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Interaction between Two Ubiquitin-Protein Isopeptide Ligases of Different Classes, CBLC and AIP4/ITCH*

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In metazoans, CBL proteins are RING finger type ubiquitin-protein isopeptide (E3) ligases involved in the down-regulation of epidermal growth factor tyrosine kinase receptors (EGFR). Among the three CBL proteins described in humans, CBLC (CBL3) remains poorly studied. By screening in parallel a human and a *Caenorhabditis elegans* library using the two-hybrid procedure in yeast, we found a novel interaction between Hsa-CBLC and Hsa-AIP4 or its *C. elegans* counterpart Cel-WWP1. Hsa-AIP4 and Cel-WWP1 are also ubiquitin E3 ligases. They contain a HECT (homologous to E6-AP C terminus) catalytic domain and four WW domains known to bind proline-rich regions. We confirmed the interaction between Hsa-CBLC and Hsa-AIP4 by a combination of glutathione S-transferase pull-down, co-immunoprecipitation, and colocalization experiments. We show that these two E3 ligases are involved in EGFR signaling because both become phosphorylated on tyrosine following epidermal growth factor stimulation. In addition, we observed that CBLC increases the ubiquitination of EGFR, and that coexpressing the WW domains of AIP4 exerts a dominant negative effect on EGFR ubiquitination. Finally, coexpressing CBLC and AIP4 induces a down-regulation of EGFR signaling. In conclusion, our data demonstrate that two E3 ligases of different classes can interact and cooperate to down-regulate EGFR signaling.

In metazoans, the ubiquitin-associated proteolytic system is a crucial mechanism that regulates signal transduction pathways involved in various cellular processes (reviewed in Ref. 1). The ubiquitination catalytic transfer to a substrate requires at least three types of enzymes. First, the ubiquitin-activating enzyme activates the ubiquitin moiety in an ATP-dependent manner. The activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2).¹ E2 associates with a third

enzyme called the ubiquitin ligase (E3), to catalyze the transfer of ubiquitin to a specific substrate. The polyubiquitinated substrate is then degraded by the 26 S proteasome complex. The E3 ubiquitin ligase plays a key role in this process by insuring a highly specific recognition of the substrate.

There are two major classes of E3s, based on structural differences (1). The first class has a HECT (homologous to E6-AP C terminus) domain that participates directly in the transfer of ubiquitin to substrates. E3 proteins of the second class carry a RING finger domain and function as adapter proteins, bringing the substrate to the ubiquitin-charged E2. These two classes are implicated in the down-regulation of multiple proteins via the 26 S proteasome.

Among the RING finger E3s, the CBL proteins play a role in the regulation of several signaling pathways involving tyrosine kinases (reviewed in Ref. 2). They have a common overall structure and display several domains: a N-terminal tyrosine kinase binding domain (hereafter named TKB) that recognizes and bind specific phosphotyrosine residues on a substrate (3), a RING finger domain involved in the ubiquitin transfer, and a C-terminal proline-rich region with potential SH3 binding sites.

In humans, three CBL proteins encoded by paralogous genes have been identified, namely CBL (or CBLA), CBLB, and CBLC (also named CBL3) (4–7). The CBL proteins play a role in the down-regulation of the EGF receptor (EGFR) tyrosine kinase family in vertebrates and nonvertebrates (8–11). Following ligand receptor activation, CBLA is tyrosine-phosphorylated by EGFR and recruited to a specific phosphotyrosine residue on EGFR (12). This direct binding between CBLA and EGFR is mediated through its TKB domain. Several groups have also demonstrated the essential role played by CBL RING finger in the ubiquitination and desensitization of endocytosed EGFR (13, 14). The three CBLs appear to induce the degradation of EGFR through ubiquitination (8, 9, 15). However, it is not known precisely whether *in vivo* the three CBLs regulate the same receptor tyrosine kinases (e.g. EGFR) or whether they have both common and different substrates. CBLA and CBLB have a quite ubiquitous tissue distribution, and their role at least in the hematopoietic lineage has been clearly demonstrated upon analysis of the phenotypes of knock-out mice (16–19). CBLC is expressed at low level in most tissues with a high expression level in the digestive tract, whereas it is not detected in the hematopoietic lineage (6, 20, 21).

A CBL protein has been characterized in protostomians *Cae-*

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¹ The abbreviations used are: E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; EGF, epidermal growth factor; EGFR,

epidermal growth factor receptor; AD, activation domain; DBD, DNA binding domain; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; HECT, homologous to E6-AP C terminus; SH, Src homology; HA, hemagglutinin; PBS, phosphate-buffered saline; TKB, tyrosine kinase binding domain.

norhabditis elegans and *Drosophila melanogaster* (10, 11, 22). In *C. elegans*, genetic studies have shown that the CBL ortholog SLI-1 exerts a negative role on vulval development by inhibiting the RAS-dependent signaling of LET-23 EGFR ortholog (10, 23). The negative role of CBL proteins on EGFR signaling in vertebrates and nematodes are largely conserved, in agreement with the similar structure of SLI-1 and mammalian CBLs.

To identify new partners for CBLC, we screened in parallel a human epithelial and a worm *C. elegans* libraries using the two-hybrid procedure in yeast. We describe here an interaction between CBLC and Hsa-AIP4/Cel-WWP1, which belongs to the HECT family of E3s (24, 25). Our data suggest that this association is involved in the ubiquitination and desensitization of EGFR.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The following antibodies were used: rabbit anti-EGFR (1005) (Santa Cruz Biotechnology, catalog no. sc-03), mouse anti-myc (9E10) (Santa Cruz Biotechnology, catalog no. sc-40), rat anti-HA High Affinity (3F10) (Roche Molecular Biochemicals, catalog no. 1867423), and mouse anti-GFP (mixture of two monoclonal antibodies) (Roche Molecular Biochemicals, catalog no. 1814460). Mouse anti-phosphotyrosine (4G10) was from Upstate Biotechnology, Inc. (Buckingham, United Kingdom (UK)). A polyclonal anti-CBLC was made against the 17 last C-terminal amino acids of Hsa-CBLC in rabbit. Goat anti-rabbit and anti-mouse IgG coupled to horseradish peroxidase were purchased from Jackson Laboratories (Bar Harbor, ME) and Dako (Botany, New South Wales, Australia), respectively. Recombinant human EGF was from PromoCell GmbH (Heidelberg, Germany), and the inhibitor of proteasome, MG 132, was from Sigma.

DNA Constructs—Two-hybrid constructs were made using pGBKT7 or pGADT7 (Clontech, Heidelberg, Germany) for the GAL4 system and pBTM116 or pVP16 for the LEXA system. To isolate the 5' of Hsa-AIP4, we derived oligonucleotides from a sequence identified in a human genomic data bank (www.ncbi.nlm.nih.gov/LocusLink/). This genomic sequence contains the Hsa-AIP4 locus and by virtue of its high similarity with the *Itch* locus (mouse *Aip4*) 5' coding sequences, we were able to derive a sense primer encompassing the putative initiating codon to clone the 5' sequence of Hsa-AIP4 cDNA. We performed PCR amplification of reverse transcribed human mammary gland poly(A) mRNA. After sequencing, the 5' cDNA of AIP4 was fused to the partial human AIP4 clone isolated in our two-hybrid screen. The myc-tagged constructs were made using the pRK5myc vector (26). To construct myc-AIP4, pRK5myc vector was converted in the Gateway version according to the recommendations of the manufacturer (Invitrogen). HA-tagged constructs were made using the pCDNA3-3HA plasmid (Invitrogen). The pGEXtag plasmid was used to produce all GST fusion proteins. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene, Amsterdam, The Netherlands). PRK5-EGFR was described in Ref. 24. pcDNA3-HA-ubiquitin was a kind gift from D. Bohmann (Heidelberg, Germany). All constructs were sequenced by Genome Express (Grenoble, France).

Two-hybrid Procedure—Full-length cDNA of Hsa-CBLC was subcloned into the pGBKT7 GAL4 DNA binding domain (DBD) plasmid (Clontech). We checked for the good expression of the GAL4 DBD-CBLC protein. The screen of the two PACT2 GAL4 activation domain (AD) fusion libraries was essentially performed according to the recommendations of the manufacturer (Clontech) in the yeast strain AH109 containing three reporter genes (*LACZ*, *HIS3*, and *ADE*). Because the CBLC bait transactivated the *HIS* reporter gene, the *ADE* gene was used as the main reporter gene in the screening. Approximately 2 million transformants were screened in the human and worm libraries and selected on plates containing minimum media lacking *LEU*, *TRP*, *HIS*, and *ADE* and supplemented with 10 mM 3-aminotriazol. Positive clones were rescued and retransformed in the AH109 strain to test for interaction with the GAL4 DBD-CBLC or control bait (GAL4-lamin). Clones that were positive interactors with GAL4 DBD-CBLC and negative with GAL4 DBD-lamin were sequenced. Alternatively, we performed the two-hybrid analysis using the LEXA system in the L40 yeast strain with pBTM116- and pVP16-derived constructs.

Cell Culture and Transfections—COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen), 200 mM 1% L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen). HeLa cells were

grown in the same conditions without penicillin/streptomycin. Cells were in 40–60% confluence the day of transfection. All cell transient transfections were made using FuGENE 6 transfection reagent (Roche, Mannheim, Germany) or PolyFect transfection reagent (Qiagen, Courtaboeuf, France), according to the recommendations from the manufacturer. For unstimulated cells, 48 h after transfection cells were washed in cold PBS and lysed in a lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg·ml⁻¹ aprotinin, 10 μg·ml⁻¹ leupeptin, 10 μg·ml⁻¹ pepstatin, and 200 μM sodium orthovanadate. Lysates were cleared by centrifugation and subjected either to Western blot analysis or to immunoprecipitation. For EGF-stimulated cells, cells were grown in 100-mm dishes, and 36 h after transfection, they were split in two 100-mm dishes and serum-starved overnight. Cells were either lysed directly or after stimulation by EGF (100 ng·ml⁻¹).

Western Blot Analysis, Immunoprecipitation, and GST Pull-down—Lysate protein content was normalized using the Bio-Rad protein assay. For immunoprecipitation, lysates were incubated with antibodies overnight at 4 °C. Protein A-agarose or Protein G-agarose was added, and immune complexes bound to beads were recovered after 1 h, washed three times with HNTG buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 0.1% Triton X-100), boiled in 1× sample buffer, separated by denaturing SDS-PAGE gels, and transferred to nitrocellulose membrane Hybond-C extra (Amersham Biosciences). After blocking, the membranes were incubated with the indicated primary antibodies. Membranes were washed twice in Tris-buffered saline containing 0.05% Tween 20 and once in Tris-buffered saline. Then, they were incubated during 1 h with horseradish peroxidase-conjugated secondary antibodies. After washing, specific signals were visualized with enhanced chemiluminescence (ECL) detection system (Pierce). For GST pull-down assay, lysates were incubated with GST fusion proteins during 2 h at 4 °C. Beads were harvested by centrifugation and washed as described for the immune complex.

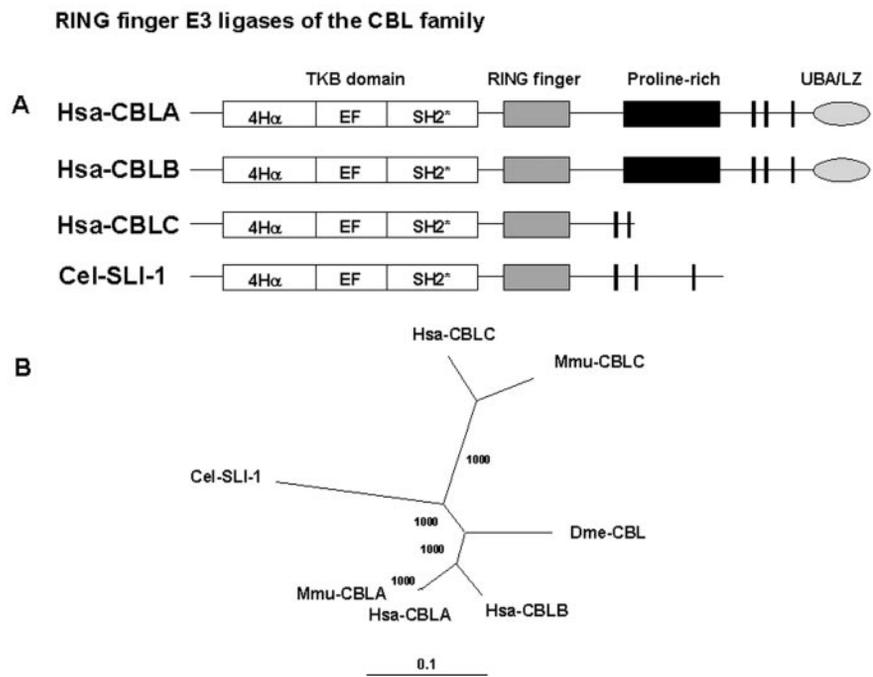
Immunofluorescence Studies—COS-1 cells were seeded on glass coverslips and cotransfected using FuGENE 6 (Roche) with the cytomegalovirus expression constructs myc-AIP4ΔC2 and EGFP-CBLC. 24 h later the cells were serum-depleted overnight in Dulbecco's modified Eagle's medium containing glutamine and 0% fetal calf serum. Cells were then treated with EGF (50 ng/ml) during 10 min and processed for immunofluorescence staining. Cells were washed once with PBS, fixed in 3% paraformaldehyde in PBS for 30 min, washed twice in PBS, then in PBS + 50 mM NH₄Cl, and again in PBS. Permeabilization and blocking were performed in 10% fetal calf serum + 0.1% saponin for 30 min. The primary antibody was incubated 1 h and diluted in the same blocking solution. After several PBS washes in PBS + saponin 0.1%, coverslips were incubated with a Texas Red-conjugated secondary antibody, rinsed in PBS + saponin, and mounted in Mowiol. The monoclonal anti-myc 9E10 was used at 0.7 μg/ml to label myc-tagged AIP4ΔC2 protein and revealed with a secondary goat Texas Red anti-mouse antibody (Molecular Probes, Leiden, The Netherlands). The cellular localization of proteins was analyzed by confocal laser system microscopy using a TCS NT Leica apparatus (Heidelberg, Germany), and fluorescent images were processed using Adobe Photoshop (Adobe System Inc., Paris, France).

Luciferase Assay—HeLa cells were cultured in 100-mm dishes. Transfection with PolyFect (Qiagen) of the indicated vectors was done with a reporter pSRE-FLuc plasmid and a control pTK-Renilla-Luc. 36 h later cells were split in six-well plates, and serum-starved overnight. Cells were then either maintained serum-starved (3 wells) or EGF-stimulated during 24 h (3 wells). SRE luciferase assay was performed with the dual-luciferase reporter assay system (Promega, Madison, WI) according to the recommendations of the manufacturer. The luciferase activity was measured in triplicate and normalized against protein concentrations and Renilla luciferase activity for each point.

Northern Blot Analysis—The multiple tissue human Northern blots (Clontech, Palo Alto, CA; 7759-1, 7760-1, and 7767-1) were hybridized according to the instructions from the manufacturer. Hybridizations were performed according to Church and Gilbert (50) with a ³²P-labeled probe (Megaprime kit RPN-1607, Amersham Biosciences, Buckinghamshire, UK) corresponding to a 1400-bp fragment from AIP4 human 5' cDNA. The β-actin probe was purchased from Clontech. The filters were washed at high stringency and imaged using a phosphorimager.

Phylogenetic Analysis—Phylogenetic trees were constructed using the distance matrix (Blosom 30 matrix) and neighbor-joining algorithms implemented in ClustalW. Sequences were retrieved from the NCBI data base (www.ncbi.nlm.nih.gov/LocusLink/list.cgi). Concatenate

FIG. 1. The RING finger E3 ligases of the CBL family. A, the CBL proteins from *H. sapiens* (Hsa-CBLA, Hsa-CBLB, and Hsa-CBLC) and *C. elegans* (Cel-SLI-1) are schematically depicted. From N terminus to C terminus, CBL proteins have in common a TKB (*white box*), a RING finger domain (*gray box*), and proline-rich region (*black boxes*). Three subdomains define the TKB: a four-helix bundle (*4H α*), a calcium-binding EF hand (*EF*), and an atypical Src homology region 2 (*SH2**) (3). In contrast to SLI-1 and CBLC, CBLA and CBLB present a long C-terminal extension with an ubiquitin-associated leucine zipper domain (*UBA/LZ*, *gray circle*) in their C-terminal region. B, an unrooted phylogenetic tree of seven well characterized CBL proteins is shown. Bootstrap values (out of 1000 replicates) are indicated at each node. Branch lengths are proportional to time (0.1 million years ago).



nated domain sequences only were used to build the trees. A total of 1000 bootstrapped replicates were run.

RESULTS

Two-hybrid Screen—CBLC and SLI-1 have significant sequence identities in their TKB, RING finger, and proline-rich region, and are both involved in conserved negative regulation of EGFR pathways in metazoans. Fig. 1 shows the structural features and phylogenetic relationships of the CBL family members. We hypothesized that a CBLC-interacting protein identified in the two species *Homo sapiens* and *C. elegans* would be the basis for a likely valid and functional interaction conserved during evolution. We thus searched for CBLC-interacting proteins using the two-hybrid procedure in yeast by screening a human mammary epithelial and *C. elegans* libraries. Both were screened using human CBLC fused to GAL4 DBD as a bait in the AH109 yeast containing three reporter genes (*LACZ*, *HIS3*, and *ADE*).

Following the analysis of positive interacting clones, we controlled the specificity of the two-hybrid interactions with a DBD-lamin fusion control. Several proteins were identified from the screen of the human library. Among them, the SH3-containing protein ponsin/SORBS1, already characterized by others as a CBL-interacting protein (Fig. 2A) (27–29) and previously called CBL-associated protein. We isolated a second SH3-containing protein named vinexin/SCAM-1 in our screen with a CBLC bait (Fig. 2A). Like ponsin, vinexin is known to be connected to the cytoskeletal network (30). The ponsin and vinexin genes are paralogous, and the two proteins belong to the same family (31). The identification of related proteins already known to bind CBL proteins indicated that our screen could reveal other valid interactions.

Only one CBLC-interacting protein was isolated from both the human and *C. elegans* libraries. This protein is an E3 ubiquitin ligase named AIP4 for the human version and WWP1 for its *C. elegans* counterpart (25). The worm and human proteins share significant (56.5%) amino acid sequence similarity. The general structure of these E3 ligases is shown in Fig. 2B. Like the NEDD4 family, these proteins belong to the HECT family of E3 ligases and are composed of several domains: a C2 N-terminal domain involved in the calcium-dependent association with the plasma membrane; four WW domains, which are

specific interacting modules binding proline-rich sequences (32); and a C-terminal HECT domain, ensuring the catalytic transfer of ubiquitin from an E2-conjugating enzyme to the substrate being ubiquitinated (33). A phylogenetic analysis showed that the NEDD4 proteins and the WWP1s constitute two subfamilies of HECT E3 ligases (Fig. 2C).

We characterized further the potential interaction between HECT E3 ligase AIP4 and CBLC. As an additional control, the CBLC/AIP4 interaction was also tested in the L40 yeast with a LEXA-CBLC fusion protein. The same results were obtained with the GAL4-derived system (data not shown). The Hsa-AIP4 cDNA isolated from the two-hybrid screen (AIP4 Δ C2) was devoid of the 5' sequence encoding the C2 N-terminal region. We thus performed a PCR experiment to clone the 5' cDNA of Hsa-AIP4 and reconstruct a full-length Hsa-AIP4. In summary, the two-hybrid analysis indicated that CBLC is able to interact with both Hsa-AIP4 and Cel-WWP1.

AIP4 mRNA Expression—The tissue distribution of AIP4 mRNA was investigated in human adult tissues by Northern blot hybridization using commercial Northern blot membranes (Fig. 3). We used a probe corresponding to the N terminus of the protein, encompassing the C2 and the WW domains because the N terminus of HECT E3 ligases is the less conserved region among proteins of the family. AIP4 was expressed as a major transcript of 6.0 kb in most tissues with the exception of bone marrow; it was also weak in spleen and thymus. A transcript of lower molecular mass was seen in testis (Fig. 3). This pattern grossly overlaps with that of CBLC mRNA, which is also weak or totally absent in the lymphohematopoietic system and is strong in the gastrointestinal tract (6, 21).

CBLC and AIP4 Interact in Mammalian Cells—To confirm the two-hybrid results, we produced recombinant GST-CBLC proteins in *E. coli* to perform pull-down experiments with lysates of COS-1 cells overexpressing the myc-tagged Hsa-AIP4 (AIP4 Δ C2) (Fig. 4A, upper panel). This experiment showed that AIP4 binds to GST-CBLC but not to a GST control protein, confirming the binding observed using the two-hybrid procedure.

To further document the interaction, proteins from COS-1 cells cotransfected with myc-AIP4 Δ C2 and either EGFP-CBLC (Fig. 4B) or EGFP-CBLA (Fig. 4C) were immunoprecipitated

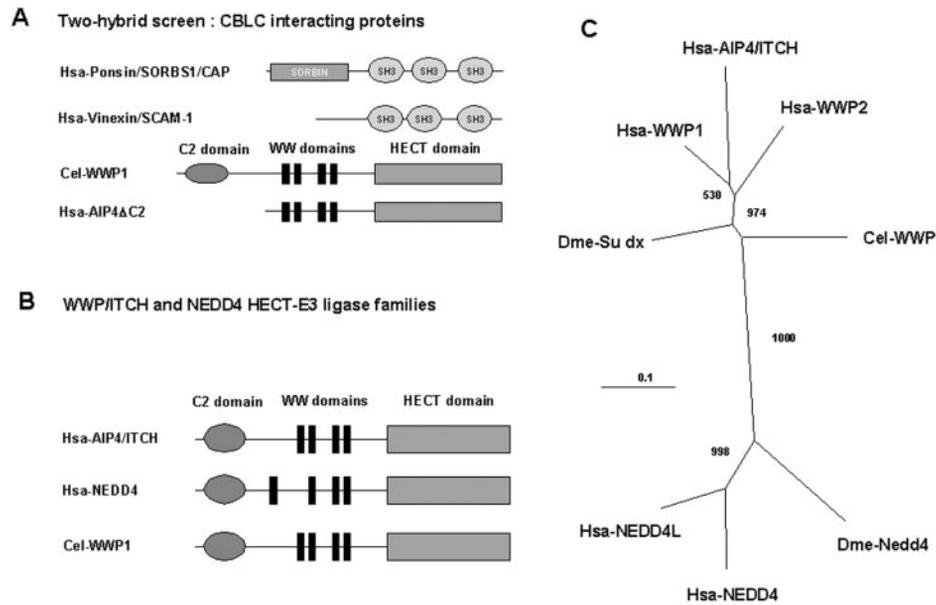
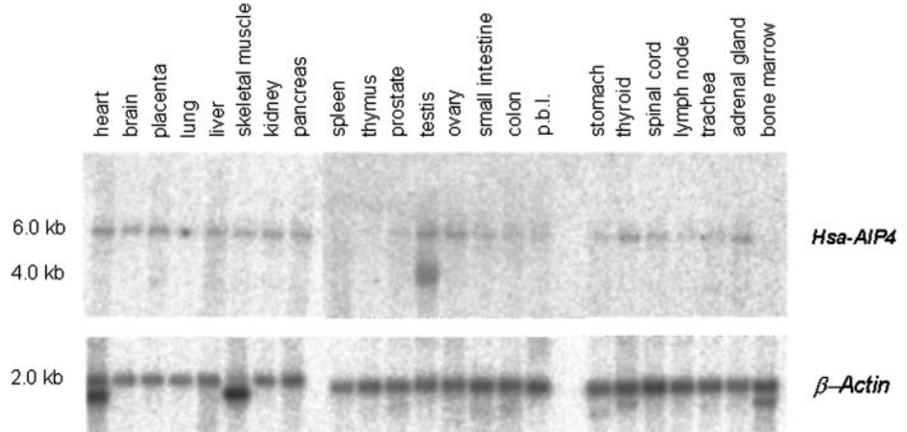


FIG. 2. Structure of CBLC-interacting proteins identified in yeast two-hybrid screen. A human mammary epithelial library and a *C. elegans* PACT2-derived library were screened using human CBLC fused to the DBD of GAL4 in the AH109 yeast containing three reporter genes (*LACZ*, *HIS3*, and *ADE*). *A*, schematic representations and domain composition of some CBLC-interacting proteins are shown: *H. sapiens* (Hsa) ponsin/SORBS1/CBL-associated protein and Hsa-vinexin/SCAM-1 two proteins known to bind CBLA protein (25–27, 29). One CBLC-interacting protein was isolated from both human and *C. elegans* libraries. This protein is an E3 ubiquitin ligase named AIP4 for the human version and WWP1 for its *C. elegans* counterpart. *B*, WWP/ITCH and some members of the NEDD4 HECT-E3 ligase family are depicted. They share a common overall structure with a C2 domain located at their N terminus, three to four WW modules, and a HECT domain in their C-terminal region. The C2 domain is responsible for Ca^{2+} -dependent binding of membrane phospholipids. WW domains are protein-protein interaction modules binding small proline-rich sequences (e.g. PPXY). The HECT domain is an ubiquitin-protein ligase domain that catalyzes the transfer of activated ubiquitin to a protein substrate. *C*, an unrooted phylogenetic tree of proteins from AIP4/WWP family and NEDD4 family is shown. Bootstrap values (out of 1000 replicates) are indicated at each node. Branch lengths are proportional to time (0.1 million years ago).

FIG. 3. Analysis of AIP4 mRNA expression. A commercial Northern blot membrane was used to establish the AIP4 mRNA expression pattern in human adult tissues. A 1400-bp probe corresponding to the N-terminal domain (C2 and WW domain) of the AIP4 protein was used. A major transcript of 6.0 kb was observed in most tissue except the bone marrow; AIP4 expression is weaker in spleen and thymus, and a transcript of 4 kb was seen in testis.



with anti-myc antibody. Blotting with anti-EGFP antibody revealed that EGFP-CBLC protein co-immunoprecipitated with AIP4ΔC2. The same result was obtained with full-length AIP4 (data not shown). EGFP-CBLA also co-immunoprecipitated with myc-AIP4, although less strongly than for CBLC (Fig. 4C).

Thus, both GST pull-down and co-immunoprecipitation experiments confirmed that the CBLC and AIP4 E3 ligases can be *bona fide* binding partners.

Colocalization of CBLC and AIP4 Proteins—We studied the subcellular distribution of overexpressed EGFP-CBLC in combination myc-AIP4ΔC2 in COS-1 cells. Cells were serum-depleted overnight and, following a 10-min stimulation with EGF, prepared for immunofluorescence. The costaining in EGF-treated cells overexpressing EGFP-CBLC and myc-AIP4ΔC2 showed that the two proteins colocalized, notably in thin wavy structures at the periphery of the cells that resemble ruffles (Fig. 4D). A similar colocalization pattern was seen

between EGFP-CBLC and Hsa-AIP4 (data not shown). This overlapping subcellular location of CBLC and AIP4 supports a potential interaction of the two E3 ligases *in vivo*.

CBLC Interacts with the WW Domain of AIP4—To delineate the region of CBLC and AIP4 involved in the binding, we carried out a two-hybrid analysis with several CBLC and AIP4 constructs (Fig. 5A). Mutant constructs abolishing the structure and function of the TKB domain (mutant Arg → Lys), or of the RING finger domain (mutant Cys → Ala) did not impair CBLC binding to AIP4. In addition, no binding was observed between the TKB of CBLC and AIP4. These data suggested that the C-terminal proline-rich region of CBLC could be responsible for the binding. Indeed, we observed that AIP4 bound to the 50-amino acid C-terminal proline-rich region of CBLC. Finally, a construct encoding the four WW domains of AIP4 was sufficient to bind CBLC proline-rich region. Using a GST-WW fusion protein and a lysate overexpressing an EGFP-CBLC protein, we

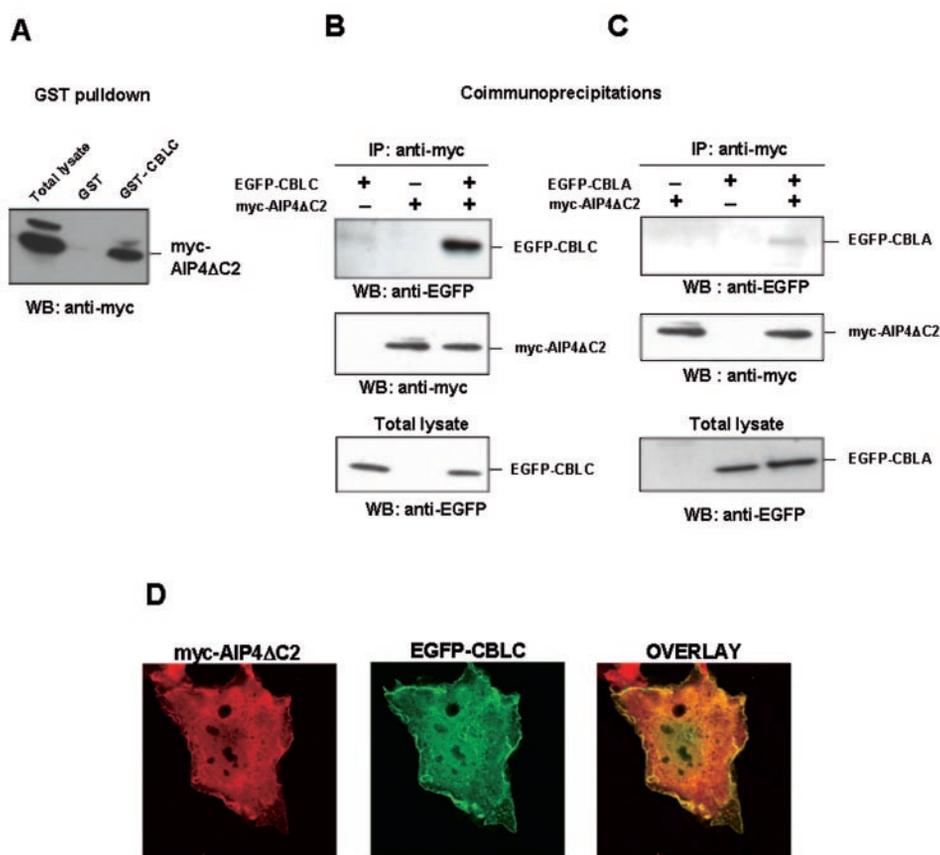


FIG. 4. **CBLC interacts and colocalizes with AIP4.** **A**, GST pull-down. Recombinant GST-CBLC and GST control proteins produced in *E. coli* were used to perform pull-down experiments with COS-1 cell lysates overexpressing myc-tagged AIP4ΔC2. **B** and **C**, co-immunoprecipitation of AIP4 with CBLC and CBLA. COS-1 cells were cotransfected with myc-AIP4ΔC2 and either EGFP-CBLC or EGFP-CBLA. AIP4ΔC2 proteins were immunoprecipitated with anti-myc antibody, and Western blot analyses were performed with anti-GFP antibody. **D**, colocalization of CBLC and AIP4 proteins. Localization of overexpressed myc-AIP4ΔC2 or EGFP-CBLC was studied in COS-1 cells. Cells were serum-starved overnight and, after 10 min of stimulation with EGF, prepared for immunofluorescence. myc-AIP4ΔC2 proteins were seen in red (left) and EGFP-CBLC in green (middle), and the colocalization between CBLC and AIP4ΔC2 is seen in yellow in the overlay image (right). WB, Western blot; IP, immunoprecipitation.

confirmed in a pull-down experiment that CBLC interacts with the WWs of AIP4 (Fig. 5B). In this experiment the EGFP-CBLC protein was also capable of binding to a GST-GRB2 fusion protein. Taken together, these experiments indicate that the proline-rich C-terminal region of CBLC and the WW domains of AIP4 are required for CBLC/AIP4 interaction.

CBLC and AIP4 Are Phosphorylated on Tyrosine Residues after Activation of EGFR—To evaluate the possible role of the CBLC/AIP4 interaction in EGFR signaling, we performed transfection experiments in COS-1 cells and measured the phosphorylation status of CBLC and AIP4 by immunoblotting with the 4G10 anti-phosphotyrosine antibody. First, as already shown by others (6), we confirmed that CBLC is phosphorylated on tyrosine residues and recruited via its TKB domain to the activated EGFR (Fig. 6A). We also demonstrated that the TKB domain was sufficient to bind to EGFR because a point mutation in the TKB domain abolished the binding of activated EGFR to CBLC (Fig. 6, A and B). Second, we show that AIP4 is also phosphorylated on tyrosine residues in cells upon EGF stimulation and that this phosphorylation increased over time (Fig. 6C). The same result was obtained with AIP4ΔC2 (data not shown).

CBLC Increases EGFR Ubiquitination and the Four WW Domains of AIP4 Inhibit EGFR Ubiquitination—The previous experiments show that the HECT domain-containing E3 ligase AIP4 interacts with CBL proteins and can be tyrosine-phosphorylated in an EGF-dependent manner. This suggests the

possible involvement of AIP4 in the ubiquitination of EGFR or EGFR-interacting proteins.

Levkowitz *et al.* (9) have shown that CBLC, like CBLA and CBLB, is able to increase ubiquitination of stimulated EGFR. We thus explored whether AIP4 constructs could also interfere with EGFR ubiquitination. To detect ubiquitinated EGFR, cells were cotransfected with an EGFR and a HA-ubiquitin construct. Following stimulation with EGF and immunoprecipitation of EGFR, we detected the level of ubiquitinated EGFR by immunoblotting with an anti-HA antibody (Fig. 7A). Cotransfection with a plasmid encoding CBLC increased the level of ubiquitinated EGFR. This is similar to what was shown in Chinese hamster ovary cells overexpressing CBLA (9). In contrast, the cotransfection of CBLC with an AIP4 construct containing only the WW domains strongly diminished the covalent attachment of HA-ubiquitin to EGFR (Fig. 7B). This negative effect was seen in the presence or absence of overexpressed CBLC. This result indicates that the region containing the WWs of AIP4 can interfere with the ubiquitination process of EGFR and suggests that AIP4, like CBL proteins, could play a role in EGFR ubiquitination and down-regulation.

CBLC and AIP4 Induce a Down-regulation of EGFR Signaling—To further evaluate the role of AIP4 in EGFR signaling, we performed luciferase assays using cotransfection experiments with a SRE-luciferase reporter construct. This approach is commonly used to monitor the level of activation of the EGFR transduction pathway (14, 34, 35). Using this assay it has been

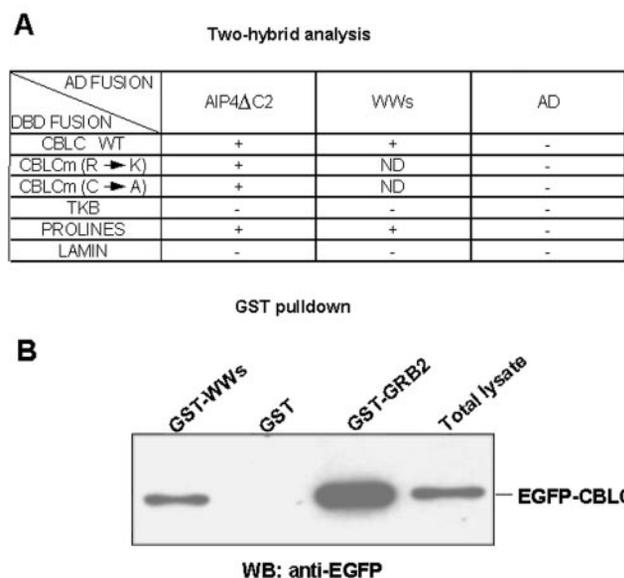


FIG. 5. CBLC interacts with the WW domains of AIP4. A, yeast two-hybrid analysis of CBLC/AIP4 interaction using several CBLC and AIP4 construct shows that the proline-rich C-terminal region (50 amino acids long) of CBLC and the WW domains of AIP4 are required for the interaction. Fusion between GAL4 DBD and CBLC wild type, mutant of the TKB (RK), mutant of the RING domain (CA), TKB, or C-terminal proline-rich region were tested for two-hybrid interaction in AH109 yeast in combination with fusion between AD of GAL4 with AIP4 Δ C2 or with the four WW domains of AIP4. GAL4 DBD fusion with lamin and GAL4 AD were used as controls. +, positive interaction; -, negative interaction; ND, not determined. B, pull-down experiment using a GST-WW domains and a lysate of COS-1 cells expressing EGFP-CBLC confirms that the four AIP4 WW domains bind to CBLC. Controls show a binding of EGFP-CBLC to a GST-GRB2 and absence of binding to GST.

shown that CBLA diminishes the luciferase signal by down-regulating activated EGFR through increased ubiquitination. Similarly, we observed that CBLC exerts a negative effect on SRE-luciferase activity (Fig. 8A). The full AIP4 protein was also able to decrease EGFR signaling (Fig. 8A). Cotransfecting CBLC and AIP4 constructs further decreased the EGF-stimulated reporter activity. Together these results indicate that CBLC and AIP4 could both contribute to the down-regulation of EGFR signaling. Additional transfection using the AIP4 Δ C2 deletion construct showed that, to exert its negative effect on EGFR signaling, AIP4 requires an intact N-terminal C2 domain (Fig. 8B).

Because overexpressing the WW domains of AIP4 is able to abrogate EGFR ubiquitination (see Fig. 7B), we expected that EGFR signaling would be positively affected by this construct in this luciferase assay. Indeed, we observed that the WWs increased EGF-stimulated reporter activity (Fig. 8B). This result confirms the dominant negative effect induced by the WW domains on EGFR ubiquitination (Fig. 7B) and suggests that an activated EGFR will continue to signal in the absence of its ubiquitination and degradation.

DISCUSSION

E3 ligases play a crucial role in the ubiquitination system because they dictate the specificity of the substrate to be ubiquitinated. The CBL proteins belong to the RING finger-containing family of E3 ligases and participate notably in the ubiquitination and down-regulation of activated EGF receptors in vertebrates as well as in nonvertebrates such as nematodes. The other major class of E3 ligases contains a HECT catalytic domain; these ligases are involved in the ubiquitination and down-regulation of sodium channel activity or in the ubiquitination of Src-like tyrosine kinases (33, 36, 37). We describe here for the first time an interaction between members of these

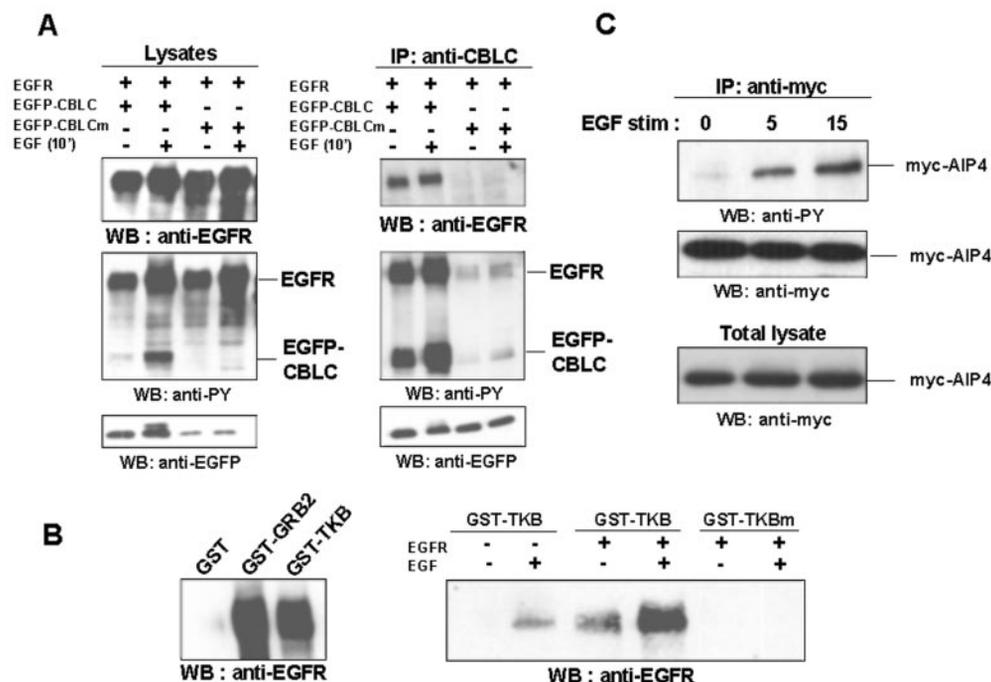


FIG. 6. Phosphorylation of CBLC and AIP4 on tyrosine after activation of EGFR. A, CBLC is phosphorylated upon EGF stimulation and co-immunoprecipitated with EGFR. EGFR and EGFP-CBLC or EGFP-CBLCm (a mutant with a Gly \rightarrow Glu point mutation in the TKB domain) were overexpressed in COS-1 cells. Cells were split and serum-starved overnight, and were stimulated or not with EGF for 10 min. Total lysates were Western-blotted with anti-EGFR, anti-phosphotyrosine, and anti-GFP antibodies (left). Immunoprecipitations using anti-CBLC antibody were also analyzed by Western blot with the same antibodies (right). B, the TKB domain of CBLC is sufficient to bind EGFR. Pull-down experiments using recombinant GST-CBLC, GST-TKB, or GST-TKB mutant proteins confirm that CBLC binds to EGFR via its TKB domain. C, AIP4 is phosphorylated on tyrosine residues upon EGF stimulation. COS-1 cells were transfected with EGFR and myc-AIP4, serum-starved overnight, and stimulated for the indicated time (0, 5, and 15 min). Myc-AIP4 proteins were precipitated with anti-myc antibody, and Western blot was revealed with anti-phosphotyrosine antibody 4G10. WB, Western blot; IP, immunoprecipitation.

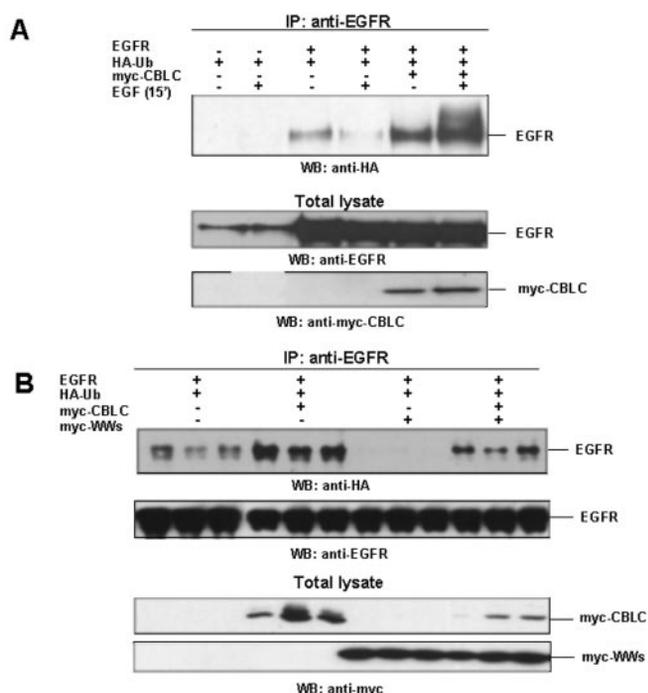


FIG. 7. CBLC and AIP4 are involved in EGFR ubiquitination. A, CBLC increases EGFR ubiquitination upon EGF stimulation. COS-1 cells were cotransfected with the indicated plasmids. Cells were split, serum-depleted overnight, and either stimulated or not with EGF (100 ng/ml) for 15 min. All cells were treated with the proteasome inhibitor MG 132. Lysates were precipitated with anti-EGFR antibody, and Western blot was performed with anti-HA antibody to detect ubiquitinated EGFR. B, the four AIP4 WW domains inhibit EGFR ubiquitination. Conditions were identical to those in A, except that cells were EGF-stimulated and the transfection was performed in triplicate. WB, Western blot; IP, immunoprecipitation; Ub, ubiquitin.

two E3 ligase families, *i.e.* CBLC and AIP4. Our data also indicate a possible role for this interaction in the modulation of EGFR ubiquitination and signaling.

CBLC and AIP4 Interact—To determine the function of human CBLC, an E3 ligase of the RING finger domain class, we sought to characterize its binding partners. Following a parallel two-hybrid screen in yeast of a human and a worm cDNA libraries, we identified Hsa-AIP4 and Cel-WWP1 as CBLC-interacting proteins. Isolating a HECT and WW-containing E3 ligase from two libraries originating from two different species strongly suggests that the interaction between a CBL and a HECT E3 ligase has been conserved during evolution. This is likely to reflect an underlying crucial role in ubiquitination or down-regulation of signaling. Two-hybrid data, GST pull-down, co-immunoprecipitation experiments, and subcellular colocalization in COS cells demonstrated that CBLC can readily interact with human AIP4. CBLC does not contain the consensus PPXY sequence recognized by WW modules. However, an interaction between NOTCH and AIP4 has been reported (38); like CBLC, NOTCH is devoid of any PPXY consensus WW binding site, and recently NEDD4-interacting proteins have been discovered that lack this recognition motif as well (39). This suggests that the E3 ligases containing a HECT and WW domains can have binding partners based on other structural requirements that will need to be delineated.

AIP4 and CBLC Are EGFR Substrates and Down-regulate EGFR Signaling—We (this study) and others (6, 20) have shown that, after EGF stimulation, CBLC is tyrosine-phosphorylated and recruited by activated EGFR. It is also known that EGFR and some of its substrates (*e.g.* GRB2, SHC) are ubiquitinated during the EGFR endocytic route toward lysosomes

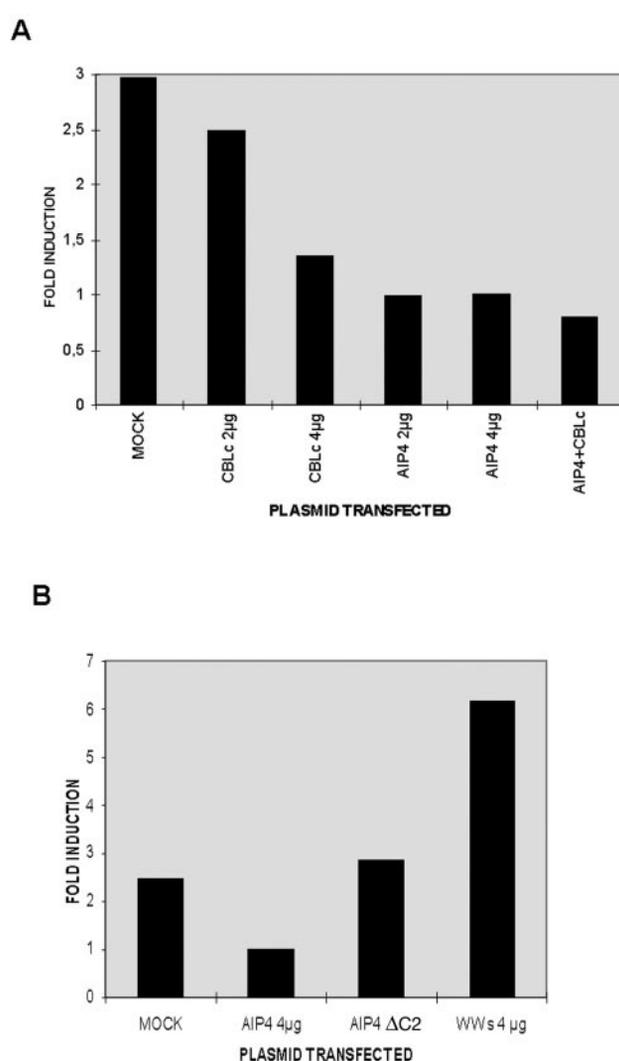


FIG. 8. CBLC and AIP4 down-regulate EGFR signaling. A, AIP4 and CBLC cooperate in the down-regulation of EGFR signaling. HeLa cells were transfected with the indicated plasmids. Control transfection was performed with empty pRK5-myc vector. The total amount of DNA was kept constant by the addition of this empty vector. All transfections were done in the presence of a pSRE-F-luciferase (serum-responsive element) and a pTK-Renilla reporter plasmid. Thirty-six hours later, cells were split and serum-starved overnight, and either kept serum-starved during additional 24 h or stimulated during 24 h with EGF (100 ng/ml). Cells were harvested for a luciferase assay, and signals obtained were normalized against protein concentrations and Renilla luciferase activity. EGF stimulation of reporter activity (in -fold activation) is shown. B, same conditions as in A; the C2 domain of AIP4 is required to down-regulate the EGFR pathway, and its WW domains increases EGFR signaling. The figure shows representative experiments.

and that CBL E3 ligases are involved in this ubiquitination process (40–42). We demonstrate here that a HECT-containing E3 ligase can also be phosphorylated on tyrosine following EGFR stimulation. We have shown that CBLC and EGFR can form a complex; however, we were unable to detect an interaction between AIP4 and EGFR (data not shown). The HECT E3 ligase may contribute to the ubiquitination of either activated EGFR itself and/or protein(s) involved in the endocytosis process. Our results using the SRE-luciferase reporter assay show that AIP4 and CBLC can cooperate to negatively regulate EGFR signaling. Analysis of the knock-out phenotype of *itchy* (*Aip4*^{-/-}) mice has shown that AIP4/ITCH is somehow involved in the inflammatory response (43). Other defects of the *itchy* mouse, such as hyperproliferation of stomach epithelium, could indeed be the result of increased EGFR signaling.

Dominant Negative Effect of WW Motifs upon EGFR Ubiquitination—A transfected construct containing the four WW domains of AIP4 inhibits the ubiquitination of EGFR. This effect may occur via binding and blocking of proteins like CBLs, normally involved in the ubiquitination process. This inhibition of EGFR ubiquitination could also reflect a direct dominant negative effect of the WW domains on endogenous HECT E3 ubiquitin ligase activity, revealing the importance of this type of E3 ligases in EGFR down-regulation.

Role of AIP4/CBL Interaction—What function(s) may the CBLC/AIP4 interaction fulfill? Three scenarios deserve to be further evaluated.

First, it is possible that CBLC and AIP4 cooperate in the catalytic covalent transfer of ubiquitin onto EGFR or other substrates. Although RING finger E3 and HECT E3 have, until now, been considered to function independently, the precise mechanism underlying ubiquitination of a substrate is still a matter of debate. The coordinated action of two members of distinct E3 classes may provide more efficiency to the ubiquitination process.

Second, it is tempting to speculate that E3 ligases of different classes may contribute to the final polyubiquitination of a substrate by acting sequentially. In this respect, it is noticeable that mono and polyubiquitination do not lead to the same substrate fate. For instance, monoubiquitination has been associated with an endocytosis signal for EGFR (44), whereas polyubiquitination allows targeting to the 26 S proteasomal degradation pathway. Different E3 ligases may therefore act at definite windows during the endocytosis route of EGFR. Such specific role of each E3 ligase would explain the apparent redundancy of the system. Indeed, genetic analysis of different transgenic SLI-1 mutant in *C. elegans* demonstrated that, even in the presence of a SLI-1 RING-defective mutant, some down-regulation of LET-23 signaling is still observed. This indicates the possible involvement of another E3 ligase in the down-regulation of LET-23 (45).

Third, the binding between CBLC and AIP4 may be involved in the down-regulation of one of the ubiquitin E3 ligase itself. Ettenberg *et al.* (42) have suggested that CBL proteins are also ubiquitinated and degraded in a proteasomal dependent pathway following endocytosis (42), but whether CBL could ubiquitinate itself or whether another E3 ligase like AIP4 is required for this process still remains to be determined.

Cooperation between RING and HECT E3 Ligases May Occur in Various Pathways—Several recent reports connecting CBLs and a HECT E3 ligase reinforce the concept that two E3 ligases of different structure may interact in numerous signaling pathways. Lafont *et al.* (46) have observed a co-compartmentalization of CBLA and NEDD4 in rafts following immunoglobulin engagement. Others have shown that E3 ligases of the RING and HECT families may also share a common substrate and could therefore cooperate in its ubiquitination (47). It is the case for NOTCH that can be ubiquitinated by AIP4 and CBLA (38, 48). AIP4, recruited to Src-like kinases through different viral proteins, could interact with CBLA, a known substrate and binding partner of these tyrosine kinases (36, 37, 49). Taken together, these data suggest that CBLs and WWP E3 ligases could interact and/or exert a cooperative effect on ubiquitination on common substrates involved in numerous pathways.

In summary, we have shown that CBLC and AIP4 can interact and that these two E3 ligases could contribute to down-regulate EGFR signaling by ubiquitination. In humans, so far, only three CBLs have been described, contrasting with many more E3 ligases of the NEDD4 type. Because CBLC and AIP4 can interact, it would be interesting to test possible interactions between other

members of these two families of E3s opening many ways to modulate signalization pathways through ubiquitination. Further studies focusing notably on endogenous proteins will help delineate the importance of the interaction between E3 ligases of the RING and the HECT classes.

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**Interaction between Two Ubiquitin-Protein Isopeptide Ligases of Different Classes,
CBLC and AIP4/ITCH**

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