Dab2 links CIN85 with clathrin-mediated receptor internalization

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Abstract CIN85 is a multidomain scaffold protein involved in downregulation of receptor tyrosine kinases. Here we show that disabled-2 (Dab2), an endocytic adaptor molecule implicated in clathrin-coat assembly, associates with CIN85 in mammalian cells. All three SH3 domains of CIN85 were able to bind to the PKPAPR peptide in the carboxyl-terminal part of Dab2, possibly enabling CIN85 to simultaneously interact with multiple Dab2 molecules. CIN85 association with Dab2 is essential for its recruitment to clathrin coat and appears to be modulated by growth factor stimulation. Dab2 and clathrin dissociated from CIN85 following growth factor treatment, enabling other molecules, such as Cbl, to bind to CIN85. Taken together, our data indicate a dynamic interplay between CIN85 and its effectors during endocytosis of receptor tyrosine kinases.

Key words: AP-2; CIN85; Clathrin; Dab2; Epidermal growth factor (receptor)

1. Introduction

Growth factor binding to receptor tyrosine kinases (RTKs) triggers signaling cascades that elicit diverse biological responses, such as cell proliferation, migration, adhesion, differentiation or apoptosis [1]. Activation of receptors also results in their internalization through clathrin-coated pits, which is considered as a main mechanism of receptor downregulation and cessation of signaling [2]. The Cbl family of ubiquitin ligases are believed to be among the most important regulators of RTK trafficking [3,4]. Cbl mediates multiple mono-ubiquitination of activated receptors at the cell membrane, targeting them for endocytosis and lysosomal degradation [5,6]. In addition, Cbl proteins, independently on their ubiquitin ligase activity, recruit an adaptor molecule CIN85, bound to endophilins, in the complex with activated RTKs [7–9]. This constitutes a critical step for receptor internalization and trafficking [8,9].

CIN85 (Cbl interacting protein of 85 kDa) is a multidomain adaptor molecule, functioning in the assembly of protein complexes that participate in numerous signaling networks [10]. It is also involved at distinct steps of clathrin-mediated receptor endocytosis, as well as being sorted together with receptors for lysosomal degradation [11]. Moreover, CIN85 Src homology 3 (SH3) domains have recently been shown to recognize a novel PxxxPR motif, present in Cbl as well as in numerous proteins implicated in endocytic trafficking [12] (and unpublished observations). Thus CIN85 has a potential to regulate the assembly of a large receptor-associated protein network upon growth factor stimulation.

Dab2/DOC-2 (disabled-2/differentially-expressed in ovarian carcinoma) is another multidomain protein, acting as an endocytic adaptor [13]. Dab2 binds to clathrin and is able to regulate its assembly in vitro, and moreover interacts with clathrin adaptor protein AP-2 and phosphoinositides [13]. Conditional deletion of Dab2 in mice leads to reduced clathrin-coated pits and impaired transport of transmembrane receptors in the proximal tubule of kidney [14].

In this report we demonstrate that Dab2 associates with the SH3 domains of CIN85 via a conserved PxxxPR motif in its carboxyl-terminus. Moreover, Dab2 links CIN85 with clathrin complexes in mammalian cells. Epidermal growth factor receptor (EGFR) activation results in increased Cbl/CIN85 association [8], which in turn leads to disruption of CIN85/Dab2 complexes. Thus CIN85 functions as an endocytic adaptor, not only by binding to RTK/Cbl complexes and numerous endocytic proteins, but also being a component of a complex protein network that accompanies clathrin-coated vesicles formation.

2. Materials and methods

2.1. Products, antibodies and expression vectors

EGF was purchased from Intergen; mouse anti-Dab2 (p96) and mouse anti-clathrin heavy chain antibodies were from Transduction Laboratories, mouse anti-HA were form Roche and mouse anti-FLAG M2 antibodies were from Sigma. Anti-Cbl (RING) and anti-CIN85 (CT) antibodies were used as previously described [8,9]. All constructs of Cbl and CIN85 were described previously [8,9,12]. Dab1 and Dab2 constructs were from Philip H. Howe.

2.2. Yeast two-hybrid system screening

The screening procedure was performed as previously described [8,9].

2.3. Site-directed mutagenesis

Dab2 R699A mutant was generated by PCR using Quick Change (Stratagene).

2.4. Cell culture and transfections

HEK293T, NIH SAA (NIH-EGFR) and NIH3T3 cells were used as described previously [8,9]. Cells were transfected with Lipofectamine reagent (Invitrogen) following manufacturer's instructions. Where indicated, 30 h after transfection cells were starved for additional 12 h

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Abbreviations: AP-2, adaptor protein 2; CIN85, Cbl interacting protein of 85kDa; Dab2, disabled-2; EGF(R), epidermal growth factor (receptor); GST, glutathione *S*-transferase; RTK, receptor tyrosine kinase; SH3 domain, Src homology 3 domain

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and stimulated with 100 ng ml^{-1} EGF for indicated times. Cells were lysed in ice-cold 1% Triton X-100 lysis buffer (pH 7.4, 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol) containing a cocktail of protease and phosphatase inhibitors. The lysates were cleared by centrifugation at 13 000 rpm for 20 min at 4°C.

2.5. Peptide synthesis and peptide binding assays

All peptides were synthesized and used as previously described [12].

2.6. Biochemical assays

Glutathione *S*-transferase (GST) binding assays, immunoprecipitation and immunoblotting were performed as reported before [9].

3. Results

3.1. Dab2 interacts with the three SH3 domains of CIN85

In order to unravel novel functions for CIN85 in formation of signaling networks, we screened human thymus cDNA library with the full size CIN85 as bait. Two of the positive clones contained the carboxyl-terminus of Dab2, encoding its proline-rich region. Subsequently, yeast clones expressing either three SH3 domains of CIN85 (SH3), a proline-rich region with the coiled-coil domain (PCc), full size CIN85 or an empty vector were transformed with the Dab2 library clone, and interactions were checked by the ability of clones to grow on selective agars and by filter lift assays. The SH3 domains and the full size molecule, but not the PCc domain of CIN85 or an empty bait vector, bound to Dab2 in yeast cells (Fig. 1A).

To confirm these results in mammalian cells, we performed pull-down assays with GST-fusion proteins encoding three SH3 domains (SH3) or the proline-rich region with the coiled-coil domain (PCc) of CIN85. To this aim we used lysates of HEK293T cells transfected with Dab2, or its neuron-specific relative Dab1 [15]. Three SH3 domains of CIN85 interacted strongly with both Dab2 and Dab1, whereas no interaction was detected with an empty GST or with the GST-fusion construct encoding the proline-rich coiled-coil part of CIN85 (Fig. 1B). In order to further characterize interactions between the SH3 domains of CIN85 and Dab2, we compared GST-fusion proteins containing individual (SH3-A, SH3-B, SH3-C), combination of two (SH3-AB and SH3-BC), or all three (SH3-ABC) SH3 domains of CIN85, for their ability to bind to Dab2 expressed in 293T cells. All single SH3 domains of CIN85 bound Dab2 to the similar extend, whereas SH3-AB, SH3-BC and a fusion protein containing all three SH3 domains (SH3-ABC), associated with Dab2 more potently. Thus multiple SH3 domains of CIN85 are required for the most efficient binding to Dab2 (Fig. 1C). These data point out that all SH3 domains of CIN85 are implicated in interacting with Dab2 molecules, which resembles a mechanism of CIN85-Cbl interaction [12].

3.2. Identification of CIN85 binding site in the carboxyl-terminus of Dab2

The carboxyl-terminal part of Dab2 was sufficient to mediate interaction with CIN85 in yeast. It contains several proline-rich sequences, and among them two PxxxPR motifs, which constitute potential recognition sites for the SH3 domains of CIN85 [12]. One of them (PKPAPR) shared high similarity with the CIN85 binding motifs in Cbl and Cbl-b (PKPFPR and PKPRPR), respectively [12]. Binding of CIN85 to the purified Dab2 peptide NKINEP**PKPAPR**QGVLLG was further assessed by in vitro peptide binding assays. In-

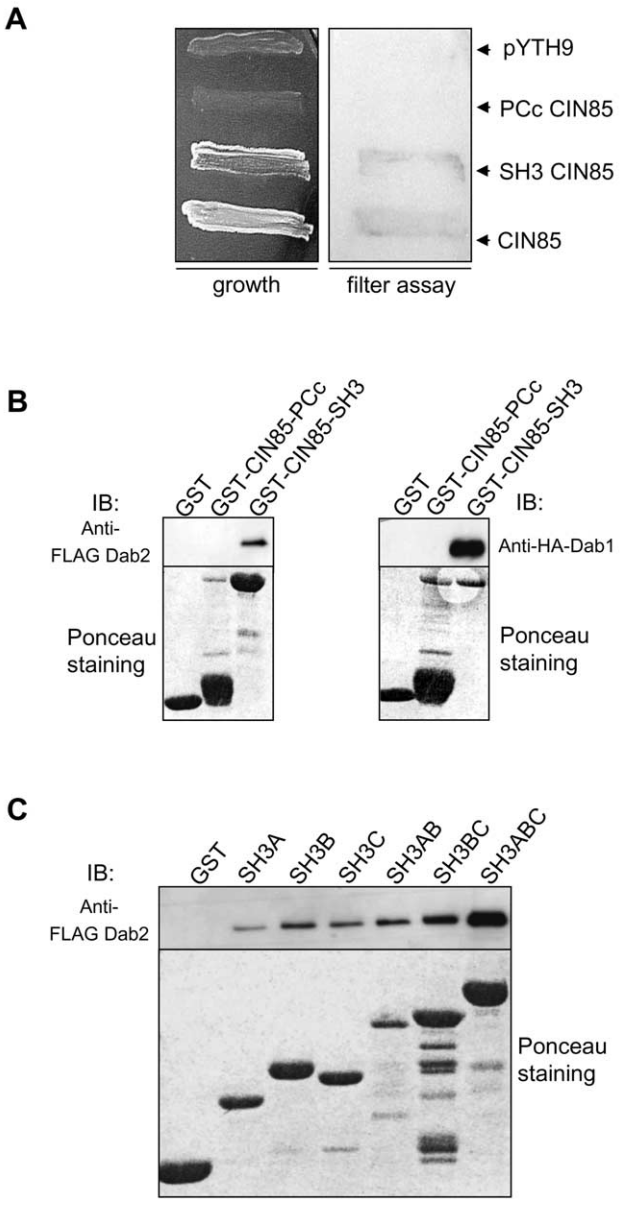

Fig. 1. Dab2 interacts with the SH3 domains of CIN85. A: Yeast clones expressing full size CIN85, 3SH3 or PCc (proline coiled-coil) domains were transformed with Dab2 cDNA and streaked on selective agar plates (-Leu-Trp-His+3-aminotriazole). Growth rate (left) and β-galactosidase activity in a filter lift assay (right) are shown. B: Equal amounts of lysates from HEK293T cells transiently transfected with Dab2 or Dab1 were incubated with GST alone or GST-fusion forms of CIN85 immobilized on glutathione Sepharose beads. Amounts of precipitated Dab1 and Dab2 are visualized in the upper panel (anti-FLAG Dab2 and anti-HA Dab1), while the lower panel shows the levels of used GST constructs. C: Equal amounts of lysates from HEK293T cells transiently transfected with Dab2 were incubated with GST alone or different GST-fusion proteins encoding all single, combinations of double or triple SH3 domains of CIN85 immobilized on glutathione Sepharose beads. Amounts of precipitated Dab2 are visualized in the upper panel (anti-FLAG Dab2), while the lower panel shows the levels of used GST constructs.

creasing amounts of CIN85 were precipitated in a dose-dependent manner with the Dab2 peptide, however the binding was much weaker when compared to the Cbl-derived peptide (Fig. 2A). The other potential CIN85-SH3 domains' recognition

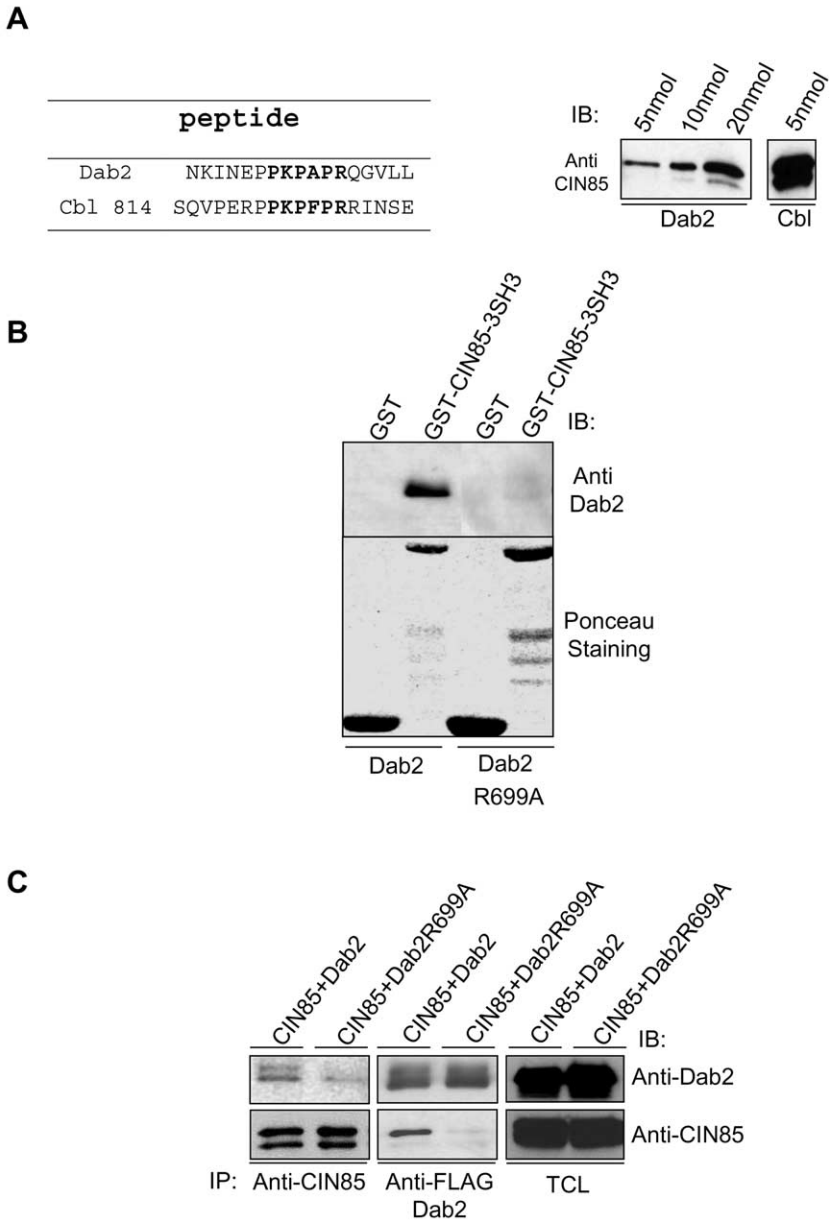

Fig. 2. The **PKPAPR** sequence in Dab2 is responsible for CIN85 binding. A: In vitro peptide binding assay. The left panel lists the peptides used: the Dab2 proline-rich peptide encompasses amino acids 707 to 724 from mouse Dab2 p96 splice form, the c-Cbl peptide (814) was previously described [12]. The amino acid sequences of both peptides are presented and the conserved PxxxPR motif is emphasized in bold. Lysates of 293T cells transfected with CIN85 were incubated with the indicated amounts of the peptides coupled to the SulfoLink gel (right panel). After recovery of the bound material, detection was performed by immunoblotting with anti-CIN85 antibodies. B: Equal amounts of lysates from HEK293T cells transiently transfected with the wild-type Dab2 or Dab2 R699A mutant were incubated with GST alone or GST-3SH3 domains of CIN85 immobilized on glutathione Sepharose beads. The amounts of precipitated Dab2 are visualized in the upper panel (anti-Dab2), the lower panel shows the levels of used GST constructs (Ponceau staining). C: 293T cells were transiently transfected with CIN85 and the wild-type Dab2 or the R699A mutant. Lysates were immunoprecipitated with the indicated antibodies, and after recovery of the bound material, detection was performed by immunoblotting with anti-Dab2 or anti-CIN85 antibodies.

site present in Dab2, PQPPPR, did not bind to CIN85 in the same assays (data not shown). Subsequently, we mutated arginine in the PKPAPR sequence and have shown that the R699A mutation (corresponding to human Dab2 sequence) completely abrogated the interaction of Dab2 with the GST-SH3 domains of CIN85 (Fig. 2B).

To further confirm that the PKPAPR sequence is critical for mediating interaction with CIN85, we disrupted this motif in full size Dab2 molecule, by mutating arginine 699 to alanine. Indeed, the co-precipitation between CIN85 and Dab2 was impaired when the R699A Dab2 mutant was used instead of the wild-type protein (Fig. 2C). Taken together, we have identified a conserved binding site for the SH3 domains of CIN85 in the C-terminal part of Dab2 and Dab1 (PxPAPR), which is highly homologous to the CIN85 interacting motif in the C-terminus of Cbl and Cbl-b [12].

3.3. *Interactions with Dab2 link CIN85 to clathrin-coated vesicles*

Dab2 has been shown to potently associate with clathrin,

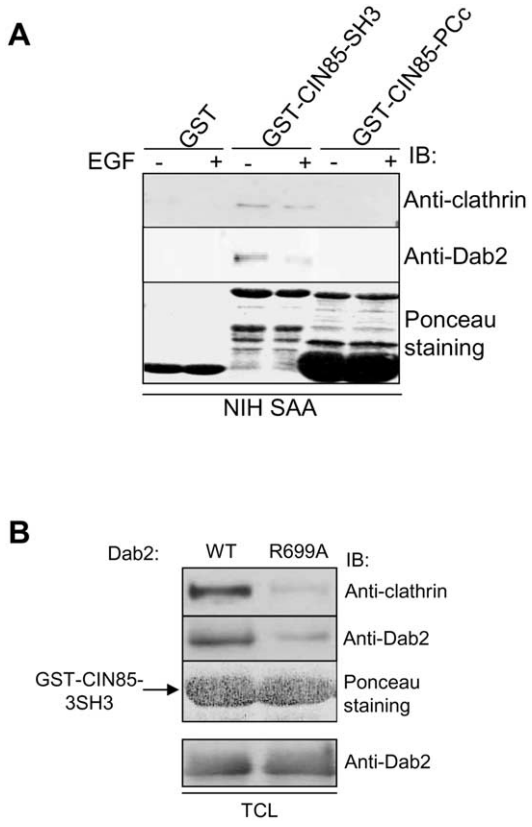

Fig. 3. CIN85 is coupled to clathrin-coated vesicles via interaction with Dab2. A: NIH SAA cells were left unstimulated (−) or stimulated with 100 ng/ml of EGF for 5 min. Cell lysates were subjected to GST-pull down with either an empty GST or GST-3SH3 and GST-PCc constructs of CIN85, and after recovery of the bound material, detection was performed by immunoblotting with anti-clathrin heavy chain and anti-Dab2 antibodies. The lower panel shows the levels of used GST constructs (Ponceau staining). B: Cell lysates of 293T cells transfected with the wild-type Dab2 or the R699A mutant were subjected to GST-pull downs with CIN85 GST-3SH3, and analyzed as in Fig. 3A.

both directly and indirectly via its interactions with AP-2 [13,16]. Therefore we tested whether Dab2 constitutes a link between clathrin and CIN85 in mammalian cells. Indeed, CIN85, Dab2 and clathrin heavy chain were detected in one protein complex. GST-fused 3SH3 domains of CIN85 were associating with endogenous clathrin in NIH SAA cells, whereas no binding was detected for CIN85 GST-PCc or an empty GST (Fig. 3A). Interestingly, there was a decrease in the amount of Dab2 as well as of clathrin heavy chain co-precipitated with GST-CIN85 3SH3 domains upon growth factor stimulation (Fig. 3A). Thus, CIN85 can be found in a complex with clathrin heavy chain, and this is at least in part due to its direct binding to Dab2. In accordance with our hypothesis, transfection of the Dab2 R699A mutant in 293T cells led to a significant decrease in the amount of endogenous clathrin heavy chain bound to the GST-fused 3SH3 domains of CIN85, as compared to the cells transfected with the wild-type Dab2 (Fig. 3B).

We have also observed that CIN85 contains three motifs in its C-terminal part, resembling the so-called 'clathrin box', found in numerous clathrin binding proteins [17]. However, we were not able to detect any direct interaction between CIN85 and clathrin heavy chain (data not shown).

3.4. Dab2 dissociates from CIN85 following EGFR stimulation and is replaced by Cbl

We have previously shown that CIN85 associates with Cbl and EGFRs following ligand stimulation, and remains in this complex during receptor trafficking to the lysosome [8,11]. Since CIN85-SH3 domain-mediated interactions can be modulated by growth factor stimulation [8], and additionally Src and Grb2 interact with the Dab2 proline-rich region in a ligand-dependent manner [18,19], we tested whether binding of CIN85 to Dab2 is affected by EGFR activation. To this aim, HEK293T cells co-expressing FLAG-tagged Dab2 and CIN85 were stimulated with EGF for indicated time periods, then lysed and subjected to immunoprecipitation with anti-FLAG or anti-CIN85 antibodies. Binding between CIN85 and Dab2 was observed in unstimulated cells, was significantly decreased after 2 min of EGF stimulation, and completely undetectable after 20 min of stimulation (Fig. 4A). Importantly, we were able to detect complex formation between endogenous Dab2 and CIN85 in NIH SAA cells (Fig. 4B). Analogously to the overexpression experiments, there was a significant decrease in the amount of Dab2 bound to CIN85 following cells' stimulation with EGF (Fig. 4B, right panel).

Since we were not able to detect tyrosine phosphorylation of Dab2 following EGF stimulation (data not shown), its ligand-dependent dissociation from CIN85 might be mediated by other mechanisms, such as Dab2/CIN85 serine–threonine phosphorylation, conformational changes within these molecules, and additionally a competition with other CIN85 bind-

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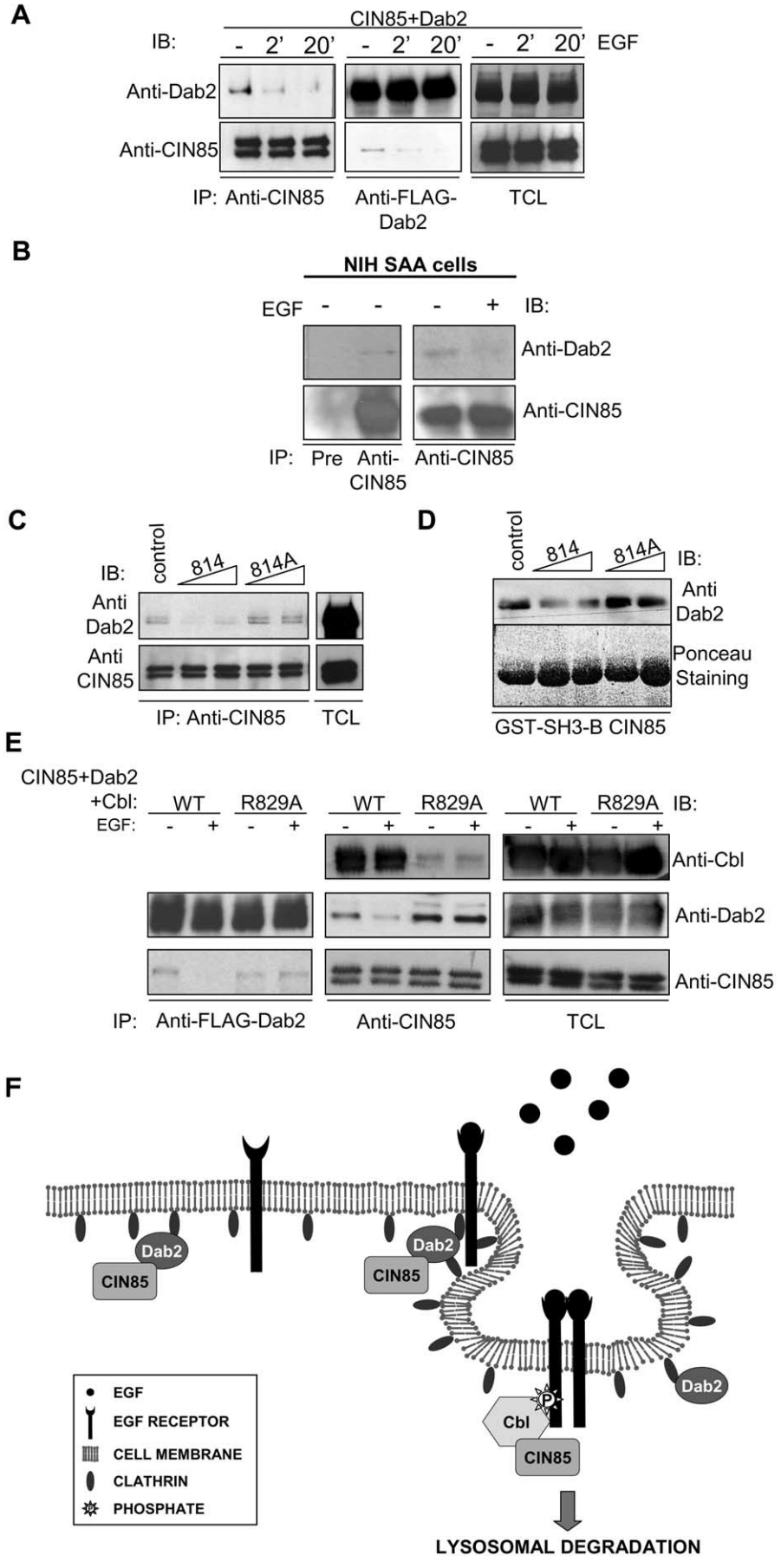
Fig. 4. Dab2 dissociates from CIN85 following ligand stimulation and is replaced by Cbl. A: HEK 293T cells transiently transfected with CIN85 and Dab2 were left unstimulated (−) or stimulated with 100 ng/ml of EGF for the indicated time periods. Cell lysates were immunoprecipitated with anti-CIN85 or anti-FLAG antibodies, and after recovery of the bound material, detection was performed by immunoblotting with the indicated antibodies. B: NIH SAA cells were left unstimulated (−) or stimulated with EGF for 10 min. Cell lysates were immunoprecipitated (IP) either with pre-immune serum (Pre) or anti-CIN85 antibodies, and detection was performed by immunoblotting (IB) with anti-Dab2 or anti-CIN85 antibodies. C,D: Peptide competition assay. Lysates of 293T cells transfected with CIN85 and Dab2 (C), or with Dab2 alone (D) were incubated with increasing amounts (100 and 200 nmol) of indicated peptides for 1 h. Subsequently, the lysates were immunoprecipitated with anti-CIN85 antibodies (C), or subjected to GST-pull down with the GST-CIN85-SH3-B construct (D), and immunoblotted with anti-CIN85 or anti-FLAG antibodies. E: HEK 293T cells were transiently transfected with CIN85, Dab2 and Cbl: wild-type (WT) or R829A mutant, impaired in its ability to interact with CIN85. Cells were left unstimulated (−) or stimulated with 100 ng/ml of EGF for 10 min (+). Cell lysates were immunoprecipitated with anti-CIN85 or anti-FLAG antibodies, and after recovery of the bound material, detection was performed by immunoblotting with indicated antibodies. F: Schematic model of Dab2/CIN85 interaction during EGFR internalization. In the absence of EGF, cell surface-localized Dab2 binds to CIN85 and clathrin. Following early phases of cell stimulation, Dab2 positions CIN85 in the vicinity of forming clathrin-coated pits where activated EGFR undergoes internalization. Activated receptors also bind to and phosphorylate Cbl, which can in turn recruit CIN85 to receptor complexes, and thus cause dissociation of Dab2-CIN85 interactions. CIN85 bound to EGFR/Cbl complexes may regulate subsequent steps in receptor endocytosis and their sorting for degradation in the lysosome.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EGF RECEPTOR
CELL MEMBRANE
CLATHRIN
PHOSPHATE
LYSOSOMAL DEGRADATION

ing proteins. To verify the last hypothesis, we checked whether Cbl-derived high affinity peptide 814 [12] is able to compete with Dab2 for binding to CIN85. Increasing amounts of peptide 814, but not of arginine mutant R829A peptide, efficiently competed for the Dab2/CIN85 interaction. The same effect was observed when the peptide was added to the lysates of cells transfected with both Dab2 and CIN85, or when it was incubated together with Dab2-transfected cell lysates and CIN85 GST-SH3B construct (Fig. 4C,D). Importantly, Dab2/CIN85 complex dissociated following EGF stimulation in the presence of ectopic Cbl, whereas no such effect was observed when Cbl R829A, impaired in its ability to bind to CIN85, was used instead (Fig. 4E). Moreover, overexpression of Cbl R829A prevented competition between endogenous Cbl and Dab2 and resulted in constitutive CIN85/Dab2 association (Fig. 4E). The binding between CIN85 and Dab2 was already impaired in the presence of overexpressed wild-type Cbl in unstimulated cells, as also in these conditions binding between CIN85 and Cbl was very strong, probably due to high tyrosine phosphorylation of overexpressed Cbl (Fig. 4E).

Taken together, our data suggest that Dab2 transiently links CIN85 with clathrin complexes during receptor internalization. Recruitment of Cbl to activated receptors, as well as potential receptor-mediated protein modifications such as phosphorylation, might lead to increased binding between CIN85 and Cbl, and disruption of a Dab2-CIN85 complex following growth factor stimulation (Fig. 4F).

4. Discussion

Clathrin-mediated endocytosis is involved in lipid and protein trafficking in eukaryotic cells from the plasma membrane, trans-Golgi network and endosomes [20]. Clathrin oligomerization is the most important force for vesicle formation, but the process requires additional interactions with multiple accessory proteins, which coordinate coat assembly and disassembly, cargo recruitment, or provide link to the cytoskeleton [21,22]. The best-characterized component of clathrin-coated vesicles is a heterotetrameric adaptor complex AP-2, which binds simultaneously to clathrin, to the internalization motifs on the cargo, and recruits numerous accessory proteins to the forming vesicles [22]. Many of these proteins exhibit themselves properties of endocytic adaptors, due to their abilities to bind both clathrin and different cargo proteins. For example, Dab2 binds to clathrin, AP-2, and additionally to the internalization motifs on the LDL receptor [13,16]. Moreover, Dab2 provides a potential link between receptor endocytosis and cytoskeleton by binding to myosin VI, a motor protein implicated in the movement of clathrin-coated vesicles along actin filaments into the cell interior [23].

Here we demonstrate that CIN85, previously shown to be critical for RTK endocytosis [8], can indirectly associate with clathrin, mainly due to its SH3 domains binding to the proline–arginine motif in Dab2 (Fig. 3A,B). Dab2 contains three proline-rich sequences in the C-terminal part. The first two motifs were found to be responsible for the interaction with Src and Grb2, respectively, while no binding partner for the third motif; PKPAPR, has been identified so far [19,24]. Here we show that this sequence, also preserved in the brain-specific Dab2 homolog: Dab1, serves as a recognition region for the SH3 domains of CIN85. All three SH3 domains of CIN85 bind to Dab2 to the similar extend, and when the double or all three CIN85-SH3 domains are included within one construct, association with Dab2 is increased (Fig. 1C). These observations suggest that CIN85 could cluster Dab2 molecules on clathrin-coated pits, which might result in the formation of high affinity CIN85-Dab2-clathrin agglomerates.

Importantly, a conserved PxxxPR motif is also present in Cbl/Cbl-b and other CIN85 effectors [12]. This implies that while some of the CIN85-SH3 domains' effectors might be components of the same protein complexes, others could compete for association with CIN85. One way to regulate the dynamic exchange of these effectors could be by growth factor-dependent protein modifications, such as phosphorylation or protein conformational changes. Dab2 dissociates from CIN85 upon growth factor stimulation (Fig. 4A,B), while CIN85-Cbl binding is dramatically increased under the same conditions [8]. Accordingly, Cbl can compete with Dab2 for binding to the SH3 domains of CIN85 (Fig. 4C–E). Therefore we propose that following receptor activation, Dab2 in complex with CIN85 can be replaced by the high affinity CIN85-SH3 domains' ligands, such as Cbl (Fig. 4F). Subsequently, the CIN85-Cbl-EGFR complex is internalized and sorted for lysosomal degradation [11], while Dab2 may remain at the plasma membrane due to its PTB domain-mediated phosphoinositide interactions [13], (Fig. 4F). In accordance with our model, Dab2 has been shown to associate with clathrin transiently, being absent from the early endosomal compartments [16]. However, the exact mechanism behind growth factor-dependent Dab2-CIN85 interaction still remains obscure, and constitutes an interesting field for future investigations.

Dab2 is known to act as a tumor suppressor, able to inhibit growth of many cancer types [25,26]. One of its growth-inhibitory effects is mediated by the ability of Dab2 to downregulate EGF-induced signaling pathways, competing for binding between SOS and Grb2, or inhibiting Src activity and thus suppressing MAP kinase activation [18,19]. It remains to be elucidated whether Dab2 exerts its growth-inhibitory functions additionally by influencing RTK endocytosis.

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