positions of CD2AP and CFBP are indicated by arrows. The whole cell lysate (WCL; one-fiftieth of the amounts used for immunoprecipitation) was applied in the first and second lanes from the left, detection of the CD2AP/CIN85-binding site in CFBP. Reciprocal co-immunoprecipitation and immunoblot studies were carried out as described in B with the indicated antibodies. C, detection of the SH3 domain of CD2AP/CIN85 and the EGF Receptor. D, schematic representation of the constructs of CD2AP. The numbers indicate the amino acid residues. E, detection of the CD2AP-binding site in CD2AP. Co-immunoprecipitation studies were carried out using the cell lysates from HEK293T cells overexpressing GFP-fused CFBP-S and each derivative molecule of FLAG-tagged CD2AP. Each FLAG-tagged molecule was immunoprecipitated by α-FLAG. Immunoblot analysis was carried out using the indicated antibodies. The expression level of GFP-CFBP or p85 in each sample was monitored by immunoblot using the whole cell lysate (lower panel). F, in vitro interaction between each SH3 domain of CD2AP/CIN85 and CFBP confirmed by a GST pull-down assay. GST fusion proteins used in this assay were visualized by Coomassie Brilliant Blue staining in the upper panel. The numbers indicate amino acid residues of CD2AP/CIN85. The amounts of bound proteins to GST fusion proteins were analyzed by immunoblotting using α-FLAG (lower panel). The lysate (one-twentieth of the amounts used for this assay) from COS7 cells expressing FLAG-CFBP was subjected in the leftmost lane.
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was compared with either endogenous or FLAG-tagged CD2AP. CFBP basically co-localized with CD2AP, especially in actin-rich structures, such as peripheral lamellipodia in transfected 293T and COS7 cells (Fig. 5, a–f). In contrast to CD2AP, which was exclusively localized in the cytosol, a minor population of CFBP was localized in the nucleus. Surprisingly, the mutant lacking the proline-rich motif was mainly localized in the nucleus and not concentrated at the leading edge of lamellipodia (Fig. 5, g–i). The nuclear localization of mutant CFBP may be caused by the loss of binding of CFBP to CD2AP, which will be discussed in more detail below. EGF receptor co-localized at the plasma membrane with CFBP (Fig. 5, j–l).

CFBP Facilitates EGF Receptor Down-regulation—Since the CD2AP/CIN85 family has been reported to play an important role in the down-regulation of growth factor receptors (4), we investigated the role of CFBP, through association with CD2AP or CIN85, in the fate of the EGF receptor. First, we have found from immunofluorescence studies that the amount of remaining EGF receptor in the cells overexpressing FLAG-CFBP (upper cell in Fig. 6A) was reduced after 2 h of exposure with EGF compared with mock-transfected cells (lower cell in Fig. 6A). To confirm this result biochemically, a time course experiment to observe the amount of EGF receptor in the cells expressing each CFBP derivative was performed. Significantly, in contrast to control cells, the amount of EGF receptor in cells overexpressing CFBP remaining at 60 min after stimulation was clearly low. Furthermore, the degradation of EGF receptor in cells expressing the Y204F mutant of CFBP was relatively suppressed (Fig. 6B). Introducing Y204F into the cells can function as a dominant negative form against the tyrosine phosphorylation of endogenous CFBP (data not shown), which may reduce the amounts of CFBP bound to CIN85. The acceleration of EGF receptor down-regulation did not occur by expressing the deletion mutant lacking the proline-rich motif (CFBP-S ΔPro) that lost the ability to bind to CD2AP/CIN85 (data not shown). These data suggest that CFBP has a positive effect on the EGF receptor down-regulation involving the Cbl interactome.

FIGURE 4. Tyrosine 204 of CFBP is the major phosphorylation site upon EGF stimulation. A, tyrosine phosphorylation of endogenous CFBP upon EGF stimulation. CFBP was immunoprecipitated (IP) using anti-CFBP(C-ter) antibody with (+) or without (−) EGF stimulation. The control experiment was carried out using control IgG. Immunoblot analysis was carried out using α-CFBP(His) (lower panel) or α-phosphotyrosine (upper panel). The positions of each molecule are indicated by arrows. B, screening of the tyrosine phosphorylation site of CFBP upon EGF stimulation. Schematic representation of the wild-type and the mutants of CFBP-L used. The positions of all six tyrosine residues in CFBP are indicated. The numbers indicate the amino acid residues. COS7 cells were transfected with each plasmid encoding the FLAG-tagged construct of CFBP, and immunoprecipitation analysis was carried out using α-FLAG and α-phosphotyrosine. The results of tyrosine phosphorylation of CFBP derivatives are indicated as (+) or (−) at the left side of each schematic diagram. C, Tyr204 is a tyrosine phosphorylation site of CFBP upon EGF stimulation. The preparation of lysate of COS7 cells transfected with the expression plasmid encoding FLAG-tagged wild-type or the Y204F mutant of CFBP-L was carried out as described in B. Immunoprecipitation and immunoblot were performed with the indicated antibodies. D, detection of the phosphorylation at Tyr204 of CFBP by phospho-specific antibody. COS7 cells were transfected with the expression plasmid described above, and EGF treatment and immunoprecipitation were performed as described above. Immunoblot analysis was carried out using the anti-FLAG (lower panel) and anti-phospho-Tyr204 (α-pY204) (upper panel) antibodies. E, association of CD2AP/CIN85 and phosphorylated CFBP upon EGF stimulation. A time course experiment of the phosphorylation at Tyr204 and association of the molecules was analyzed by immunoprecipitation and immunoblots. COS7 cells transfected with FLAG-tagged CFBP-S were treated with EGF for the indicated times, and immunoprecipitation was performed as described above. Immunoblot analysis was carried out using the indicated antibodies. F, association of CFBP and CIN85/CD2AP is dependent on the phosphorylation at Tyr204 of CFBP. COS7 cells expressing FLAG-tagged CFBP-S derivatives were treated with EGF for 10 min, and immunoprecipitation was performed as described above. Immunoblot analysis was carried out using the indicated antibodies.
and a substantial amount of the receptor remained in the knockdown cells. Therefore, ligand-induced down-regulation of EGF receptor is partially inhibited by decreasing the amounts of CFBP.

To study the effect of CFBP on complex formation of the Cbl interactome, immunoprecipitation was carried out using COS7 cells overexpressing HA-tagged Cbl and FLAG-tagged CFBP derivatives with EGF treatment. Interestingly, comparing the mock-transfected and Y204F mutant of CFBP-transfected cells, the amount of CIN85 bound to Cbl was increased in the cells expressing wild-type CFBP (Fig. 6D), which might promote the ligand-induced down-regulation of EGF receptor (Fig. 6, A and B). The mechanisms of this effect will be discussed below.

Morphological Alteration of A431 Cells Stably Expressing CFBP—As we have shown in Fig. 1B, human epidermoid carcinoma cells, A431, might be expressing a functionally null mutant of CFBP that lacks the ability to bind to the CD2AP/CIN85 family due to a deletion of the PX(P/A)XXR sequence. To make certain of this correlation between the mRNA and a protein analysis of CFBP in A431 cells, we next investigated and compared the protein expression level of CFBP among various mammalian culture cells. In most of the cell lines tested, two bands corresponding to the full-length and the splice variant lacking exon 8 were observed. In A431 cells, the protein expression level of CFBP was extremely low (Fig. 7A), corresponding to the low transcriptional level (Fig. 1B). We chose this cell for establishing the stable cell line expressing exogenous CFBP to investigate the effects on the EGF receptor. A total of eight cell lines expressing FLAG-tagged CFBP-S were established, and two representative clones were used in the following experiments (Fig. 7B). It is notable that the amounts of EGF receptor in the cells expressing CFBP decreased rather drastically (clones C8 and E3) compared with the mock-transfected cells. On the other hand, CD2AP expression was not affected to any significant extent. Next, we examined the time course of EGF receptor down-regulation upon ligand stimulation by measuring the amount of EGF receptor in the whole cell lysate. In contrast to control cells, the down-regulation of EGF receptor in CFBP-expressing cells was accelerated (Fig. 7, C and D). To study the effect of CFBP on the complex formation between CD2AP and Cbl, immunoprecipitation was carried out using anti-CD2AP antibody with or without EGF stimulation. Although little, if any,
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In addition to the EGF receptor down-regulation, CD2AP has been implicated in cytoskeletal rearrangement through its binding to F-actin (19, 20). As shown in Figs. 5C and 6A, FLAG-CFBP expressed in COS7 cells concentrated with actin filaments on lamellipodia and stress fibers. Because of the co-localization with filamentous (F)-actin, we carefully compared phenotypes of cell lines stably expressing CFBP. In general, A431 cells grow with good cell-cell adhesion on normal culture dishes as shown in Fig. 7F (upper panels). However, stable cell lines expressing CFBP become flat with extensive membrane ruffling (Fig. 7F, lower panels). It has been reported that the suppression of EGF receptor kinase activity results in the morphological changes of A431 cells into the form of a flat cell phenotype with actin cytoskeletal rearrangement (21). The effects of CFBP overexpression can be explained by the facilitated down-regulation of EGF receptor. On the other hand, the CD2AP/CIN85 family is known to be a scaffold protein bound to large numbers of protein partners involved in the actin reorganization regulated by growth factor receptor signaling (18). Moreover, the overexpression of CD2AP in COS7 cells induced formation of lamellipodia (22). Therefore, the effects of exogenous expression of CFBP in A431 cells may be due to the perturbation of the CD2AP/CIN85 complex.

DISCUSSION

One of the earliest reports on the proteomic analysis of EGF receptor signaling identified a single novel protein, VAV-2, as its substrate (23). Successive improvements in the methodology increased significantly the numbers of proteins identified (3–5). Our present approach employs not only a sensitive analytical method that enables the detection of proteins below the limit of detection by highly sensitive silver staining but also highlights the utility of a combination of multiple types of anti-phosphotyrosine antibodies and the scaling up of samples. The fact that one of the newly identified proteins in our study, CFBP, is scarcely expressed in A431 cells used for proteomic analysis demonstrates the ability of our approach to detect much less abundant proteins.

In the present study, we have shown that CFBP binds to the CD2AP/CIN85 family scaffold proteins and that the interaction affects the CIN85-mediated down-regulation of EGF receptor, probably recruiting Cbl to the CIN85 complex. Recent studies show that the CD2AP/CIN85 family proteins play important roles in many aspects of cellular functions, such as recruiting the complex of Cbl protein and the growth factor receptor to endosomes for its down-regulation (18), cytoskeletal rearrangements involving F-actin or other actin-related proteins (22, 25–27), and cell death signaling (28). Most recently, CD2AP has been reported as a regulator of cytokinesis (29). The wide spectrum of binding partners reflects the versatility of the CD2AP/CIN85 family (18). Almost all of these functions have been found and proven by identifying its binding proteins. Because the CD2AP/CIN85 family members are scaffold proteins containing multiple functional domains, including SH3 domains, proline-rich motifs, and a coiled-coiled domain, numerous partners have been isolated and characterized as binding proteins. Furthermore, fine mapping of each binding molecule to its structural domains has been analyzed. For
FIGURE 7. Ectopic expression of CFBP induces morphological change in A431 cells. A, comparison of the protein expression levels of EGF receptor (EGFR, top), CD2AP (middle), and CFBP (bottom) in various mammalian cultured cells. Each whole cell lysate (WCL) prepared from the indicated cells was subjected to SDS-PAGE and analyzed by immunoblot (IB) with each antibody. HEK293T cells were transfected with the empty vector (first lane from the left) or the expression vectors for overexpressing the recombinant proteins of EGF receptor and CFPB-S (second lane from the left). B, protein expression levels in stably expressing FLAG-CFBP-S A431 cells. Two independent representative clones (clones C8 and E3) stably expressing FLAG-CFBP are shown. Control cells were mock-transfected with the empty vector. For monitoring the amount of proteins, 4 μg (for the EGF receptor) or 20 μg of protein (for CD2AP and CFBP) of whole cell lysate was used and analyzed by immunoblot using each specific antibody. C, effect of the expression of CFBP in response to EGF stimulation. Comparison of the amount of each protein was monitored by immunoblot with specific antibodies. Twenty micrograms of protein of each whole cell lysate prepared from control (left panel set) and clone E3 (right panel set) cells stimulated with EGF for the indicated times was applied. D, densitometric analysis of data in C. The results represent three independent experiments. Error bars show the S.D. The amounts of EGF receptor at each time point are normalized to the control (lane of each 0 time). E, effect of the expression of CFBP on the CD2AP-Cbl complex. The complex was monitored by immunoprecipitation in A431 cell lines. The control and E3 cell lines with or without EGF stimulation were lysed, and the immunoprecipitation was performed with anti-CD2AP antibody. Immunoprecipitated CD2AP and co-precipitated Cbl are indicated in the lower and upper panels, respectively. F, expression of CFBP induced morphological alteration of A431 cells. Images show representative colonies, each comprising of 10 –20 cells of the A431 cell line. Images were taken at 30 h of the last passage of the cells on the culture dish in normal growth medium. Images of control A431 and the A431 clone C8 cells stably expressing FLAG-CFBP-S are shown in the top and bottom rows of panels, respectively. The left, middle, and right columns show the images of phase contrast, actin, and the nucleus, respectively. Scale bar, 50 μm. G, graph showing the average area of each cell in the colony. This value was calculated by measuring the area of a colony in (F) and divided by the number of cells contained within that colony. Data were taken from 30 randomly selected colonies in one experiment, and three independent sets of experiments were carried out. Error bars, S.D.
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example, the CD2AP/CIN85 family has a stretch of three SH3 domains at its N terminus. However, proline-rich motifs binding to the classical SH3 domain with a consensus sequence, such as (R/K)XXPXXP (Class I) and PXAAXXX (Class II; X denotes any amino acid residue), are not suitable for binding to the SH3 domain of the CIN85 family. Kurakin et al. (16) reported the affinity of each SH3 domain in CIN85 for many kinds of proline-rich sequences and classified them as Class IIA (atypical) with a consensus sequence, PX(P/A)XXR (37). The PVPKPR sequence found in CFBP clearly falls within this category.

One of the characteristics of the interaction with the CD2AP/CIN85 family members is the dependence on the phosphorylation at Tyr204 in CFBP evoked by EGF stimulation. This binding property is similar to those of other partner proteins, CD2 (11) and Cbl (26), whose binding to CD2AP or CIN85 is dependent on various ligand stimulations. In the case of Cbl, not only its phosphorylation, but also the tyrosine phosphorylation of CD2AP, has been shown to be necessary for the interaction (26). From our observations, CFBP prefers the most N-terminal SH3 domain of CIN85/CD2AP. In the case of CD2AP, SH3A is known as a binding site of CD2 in T cells, whereas Cbl binds to SH3B (32). In contrast, SH3A in CIN85 plays an important role for its intramolecular interaction (32, 33). According to the functional model of Ruk/CIN85 reported by Borthwick et al. (33), a member of the CIN85/CD2AP family basically keeps a homodimeric formation via its C-terminal coiled-coiled domain. Furthermore, intramolecular association could occur in Ruk/CIN85 through the SH3A and the internal PXXPXXP motif that is not present in CD2AP. Therefore, CD2AP is not able to participate in this formation. Our result reveals that Tyr204-phosphorylated CFBP is able to form a ternary complex with CIN85 and Cbl induced by EGF stimulation and facilitates the recruitment of Cbl to the CIN85 complex. Consequently, the EGF receptor might be internalized to the endosomal degradation pathway rapidly. Although the reasons remain unclear, it is possible to speculate that phosphorylation of CFBP is induced by its conformational change for exposing the PX(P/A)XXPR motif to bind to SH3A in CIN85, and this process would function to convert an active form of CIN85 for accessing other factors, such as Cbl. Further study is needed to classify each SH3 binding affinity between CIN85 and CD2AP for proving this hypothesis.

Recently, it has been reported that herpes simplex virus 1-infected cell protein 0 containing several PX(P/A)XXR motifs binds to CIN85, and the herpes simplex virus 1-infected cell protein 0-CIN85 complex has an acceleration effect on the c-Jun-mediated degradation of the EGF receptor (34). Moreover, exogenously expressed ASAP1 (Arf-GTPase-activating protein 1) constitutively binds to CIN85 and induces an increase in EGF receptor recycling (31, 35). These results suggest that a similar mechanism operates in the CFBP/CIN85-mediated down-regulation of the EGF receptor. The expression level of mRNA of CFBP is relatively low, although it is almost ubiquitously expressed among various tissues. We also found that in addition to the full-length isoform, at least two spliced variants of CFBP mRNA are expressed. It is of interest to note that the variant lacking exon 5 (Δex5) incapable of binding to CD2AP was found only in A431 carcinoma cells. The A431 cells are known as EGF receptor-overexpressing cells. Moreover, in these cells, the total amounts of CFBP protein are also very low. It is possible that not only the gene amplification of the EGF receptor but also the lack of the CFBP expression in A431 cells contributes to maintain the high level expression of EGF receptor in contrast to normal cultured cells.

The regulation of the EGF receptor down-regulation may not be the sole physiological function of CFBP. Morphological changes of CFBP-expressing cells present as a flat cell phenotype with extensive lamellipodia. This is not surprising, since CD2AP is well known to be involved in cellular adhesion, motility, and morphology, depending on the actin cytoskeleton (22). It has been reported that overexpression of CD2AP induces membrane ruffles cooperating with PI3Kα in COS7 cells (22, 26). Cbl has also been known as a regulator of lamellipodia formation and cell morphology (36). CFBP has a 101KKKR sequence, which is a putative nuclear localization signal sequence, and a CFBP homologous protein, FLJ00022, also has the same sequence. A large amount of FLJ00022 expressed in COS7 cells was localized in the nucleus (data not shown). Since FLJ00022 lacks the proline-rich motif, it is incapable of binding to the CD2AP/CIN85 family proteins. Interestingly, the mutant CFBP lacking the proline-rich domain is localized in the nucleus (Fig. 5g). These results suggest that the CFBP family proteins may play a role in the nucleus, and the subcellular localization of CFBP is controlled by the interaction with CD2AP/CIN85. In summary, a newly identified tyrosine-phosphorylated protein, CFBP, may function as a multifunctional protein related to several biological phenomena induced by EGF signaling with CD2AP/CIN85-dependent or -independent mechanisms, and its functionally null mutant is expressed in A431 carcinoma cells by alternative splicing. Finally, the function of CFBP at a normal physiological level remains to be elucidated. We are currently analyzing the amounts of the CFBP protein and the level of its Tyr204 phosphorylation in various carcinoma cells to understand the relationship between CFBP expression and tumorigenesis. Furthermore, screening of other interacting molecules in other cells derived from podocyte should help in understanding its physiological functions in more detail.

REFERENCES

1. Aebersold, R., and Mann, M. (2003) *Nature* **422**, 198–207
2. Hynes, N. E., and Lane, H. L. (2005) *Nat. Rev. Cancer* **5**, 341–354
3. Blagoev, B., Ong, S. E., Kratchmarova, I., and Mann, M. (2004) *Nat. Biotechnol.* **22**, 1139–1145
4. Rush, J., Moritz, A., Lee, K. A., Guo, A., Goss, V. L., Spek, E. J., Zhang, H., Zha, X. M., Polakiewicz, R. D., and Comb, M. J. (2005) *Nat. Biotechnol.* **23**, 94–101
5. Thelemann, A., Petti, F., Griiffin, G., Iwata, K., Hunt, T., Settinari, T., Fenyo, D., Gibson, N., and Halej, J. D. (2005) *Mol. Cell. Proteomics* **4**, 356–376
6. Konishi, H., Yamauchi, E., Taniguchi, H., Yamamoto, T., Matsuzaki, H., Takemura, Y., Ohnmae, K., Kikkawa, U., and Nishizuka, Y. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6587–6592
7. Johnson, S. A., and Hunter, T. (2005) *Nat. Methods* **2**, 17–25
8. Szymkiewicz, I., Shupliakov, O., and Dikic, I. (2004) *Biochem. J.* **383**, 1–11
9. Yamauchi, E., Nakatsu, T., Matsubara, M., Kato, H., and Taniguchi, H. (2003) *Nat. Struct. Biol.* **10**, 226–231
10. Matsubara, M., Nakatsu, T., Kato, H., and Taniguchi, H. (2004) *EMBO J.* **23**, 712–718
11. Dustin, M. L., Olszowy, M. W., Holdorf, A. D., Li, J., Bromley, S., Desai, N., Widder, P., Rosenberger, F., van der Merwe, P. A., Allen, P. M., and Shaw, A. S. (1998) *Cell* 94, 667–677.

12. Kim, J. H., Wu, E., Green, G., Winkler, C. A., Kopp, J. B., Miner, J. H., Unanue, E. R., and Shaw, A. S. (2003) *Science* 300, 1298–1300.

13. Soubeyran, P., Kowanetz, K., Szymkiewicz, I., Langdon, W. Y., and Dikic, I. (2002) *Nature* 416, 183–187.

14. Taniguchi, H., Suzuki, M., Manenti, S., and Titani, K. (1994) *J. Biol. Chem.* 269, 22481–22484.

15. Kurakin, A. V., Wu, S., and Bredesen, D. E. (2003) *J. Biol. Chem.* 278, 34102–34109.

16. Schmidt, M. H., and Dikic, I. (2005) *Nat. Rev. Mol. Cell. Biol.* 6, 907–919.

17. Lehtonen, S., Zhao, F., and Lehtonen, E. (2002) *Am. J. Physiol.* 283, F734–F743.

18. Welsch, T., Endlich, N., Kriz, W., and Endlich, K. (2001) *Am. J. Physiol.* 281, F769–F777.

19. Nelson, J. M., and Fry, D. W. (1997) *Exp. Cell Res.* 233, 383–390.

20. Kirsch, K. H., Georgescu, M., Shishido, T., Langdon, W. Y., Birge R. B., and Hanafusa, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 6211–6216.

21. Pandey, A., Podtelejnikov, A. V., Blagoev, B., Bustelo, X. R., Mann, M., Lodish, H. F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 179–184.

22. Dikic, I., and Giordano, S. (2003) *Curr. Opin. Cell Biol.* 15, 128–135.

23. Hutchings, N. J., Clarkson, N., Chalkley, R., Barclay, A. N., and Marion, B. H. (2003) *J. Biol. Chem.* 278, 22396–22403.

24. Kirsch, K. H., Georgescu, M., Shishido, T., Ishimaru, S., and Hanafusa, H. (2001) *J. Biol. Chem.* 276, 4957–4963.

25. Lynch, D. K., Winata, S. C., Lyons, R. J., Hughes, W. E., Lehrbach, G. M., Wasinger, V., Corthals, G., Cordwell, S., and Daly, R. J. (2003) *J. Biol. Chem.* 278, 21805–21818.

26. Schiffer, M., Mundel, P., Shaw, A. S., and Bottinger, E. P. (2004) *J. Biol. Chem.* 279, 37004–37012.

27. Monzo, P., Gauthier, N. C., Keslair, F., Loubat, A., Field, C. M., Le Marchand-Brustel, Y., and Cormont, M. (2005) *Mol. Biol. Cell* 16, 2891–2902.

28. Mayer, B. J. (2001) *J. Cell Sci.* 114, 1253–1263.

29. Liu, Y., Yerushalmi, G. M., Grigera, P. R., and Parsons, I. T. (2005) *J. Biol. Chem.* 280, 8884–8892.

30. Kowanetz, K., Szymkiewicz, I., Haglund, K., Kowanetz, M., Husnjak, K., Dikic, I. (2004) *Mol. Biol. Cell* 15, 3455–3466.

31. Thien, C. B., and Langdon, W. Y. (2001) *Nat. Rev. Mol. Cell. Biol.* 2, 294–307.

32. Kowanetz, K., Szymkiewicz, I., Haglund, K., Kowanetz, M., Husnjak, K., Taylor, J. D., Soubeyran, P., Engstrom, U., Ladbury, J. E., and Dikic, I. (2003) *J. Biol. Chem.* 278, 39735–39746.