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13. ABSTRACT ***(Maximum 200 Words)***

Signals to multiply, migrate and outgrow blood vessels are mediated by growth factors of the EGF/neuregulin family. Concentrating on the membrane receptors for EGF and neuregulins, namely tyrosine kinases of the ErbB/HER family, our first task is to resolve mechanisms that normally restrain ErbB receptors. These efforts have led to the identification of a regulatory loop that allows collaboration between ErbB receptors and c-Src, a major oncoprotein of breast cancers. According to our findings, c-Src phopshorylates c-Cbl, a major restrainer of ErbB signaling, and leads to its proteasomal destruction. Consequently, tumor cells overexpressing c-Src or active Src mutants are unable to down-regulate ErbB proteins. Our Task 2 relates to a putative particle that modulates recycling of ErbBs. We report on the function of two components, Hgs and STAM, and their regulation by an ubiquitin ligase called Need4. Collectively, these studies shed light on ligand-independent endocytosis of ErbB proteins, and its dependence on lipids. In an effort to translate our findings to clinical protocols, our Task 3 involves in vitro studies and assays in animals testing combinations of monoclonal antibodies to ErbBs, inhibitors of their intrinsic tyrosine kinase activity, and blockers of c-Src.

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INTRODUCTION

The objective of our study is to provide molecular understanding of the mechanisms underlying negative regulation of ligand- activated growth factor receptors in cancer cells. This understanding will likely be translated into effective strategies to inhibit tumors whose growth and metastasis depend on growth factors. Our work concentrates on one growth factor receptor, EGFR (also called ErbB-1 and HER1), and its family members. Specifically, we focus on a major regulator of EGFR signaling, namely the c-Cbl ubiquitin ligase. Task 1 of the original Statement of Work deals with the identification of phosphotyrosine-binding proteins that regulates c-Cbl activity. In the last year we reported on Grb2 as an effective collaborator of c-Cbl. The studies we performed over the last 12 months concentrated on c-Src, an oncogenic tyrosine kinase, which is often co-expressed with ErbB proteins in mammary gland tumors. Because our studies indicate that c-Src eliminates c-Cbl and consequently elevates ErbB expression, we predict that c-Src blockers will synergize with antibodies and other antagonists of ErbBs. In the last year we reported on the initial characterization of an endocytic multi-protin complex that regulates ErbB trafficking. The present report describes in details how this complex is regulated by an ubiquitin ligase (Nedd4), an interacting partner (STAM) and lipid binding. Last, on the basis of the results we accumulated, our current efforts are concentrated on in vitro and in vivo tests of tumor growth inhibition by combinations of drugs specific to ErbBs, Src and downstream pathways.

BODY

Specific Aims **(as stated in the original grant application)**

(i) To isolate and molecularly clone the hypothetical phosphotyrosine-binding sorting protein by using its identified binding site on Cbl. The functional relationships to ErbB proteins and the recycling machinery will be studied in details.

(ii) To characterize a putative multi-protein complex, the recyclosome, and its individual components, including PI3K, Cbl, and Src, as well as 14-3-3 and small GTP-binding proteins. The functional relationships within the recyclosome and their relative order of action will be worked out.

(iii) To evaluate the therapeutic potential of inhibitors of specific components of the recyclosome. The ability of tumor-inhibitory anti-ErbB-2 antibodies to interfere with recycling will be examined in an attempt to improve existing immunotherapy or identify efficient combinations of antibodies and chemotherapeutic drugs.

Task 1: *To isolate and molecularly clone a hypothetical phosphotyrosine- binding protein responsible for degradation of ErbB proteins (see appended manuscript by Bao and Yarden).*

Cellular Src (c-Src) and epidermal growth factor receptor (EGFR) collaborate in the progression of certain human malignancies. To address the underlying mechanism, c-Src proteins were ectopically expressed together with EGFR in fibroblasts devoid of ubiquitous Src family proteins. We report that EGFR accumulates in cells overexpressing either wild-type c-Src or its transforming mutant. Up-regulation of EGFR requires its kinase function, as well as an auto-phosphorylation site known to bind c-Cbl, a major negative regulator of active receptors. Apparently, c-Src phosphorylates c-Cbl, and consequently accelerates its proteasomal destruction. Although ubiquitylation of c-Cbl is enhanced by c-Src, the intrinsic ubiquitin ligase function of c-Cbl may not be necessary for Src-induced degradation. Hence, by promoting destruction of c-Cbl, c-Src up-regulates growth factor signals, which may explain its synergy with EGFR in oncogenesis.

Task 2: *To characterize a putative multi-protein complex, the recyclosome, and its individual components (see appended manuscript by Katz et al.).*

Our Task 2 aims at resolving the mechanisms underlying the action of a putative multi-protein complex that controls endocytosis of growth factor receptors. Ligand-dependent endocytosis of the epidermal growth factor receptor (EGFR) involves recruitment of an ubiquitin ligase, and sorting of ubiquitylated receptors to lysosomal degradation. By studying Hgs, a mammalian homologue of a yeast vacuolar-sorting adaptor, we provide information on the less understood, ligand-independent pathway of receptor endocytosis. Constitutive endocytosis involves receptor ubiquitylation and translocation to Hgs-containing endosomes. Whereas the lipid binding motif of Hgs is necessary for receptor endocytosis, the ubiquitin-interacting motif (UIM) negatively regulates receptor degradation. We demonstrate that the UIM is endowed with two functions: it binds ubiquitylated proteins and it targets self-ubiquitylation by recruiting Nedd4, an ubiquitin ligase previously implicated in endocytosis. Based upon the dual function of the UIM and its wide occurrence in endocytic adaptors, we propose a UIM network that relays ubiquitylated membrane receptors to lysosomal degradation through successive budding events.

Task 3: *To evaluate the therapeutic potential of inhibitors of specific components of the recyclosome.*

Detailed understanding of receptor desensitization mechanisms will likely identify potential targets for drug intervention. Our working hypothesis assumes that antibody-induced endocytosis contributes to tumor inhibition by immunotherapeutic drugs. Likewise, ligand-induced endocytosis of ErbB proteins underlies cytotoxicity of drug-neuregulin conjugates. Hence, efforts to enhance receptor endocytosis or inhibit recycling will likely improve drug efficacy. Along this line, we began testing the following combinations of drugs on breast cancer cells growing both in vitro, as well as in the form of tumor xenografts.

A pan-ErbB tyrosine kinase inhibitor (CI-1033, from Pfizer) and Src-kinase inhibitors (PP2).

Specific monoclonal antibodies to ErbB proteins (generated in our laboratory) and kinase antagonists.

Blockers of molecular chaperones (e.g., geldanamycin and radicicol) in combination with antibodies or tyrosine kinase inhibitors.

KEY RESEARCH ACCOMPLISHMENTS

♦ Identification of a molecular mechanism underlying oncogene collaboration in breast cancer cells. This regulatory loop involves c-Src and c-Cbl, adaptors that antagonistically regulate ErbB trafficking.

♦ Uncovering the mode of action of the endocytic adaptor Hgs, including regulation by an ubiquitin ligase (Nedd4) and a network of adaptors containing an ubiquitin interacting motif (UIM; e.g., STAM).

♦ Initial characterization of the function of a lipid-binding domain (the FYVE) in receptor endocytosis. Our results indicate that PI3P, a product of inositol lipid kinases, is essential for effective endocytosis of ErbB proteins.

♦ Definition of potentially synergistic pairs of drugs, including Src and ErbB inhibitors (low molecular weight kinase blockers and monoclonal antibodies).

REPORTABLE OUTCOMES:

Manuscript: *Src promotes destruction of c-Cbl: implications to oncogenic synergy between Src and EGF-receptors.*
Manuscript submitted for publication. See appended material.

Manuscript: *Ligand-independent endocytosis involves receptor ubiquitination and Hgs, an adaptor whose UIM domain autonomously mediates self-ubiquitination.*
Manuscript submitted for publication. See appended material.

CONCLUSIONS

The results reported fulfill in part Tasks 1 through 3 of the original Statement of Work. An enormously complex machinery emerges from our studies: Multiple proteins seem to collectively regulate desensitization of growth factor receptors. Not only their protein ligands but also their lipid interactors and post-translational modifications (primarily phosphorylation and ubiquitination) are involved, along with vactorial translocation through successive budding events and vesicular compartments. Two major routes seem to channel membrane receptors to degradation in the lysosome. The ligand-dependent mechanism involves recruitment of a ubiquitin ligase, called c-Cbl, and a set of adaptors like Grb2 and c-Src. While Grb2 allows robust coupling of c-Cbl to active receptors, the action of c-Src leads to effective destruction of c-Cbl and consequent inhibition of receptor degradation. In the last year of research we will attempt to learn more on the mechanisms that underlie ligand-indiced and c-Cbl-mediated pathways. This includes involvement of molecules like PI3K, PKC and GTP-binders.

The alternative route of receptor endocytosis is slower and occurs even in the absence of growth factors. Our lead molecule to this pathway is Hgs/Hrs, and while this pathway is much less understood, the results we obtained over the last year of research implicate an ubiquitin ligase and several adaptors with UIM, coliled coil and EH domains. In the last year of research supported by this program we will extend our analysis of UIM-containing adaptors, resolve the mode of recruitment of Nedd4 and study in details the role of tyrosine phosphorylation and lysine ubiquitination.

Finally, in the next 12 months we will test the emerging models by using a pharmacological approach. Antagonists of specific molecules and enzymatic processes will be assayed and endocytic readouts will be followed. For example, our studies suggest that Src inhibitors will augment the action of anti-ErbB antibodies, while PI3K inhibitors may inhibit endocytosis, and therefore they may restrict the action of endocytosis-based drugs (e.g., toxin conjugates). To test these predictions we will utilize tumor cells and assays of in vitro growth. In addition, cancer cells will be grown in animals treated with specific drug combinations to reveal potential combinations of drugs with added effectiveness.

APPENDICES:
Manuscript by J. Bao and Y. Yarden (submitted for publication)
Manuscript by M. Katz et al. (submitted for publication)

Src promotes destruction of c-Cbl: implications to oncogenic synergy between Src and EGF-receptors

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Running title: Src-induced destruction of c-Cbl

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Abstract

Cellular Src (c-Src) and epidermal growth factor receptor (EGFR) collaborate in the progression of certain human malignancies. To address the underlying mechanism, c-Src proteins were ectopically expressed together with EGFR in fibroblasts devoid of ubiquitous Src family proteins. We report that EGFR accumulates in cells overexpressing either wild-type c-Src or its transforming mutant. Up-regulation of EGFR requires its kinase function, as well as an auto-phosphorylation site known to bind c-Cbl, a major negative regulator of active receptors. Apparently, c-Src phosphorylates c-Cbl, and consequently accelerates its proteasomal destruction. Although ubiquitylation of c-Cbl is enhanced by c-Src, the intrinsic ubiquitin ligase function of c-Cbl may not be necessary for Src-induced degradation. Hence, by promoting destruction of c-Cbl, c-Src up-regulates growth factor signals, which may explain its synergy with EGFR in oncogenesis.

Introduction

The essential role of polypeptide growth factors in inductive cell fate determination and in tissue remodeling is reflected in neoplastic processes. One example is the ErbB family of receptor tyrosine kinases and their ligands, all containing an epidermal growth factor (EGF) motif [reviewed in (Yarden and Sliwkowski, 2001)]. Many human tumors, including glioblastomas and carcinomas display increased expression of ErbB-1 (also called EGFR). Surprisingly, the mitogenic ability and transforming potential of EGFR are relatively weak, but heterodimerization enhances its oncogenic power (Yarden and Sliwkowski, 2001). Several lines of evidence unveiled another important oncogenic partner of EGFR, namely the cytoplasmic tyrosine kinase c-Src [reviewed in (Abram and Courtneidge, 2000; Biscardi et al., 1999)]. Src activity is modulated by two phosphorylation events that modify tyrosine 418 within the kinase domain and tyrosine 529 in the C-terminal tail. Elevated levels of Src family kinases, mutational activation, or increased intrinsic kinase activity were observed in colon carcinoma (Irby et al., 1999) and similar findings were reported for lung, breast and brain tumors [reviewed in (Biscardi et al., 1999)]. Consistent with functional interactions with EGFR, overexpression of wild-type c-Src in murine fibroblasts potentiated EGF-induced mitogenesis, whereas overexpression of dominant-negative variants of c-Src not only ablated the enhanced mitogenic response, but also interfered with the normal response to EGF (Tice et al., 1999; Wilson et al., 1989). Likewise, inactivation of Src family kinases inhibited platelet-derived growth factor- (PDGF-) stimulated DNA synthesis (Erpel et al., 1996; Twamley-Stein et al., 1993).
Subsequent studies reported that co-overexpression of c-Src and EGFR leads to synergistic increases in cell growth in vitro, and tumor formation in nude mice (Maa et al., 1995). The mechanism underlying this form of oncoprotein collaboration remains incompletely understood. Similar to the case of PDGF, treatment with EGF leads to two- to three-fold increases in Src activity (Weernink and Rijksen, 1995). On the other hand, c-Src phosphorylates EGFR at sites that further stimulate its catalytic activity (Maa et al., 1995). By contrast, another set of reports attributed to c-Src restraining effects on EGFR. Thus, overexpression of c-Src in fibroblasts accelerated internalization of EGFR (Ware et al., 1997). Apparently, subsequent to c-Src activation by EGFR, clathrin is phosphorylated, and redistributed to the cell periphery, where it enhances EGFR internalization (Wilde et al., 1999). Because internalization of EGFR terminates signaling and reduces EGF-induced mitogenicity (Vieira et al., 1996; Waterman et al., 2002; Wells et al., 1990), c-Src may restrict, rather than increase signaling downstream to EGFR.

To address the mechanism of Src-EGFR collaboration we employed Src-deficient cells, and noted that EGFR is accumulated in cells expressing an active form of c-Src. Analysis of a series of EGFR mutants revealed that a receptor form incapable of interacting with c-Cbl, a regulator of EGFR endocytosis [reviewed in (Thien and Langdon, 2001)], undergoes no up-regulation by c-Src. We therefore analyzed the interaction between c-Src and c-Cbl and unveiled a previously unknown destructive effect of c-Src. Thus, by destabilizing c-Cbl, c-Src limits the process of ligand-induced receptor desensitization, thereby prolonging growth factor signaling in tumors co-expressing EGFR and an active form of Src kinases.

Results

c-Src up-regulates EGFR levels, whereas a kinase-defective c-Src mutant decreases EGFR accumulation

To study the possibility that c-Src collaborates with EGFR by influencing the multi-step process of receptor desensitization [reviewed in (Waterman and Yarden, 2001)], we co-expressed in cultured cells the human EGFR together with one of the following forms of human c-Src: wild-type c-Src, an active mutant whose inhibitory tyrosine phosphorylation site at position 529 has been replaced by a phenylalanine (Y529F), and a mutant whose kinase activity has been inactivated (K279R). Chinese hamster ovary (CHO) cells were chosen as a cellular system because these cells express no endogenous EGFR. Forty-eight hours post-transfection, cells were stimulated with EGF for 10 minutes, and EGFR levels were analyzed by electrophoresis and immunoblotting (Fig. 1A, top panel). The results showed that EGFR levels are significantly increased in cells expressing either wild-type c-Src or its active form. In contrast, cells expressing a kinase-defective dominant-negative mutant of c-Src displayed reduced expression relative to cells transfected with an empty vector. Control analyses performed with antibodies to c-Src confirmed ectopic expression of the three c-Src proteins. The relative levels of EGFR in Fig. 1A are presented in Fig. 1B. Evidently, despite Src-induced up-regulation of EGFR, EGF was still able to slightly reduce expression of its receptor, suggesting that receptor down-regulation was not severely impaired by c-Src.
To analyze the interaction between c-Src and EGFR in a null cellular background we used mouse embryo fibroblasts, denoted SYF cells (Klinghoffer et al., 1999), lacking the three ubiquitous Src-family kinases: Src, Yes and Fyn. Expression of an active c-Src protein in SYF cells resulted in significant up-regulation of co-expressed EGFR molecules, and conservation of ligand-induced receptor down-regulation (Fig. 1C), confirming the suitability

of the SYF experimental system. We next analyzed the effect of c-Src by using pulse-chase metabolic labeling of SYF cells (Fig. 1D). Evidently, an active form of c-Src exerted no significant effect on receptor synthesis or maturation. In this seemingly sensitized assay system, receptor molecules almost completely disappeared following stimulation with EGF, but c-Src fully prevented receptor down-regulation while allowing band up-shift due to hyperphosphorylation. Conceivably, c-Src stabilizes the mature, cell surface-expressed receptor. This tenet was supported by the results of a receptor down-regulation assay (Fig. 1E). Although expression of Y529F-Src increased EGFR expression by 60% (as determined by ^{125}I-EGF binding), we detected no effect on the initial rate of receptor removal from the surface of SYF cells (first 10 minutes). This observation is consistent with a previous report showing that the effect of c-Src is limited to the first one or two minutes of EGFR internalization (Wilde et al., 1999). At later time intervals, however, c-Src reduced the extent of receptor down-regulation, probably by enhancing the rate of recycling to the cell surface (Levkowitz et al., 1998). Taken together, the results presented in Figure 1 led us to conjecture that one of the many substrates of c-Src is involved in EGFR endocytosis.

An EGFR mutant incapable of c-Cbl recruitment is refractory to Src-induced up-regulation

The mechanism underlying Src-induced stabilization of EGFR was addressed by utilizing a series of receptor molecules mutated in the cytoplasmic domain. Unlike wild-type EGFR, expression levels of a kinase-defective mutant of EGFR were only minimally up-regulated upon co-expression of an active c-Src (Fig. 2A), suggesting that the catalytic activity of EGFR is essential for the effect of c-Src. This possibility was supported by comparing two catalytically intact forms of EGFR; unlike the dramatic up-regulation of the wild type receptor (Fig. 2B), a mutant receptor lacking the whole carboxyl terminal domain [ΔCT; (Kornilova et al., 1996)] underwent no up-regulation. Notably, we reproducibly observed relatively high expression levels and a longer half-life for the carboxyl-terminally mutated receptor (data not shown), probably because it lacks a lysosome targeting domain (Kornilova et al., 1996). Since major tyrosine auto-phosphorylation sites are localized to the carboxyl terminus of EGFR, we inferred that stabilization by c-Src requires modification at one or more tyrosine auto-phosphorylation sites. In experiments that are not presented we analyzed receptor molecules harboring mutations at individual tyrosine auto-phosphorylation sites. However, only one mutant of the six analyzed, namely a receptor whose tyrosine residue 1045 has been replaced by a phenylalanine (Y1045F), was refractory to c-Src (Figs. 2C). This mutant, whose binding to c-Cbl is defective (Levkowitz et al., 1999), and half-life relatively long (Waterman et al., 2002), displayed limited up-regulation when co-expressed together with an active c-Src. Moreover, Y1045F underwent no up-regulation by a kinase-defective c-Src (Fig. 2D). Because c-Cbl negatively regulates EGFR signaling by enhancing receptor degradation [reviewed in (Thien and Langdon, 2001)], we assumed that the ability of c-Src to regulate EGFR expression involves an effect on basal, as well as ligand-induced Cbl-EGFR interactions.

c-Cbl-induced ubiquitylation and degradation of EGFR are diminished by c-Src

As a negative regulator and an E3 ubiquitin protein ligase, c-Cbl enhances ubiquitylation, endocytosis and degradation of several receptor tyrosine kinases (Joazeiro et al., 1999; Levkowitz et al., 1999; Waterman et al., 1999; Yokouchi et al., 1999). To study the role of c-Src in these c-Cbl-mediated processes, we analyzed receptor ubiquitylation in SYF cells transiently expressing a hemagglutinin peptide-tagged ubiquitin (HA-ubiquitin). Over-expression of either wild-type c-Src, or an active mutant (Y529F) significantly diminished ligand-induced receptor ubiquitylation (Fig. 3A). In addition, as we previously reported for CHO cells (Levkowitz et al., 1998), ectopic expression of c-Cbl reproducibly enhanced removal of EGFR from the surface of SYF cells (Fig. 3B). When singly expressed in SYF cells, Y529F-Src partially inhibited receptor down-regulation (data not shown, and Fig. 1E). However, this active form of c-Src almost completely abolished the effect of c-Cbl on receptor down-regulation (Fig. 3B), in line with the inhibitory effect of c-Src on receptor ubiquitylation (Fig. 3A).

To ubiquitylate EGFR, c-Cbl must physically associate with the receptor and subsequently undergo phosphorylation, presumably on tyrosine 371 (Levkowitz et al., 1999; Yokouchi et al., 2001). Examination of EGF-induced phosphorylation of c-Cbl on tyrosine residues confirmed rapid and sustained modification of the adaptor protein (Fig. 3C). Co-expression of Y529F-Src significantly elevated both basal and EGF-induced phosphorylation of c-Cbl. However, the level of c-Cbl expression was dramatically reduced in cells transfected with an active c-Src (Figs. 3C). As expected, we observed EGF-induced physical associations between EGFR and c-Cbl, but this interaction was abolished upon expression of an active c-Src (Fig. 3D). Once again, we noted that Y527F-Src diminished c-Cbl expression, which may explain why less c-Cbl was detectable in EGFR immunoprecipitates. Since no parallel change in tubulin expression was detectable (Figs. 3C and 3D), we inferred specificity to c-Cbl. These observations implied that c-Src up-regulates EGFR by inhibiting formation of a Cbl-EGFR complex and reducing c-Cbl expression, a possibility we addressed by subsequent experiments.

c-Src promotes degradation of c-Cbl

To address the relevance of the observed association between c-Src and decreased expression of an ectopic human c-Cbl in SYF cells (Figs. 3C and 3D), we examined the effect of an active c-Src on the endogenous hamster c-Cbl of CHO cells (Fig. 4A). As expected, expression of Y529F-Src led to partial disappearance of c-Cbl. More extensive elimination of c-Cbl expression was observed in SYF cells transfected with increasing amounts of the Y529F-Src expression vector (Figs. 4B and 4C). Lastly, we confirmed that Src-induced degradation of c-Cbl was independent on the tagging method of c-Cbl; as an alternative tag of we utilized a recombinant protein comprised of c-Cbl fused to the green fluorescence protein (GFP-Cbl). Robust elimination of GFP-Cbl expression was observed and it displayed no dependency on the state of stimulation of this receptor (Fig. 4D).

The ability of an active c-Src to reduce c-Cbl expression was confirmed by using metabolic labeling. A pulse of radioactive labeling of SYF cells over-expressing c-Cbl was followed by a four hour-long chase. In the absence of an active c-Src we observed slow disappearance of c-Cbl, but Y529F-Src significantly enhanced c-Cbl s disappearance (Fig. 5A, and data not shown). The intrinsic kinase activity of c-Src was essential for enhanced c-Cbl disappearance, as indicated by experiments comparing the effect of Y529F-Src with that of a kinase-defective c-Src (K279R; Fig. 5B), and a protein mutated at tyrosine 418 (Y418F-Src; Fig. 5C), whose phosphorylation is important for full kinase activation. It is interesting to note that both c-Cbl and c-Src are localized to cytoplasmic vesicular structures (Kaplan et al., 1992; Levkowitz et al., 1998), but their co-localization has not been reported. As expected, ectopic Y529F-Src molecules localized to vesicular structures in SYF cells (Fig. 5D). In the absence of Y529F-Src, c-Cbl displayed reticular cytoplasmic distribution, but in cells co-expressing c-Cbl and the active c-Src mutant the two proteins co-localized to vesicular structures (note the merge panel i in Figure 5D). This translocation is reminiscent of an EGF-induced recruitment of c-Cbl to early endosomes containing another active tyrosine kinase, namely EGFR (de Melker et al., 2001; Levkowitz et al., 1998), suggesting that c-Src similarly recruits c-Cbl into vesicular structures.

c-Cbl destruction by an active c-Src is independent of c-Cbl s ubiquitin ligase function, but it requires tyrosine phosphorylation at the carboxyl terminus of c-Cbl

To study the structural requirements of c-Src-induced destabilization of c-Cbl, we examined a series of mutant c-Cbl proteins (see Fig. 6A). In G306E-Cbl, the glycine residue at position 306 of the PTB domain is substituted by a glutamate. This residue is essential for phosphotyrosine binding, and its mutagenesis in v-Cbl ablates oncogenesis (Thien and Langdon, 1997). Unlike v-Cbl, which was refractory to c-Src (Fig. 6B), the PTB-defective mutant retained susceptibility to the destructive action of c-Src (Figs. 6C). 70Z-Cbl is another oncogenic mutant that negatively modulates c-Cbl, because it no longer binds E2 ubiquitin-conjugating enzymes (Yokouchi et al., 1999). Upon testing in SYF cells we learned that 70Z-Cbl is down-regulated by c-Src (Figs. 6C, and 6D), indicating that E2 binding to c-Cbl is unnecessary for c-Src-mediated decreases in c-Cbl levels. This conclusion was further supported by testing two additional mutants of c-Cbl (Figs. 6C and 6D): C381A-Cbl, an ubiquitylation-defective mutant whose cysteine bridging within the RING finger is tangled (Waterman et al., 1999), and Y371F-Cbl, whose linker s tyrosine involved in receptor desensitization (Levkowitz et al., 1999) and transformation (Thien et al., 2001) was mutated. Another domain of c-Cbl possibly associated with protein ubiquityaltion is the carboxyl-terminally located UBA domain, which is involved in dimerization and regulation of receptor ubiquitylation (Bartkiewicz et al., 1999). However, a UBA domain deletion mutant (ΔUBA) displayed sensitivity to an overexpressed c-Src (Fig. 6E), indicating independence on the UBA domain.

We next examined the possibility that phosphorylation at the carboxyl terminal half of c-Cbl confers sensitivity towards an active c-Src. Phosphorylation by Src family kinases affects three major sites of c-Cbl, and the corresponding 3YF mutant (tyrosines 700, 731 and 774 substituted for phenylalanines) displays not only reduced phosphorylation, but also reduced binding to c-Src (Feshchenko et al., 1998). Unlike other mutants of c-Cbl, expression of 3YF-Cbl was not affected by an active c-Src (Fig. 6F). Likewise, a mutant whose five tyrosine phosphorylation sites were mutated [5YF; (Feshchenko et al., 1998)] also exhibited insensitivity to c-Src (data not shown). Taken together, these observations imply that phosphorylation at the carboxyl terminus, and/or stable binding to c-Src, is essential for accelerated turnover of c-Cbl. In contrast, neither phosphotyrosine binding to c-Cbl, nor ubiquitin ligase activity or dimerization of c-Cbl, are necessary for enhanced turnover mediated by an active c-Src.

c-Src increases self-ubiquitylation of c-Cbl, but subsequent proteasomal destruction may be independently regulated

To gain insights into the mechanism of c-Cbl destabilization by an active c-Src, we utilized a weak base that inhibits degradation in lysosomes (chloroquine), and an effective blocker of proteasomal proteases (MG132). Sensitization

to the pharmacological inhibitors was attained by employing a zinc-inducible Src-Y529F expression plasmid (pMT-CB6+). Some ectopic expression of Src-Y529F was detectable even in the absence of zinc. Nevertheless, following two hours of incubation with $ZnCl_2$ we observed increased expression (Fig. 7A, upper panel). Utilizing these experimental parameters, we observed c-Cbl disappearance even under conditions known to completely block lysosomal degradation (Fig. 7A, middle panel). However, no destabilization of c-Cbl occurred when proteasomal degradation was blocked by MG132 (Fig. 7A, lower panel), suggesting that c-Src directs c-Cbl to proteasomal degradation. Interestingly, MG132 enhanced expression of c-Src (Fig. 7A), in agreement with a previous report on ubiquitin- and proteasome-dependent degradation of active c-Src (Hakak and Martin, 1999).

Because tetra-ubiquitin is the minimum signal for proteasomal destruction of ubiquitylated proteins (Thrower et al., 2000), we predicted that c-Src-induced degradation of c-Cbl is preceded by poly-ubiquitylation. In line with a previous report (Ettenberg et al., 2001), we found that EGF enhances poly-ubiquitylation of not only EGFR but also c-Cbl (Fig. 7B). Both poly-ubiquitylation processes were undetectable when testing the 70Z-Cbl oncoprotein, indicating that the RING finger of c-Cbl is responsible for both self- and trans-ubiquitylation. In analogy, expression of an active c-Src elevated both mono- and poly-ubiquitylation of c-Cbl (Fig. 7B). Further, ubiquitylation of 70Z-Cbl was not affected by c-Src, implying that this kinase, like EGFR, activates the intrinsic ubiquitylating function of c-Cbl, consistent with the results of an in vitro study (Yokouchi et al., 2001). In contrast with the stimulatory effect on c-Cbl s ubiquitylation, c-Src reduced EGFR ubiquitylation (Fig. 7B, see also Fig. 3A), reminiscent of the observed Src-induced defect in receptor down-regulation (Fig. 3B). Lastly, because 70Z-Cbl is susceptible to the destructive action of c-Src (Fig. 6D), but no Src-induced ubiquitylation of 70Z-Cbl was detectable (Fig. 7B), we predict that Src mediates transient or weak ubiquitylation of c-Cbl by recruiting an E3 ligase distinct from c-Cbl. This hypothesis and its relevance to oncogene cooperation in human cancer are discussed below.

Discussion

Our results may be summarized as follows: c-Src up-regulates EGFR (Fig. 1), but a receptor incapable of c-Cbl binding is refractory to the effects of Src (Figs. 2C and 2D). Concurrent with Src-induced inhibition of receptor down-regulation (Fig. 1E), ubiquitylation of EGFR is reduced (Figs. 3A and 7B), an effect attributable to the disappearance of the c-Cbl protein (Figs. 4 and 5). One interpretation of these observations is presented in Figure 7C: Normally, activation of EGFR is followed by self-phosphorylation and recruitment of c-Cbl, primarily through tyrosine 1045 (Waterman et al., 2002). By enhancing ubiquitylation, c-Cbl sorts receptor molecules for internalization, and later on it allows entry into the multi-vesicular body (Longva et al., 2002). This robust desensitization process is inhibited when c-Src is activated: Src binds to and phosphorylates c-Cbl (Feshchenko et al., 1998; Tanaka et al., 1996; Tanaka et al., 1995). In addition, it enhances Cbl s self-ubiquitylation [Fig. 7B and (Yokouchi et al., 2001)], and leads to accelerated destruction by proteasomal proteinases (Fig. 7A). Therefore, the efficacy of receptor down-regulation is reduced, whereas the rate of receptor recycling may be increased when engagement of c-Cbl is reduced (Waterman et al., 2002). Consequently, signaling by multiple mitogens, including EGF, whose receptors are regulated by c-Cbl is enhanced and prolonged. Thus, the present study defines a novel regulatory loop enabling collaboration between two major oncogenic proteins, Src and EGFR. Since c-Cbl acts as a tumor suppressor (Levkowitz et al., 2000), and collaboration between Src and EGFR is widely implicated in human cancer [reviewed in (Biscardi et al., 1999)], the uncovered regulatory mechanism may bear oncological implications.

Mechanisms underlying collaboration between c-Src and EGFR in cancer cells

Src family members are commonly activated by growth factors like PDGF and EGF, and both pathways involve formation of a physical receptor-Src complex (Maa et al., 1995; Mori et al., 1993). Within the complex, Src phosphorylates the associated receptor at a site located in the kinase domain (Biscardi et al., 1998; Hansen et al., 1996), a modification known to enhance catalytic activity of growth factor receptors. In line with the possibility that these interactions enable synergy between c-Src and various receptors in human cancer (Brunton et al., 1997; Irby et al., 1999), as well as in model systems (Maa et al., 1995), inactivation of c-Src by micro-injected antibodies blocks DNA synthesis by PDGF (Twamley-Stein et al., 1993), and mutational inactivation of the c-Src s phosphorylation site on EGFR ablates EGF-induced mitogenicity (Tice et al., 1999). Hence, the observed Src-mediated stabilization of signaling-competent receptors at the cell surface is expected to enhance interactions between c-Src and its target receptors. As a result, Src family kinases may confer to tumor cells enhanced responsiveness to autocrine or stroma-derived growth factors. If supported by additional studies, the inferred selective advantage can explain why several types of advanced tumors, including breast and colon carcinomas, exhibit simultaneous activation of both EGFR and Src family members.

Given the role of c-Cbl in enhancing receptor internalization and reducing the extent of recycling from early endosomes [reviewed in (Thien and Langdon, 2001; Waterman and Yarden, 2001)], an inhibitory effect of c-

Src on receptor endocytosis is predictable. Contrary to this prediction, studies performed with 10T1/2 cells found that over-expression of c-Src causes no change in the half-life of EGFR, but it does elicit an increase in the internalization rate constant when the endocytic apparatus is not stoichiometrically saturated (Ware et al., 1997). Interestingly, this effect disappeared at high receptor occupancy, which is closer to the conditions we used in the present work. Another study concluded that c-Src activation by EGFR is needed for subsequent phosphorylation of clathrin, which redistributes to the cell periphery and enhances receptor internalization (Wilde et al., 1999). Notably, these authors reported that the effect of Src is strictly limited to the first two minutes of ligand internalization, a time window we have not addressed in the present study. In addition, it is worth noting that several distinct routes mediate internalization of EGFR (Waterman and Yarden, 2001), and therefore measurements of ligand internalization rates reflect more than one pathway. Of note, Src kinases are involved in both activation of dynamin s GTPase activity (Herskovits et al., 1993), and caveolin phosphorylation (Li et al., 1996), two effects that may regulate non-clathrin coated pit endocytosis. We are currently examining various kinetic parameters to determine which aspects of EGFR endocytosis are affected in SYF cells expressing an active form of c-Src.

An interplay between c-Src and c-Cbl

A series of recent reports unveiled complex interactions between c-Src and c-Cbl [reviewed in (Sanjay et al., 2001a)]. Two lines of evidence indicate that the interactions involve physical contacts: c-Cbl and c-Src are co-localized to vesicular structures [(Tanaka et al., 1996) and Fig. 5D], and at least three domains of c-Cbl can directly bind to c-Src, including PTB domain binding to phosphorylated tyrosine 418 of c-Src (Sanjay et al., 2001b). This interaction underlies short-term catalytic inactivation of c-Src, but long-term inactivation involves degradation of active Src family kinases by c-Cbl (Andoniou et al., 2000). In analogy, our results portray a reciprocal pattern in which c-Src negatively regulates c-Cbl by enhancing its degradation in proteasomes (Figs. 6 and 7A). Nevertheless, the exact mechanism by which c-Src sorts c-Cbl for degradation is still unclear. Evidently, c-Src increases ubiquitylation of c-Cbl both in vitro (Yokouchi et al., 2001) and in living cells (Fig. 7B). Moreover, Cbl s ubiquitylation is likely mediated by its own RING finger, and it may require prior phosphorylation of a proximal tyrosine residue (tyrosine 371) by either c-Src or EGFR (Levkowitz et al., 1999; Yokouchi et al., 2001). However, unlike Src-induced ubiquitylation of c-Cbl, which requires an intact RING finger and a tyrosine at position 371, c-Cbl mutants defective at tyrosine 371 or the RING domain retain sensitivity to active Src proteins (Fig. 6). It is therefore conceivable that c-Src sorts c-Cbl to proteasomal destruction by mobilizing a mechanism distinct from the ubiquitin ligase function of c-Cbl. Notably, EGFR and the receptor for the macrophage growth factor elevate self-ubiquitylation of Cbl-b and c-Cbl, and subsequently sort the respective E3 ligase to either degradation or recycling (Ettenberg et al., 2001; Wang et al., 1999). In conclusion, c-Cbl maintains a complex network of interactions with its substrates, but detailed elucidation of the molecular intricacy, as well as the roles of distinct ubiquitylation events, necessitates additional studies.

Src-transformed cells exhibit a long list of unique phenotypic characteristics, which may reflect the multiple phosphorylation targets of Src family kinases. These targets are mostly involved in the regulation of cell cycle entry, actin cytoskeleton and adhesive properties (Abram and Courtneidge, 2000). Likewise, the pleiotropic cellular responses to growth factors like EGF and PDGF resemble many of the Src-mediated cellular characteristics. Our present study explains this similarity by the ability of c-Src, and presumably other members of its family, to block a major pathway leading to signaling desensitization, namely receptor down-regulation. Evidently, Src executes this function by enhancing destruction of c-Cbl, an evolutionary conserved regulator of growth factor receptors. Interestingly, c-Src accelerates destruction of another negative regulator of signaling, namely protein kinase C delta (Blake et al., 1999), raising the possibility that blocking negative regulatory pathways may be a common feature of Src family kinases. The exact mechanism by which Src kinases regulate stability of c-Cbl and other negative regulators is a matter for future investigation.

Materials and methods

Materials, buffers and antibodies

Radioactive materials were purchased from Amersham (Buckinghamshire, UK). Monoclonal antibodies (mAbs) to EGFR were generated in our laboratory. Other antibodies were from Santa-Cruz Biotechnologies (Santa Cruz, CA). Materials were purchased from Sigma (St Louis, MO), except for MG132 (Biomol; Plymouth Meeting, PA). Binding buffer contained RPMI-1640 medium supplemented with 0.5% bovine serum albumin and 20 mM HEPES. Solubilization buffer contained 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 10 μg/ml pepstatin A, 10 μg/ml aprotinin and 10 μg/ml leupeptin.

HNTG buffer contained 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol. EGF was labeled with ^{125}I by using the Iodogen reagent (Pierce, Rockford).

Plasmid construction, transfection and preparation of cell extracts

EGFR mutants, HA-tagged Cbl mutants, and a GFP-Cbl constructs were described previously (Levkowitz et al., 1998; Waterman et al., 1999). HA-Ub expression vector was obtained from Dirk Bohmann (EMBL, Heidelberg, Germany). A Flag peptide-tagged ubiquitin expression vector was constructed by using a pEF1α promoter-based expression vector and specific primers. A zinc-inducible Src-Y529F expression plasmid (pMT-CB6+) was obtained from Tona Gilmer (Glaxo Wellcome, Research Triangle Park, North Carolina). To generate the UBA domain deletion mutant, a stop codon was introduced upstream to the UBA-encoding segment using Stratagene's (La Jolla, California) Quick-change mutagenesis kit. Src expression vectors and the control pUSE vector were purchased from Upstate Biotechnology (Lake Placid, NY). Cells were transfected by using the Lipofectamine 2000 method. Unless otherwise indicated, we used the following amounts of plasmid DNA for transfection of cells in 90-mm plates: EGFR, and HA-tagged ubiquitin: 1 μg/plate; c-Src and c-Cbl: 2 μg/plate. The total amount of plasmid DNA in each transfection, including control plates, was normalized with a respective empty plasmid. Forty-eight hours post-transfection, cells were exposed to the indicated treatments.

Immunoprecipitation and immunoblotting analyses

Essentially we used previously described protocols (Levkowitz et al., 1998). Immunoprecipitates were resolved by gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in phosphate-buffered saline containing 0.5% Tween-20 and 1% milk, blotted for 2 h with a primary antibody (1 μg/ml), followed by a secondary antibody (0.5 μg/ml) linked to horseradish peroxidase. Immunoreactive protein bands were detected with an enhanced chemiluminescence reagent (Pharmacia-Amersham, Piscataway, NJ). Where indicated, membranes were stripped and re-probed with additional antibodies.

Receptor down-regulation assay

Cells were treated without or with EGF for various time intervals at 37¡C. The medium was then removed, and the cells washed once with binding buffer and twice with an acidic buffer (135 mM NaCl, 2.5 mM KCl and 50 mM acetic acid) at room temperature to remove surface-bound ligand. The cells were then washed twice in ice-cold binding buffer and incubated with ^{125}I-EGF (0.5 nM) for 4 h at 4¡C. The cells were then washed twice, solubilized, and radioactivity determined by using a γ-counter.

Immunofluorescence

Cells were fixed for 30 minutes with 3% paraformaldheyde, and permeabilized for 10 minutes at 22¡C in saline containing 1% albumin and 0.2% Triton X-100. Cover slips were then incubated for 1 hour at room temperature with an antibody to HA, c-Src, or with a mixture of both antibodies (each at 10 g/ml). Antibody detection was performed by using fluorescently labeled secondary antibodies (from Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, cover slips were mounted in Elvanol (Hoechst, Frankfurt) and examined by using a Zeiss Axiovert confocal microscope (Oberkochen, Germany).

Pulse-chase analysis of protein turnover

Cells were pulse-labeled at 37¡C by incubation with ^{35}S-methionine (250 Ci/ml) in methionine-free medium supplemented with 2% dialyzed fetal bovine serum. Thereafter, cells were washed, cultured in chase medium containing unlabeled methionine and lysed for immunoprecipitation.

Legends to figures

Fig. 1. Src up-regulates EGFR expression levels and decelerates receptor down-regulation

(A) CHO cells were transfected with a plasmid encoding EGFR, along with a control plasmid, or vectors encoding the indicated Src proteins. Forty-eight hours post-transfection, monolayers were untreated or treated with EGF (100 ng/ml) for 10 minutes. Thereafter, cell extracts were analyzed by immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies.

(B) Relative expression levels of EGFR (panel A) were quantified by scanning densitometry and the values expressed as fold induction relative to control.

(C) SYF cells were transfected with a plasmid encoding EGFR together with a plasmid encoding Y529F-Src or an empty plasmid. Forty-eight hours post-transfection cells were untreated or treated with EGF (100 ng/ml) for 20 minutes, and cell extracts analyzed.

(D) SYF cells transfected with plasmids encoding EGFR and Y529F-Src, or a control vector, were subjected to a 4 hour-long metabolic labeling with ^{35}S-labeled amino acids. Thereafter, cells were incubated for the indicated time intervals with medium containing unlabeled amino acids in the absence or presence of EGF (100 ng/ml). The autoradiogram shows results of EGFR immunoprecipitation (IP).

(E) CHO cells were transfected with an EGFR expression vector together with a plasmid encoding wild type c-Src or an empty vector. Forty-eight hours later, triplicate cultures were incubated at 37°C with an unlabeled EGF (100 ng/ml) for the indicated time intervals. Thereafter, cell-bound ligand was removed, and the level of surface receptors was determined by binding of a radioactive EGF at 4°C. Averages and the corresponding standard deviations (bars) are shown.

Fig. 2. Structural requirements for Src-induced accumulation of EGFR

(A) SYF cells were transfected with plasmids encoding EGFR, either wild type (WT) or a kinase-defective mutant (Kin$^-$), together with an empty plasmid or a plasmid encoding Y529F-Src. Forty-eight hours post-transfection, cell lysates were prepared and processed for analyses.

(B) SYF cells were transfected with increasing amounts of an expression vector encoding Y529F-Src, together with plasmids encoding the indicated forms of EGFR. Forty-eight hours post-transfection, cell extracts were prepared and processed for analyses.

(C) SYF cells were transfected and analyzed as in B by using plasmids encoding EGFR, either wild type (WT) or Y1045F.

(D) CHO cells were transfected with a plasmid encoding a mutant form of EGFR (Y1045F) together with an empty vector, or vectors encoding wild type c-Src, Y529F-Src, or a kinase-defective mutant (K279R). Forty-eight hours post-transfection, cells were treated with EGF (100 ng/ml) for 15 minutes and cell extracts processed for analyses.

Fig. 3. Src inhibits c-Cbl-induced ubiquitylation, down-regulation and degradation of EGFR

(A) SYF cells were transfected with plasmids encoding c-Src, either the wild type form or Y529F-Src. For control we used the corresponding empty vector. All plates were co-transfected with vectors driving expression of EGFR, HA-tagged ubiquitin, and c-Cbl. Forty-eight hours post-transfection, monolayers were untreated or treated with EGF (100 ng/ml) for 15 minutes. Thereafter, cell extracts were analyzed as indicated.

(B) SYF cells transiently expressing EGFR were analyzed for ligand-induced down regulation of EGFR as in panel E of Fig. 1. Prior to analysis, cells were transfected with vectors driving expression of c-Cbl (squares), or a combination of c-Cbl and an active c-Src (Y529F; triangles). As control, we transfected cells with empty vectors (circles).

(C) SYF cells transiently expressing EGFR and HA-tagged c-Cbl were transfected with a plasmid encoding Y529F-Src, or the respective empty plasmid. Following 48 hours of incubation, cells were either left untreated or they were treated at 37°C with EGF (100 ng/ml) for the indicated time intervals. The state of c-Cbl phosphorylation was analyzed by immunoblotting with anti-phosphotyrosine (P-Tyr) antibodies.

(D) SYF cells were transfected and treated with EGF as in C. Cell lysates were tested for Cbl-EGFR association by using the indicated antibodies.

Fig. 4. c-Src down regulates c-Cbl expression

(A) CHO cells were transfected with a plasmid encoding EGFR, along with a vector driving expression of Y529F-Src, or a control empty vector. Forty-eight hours later, cells were stimulated with EGF (10 minutes; 100 ng/ml) and expression levels of c-Cbl and c-Src were determined by immunoblotting.

(B) SYF cells were transfected with plasmids encoding HA-tagged c-Cbl, together with increasing amounts of either a plasmid encoding Y529F-Src, or an empty expression vector. Whole cell extracts were analyzed by immunoblotting forty-eight hours post-transfection.
(C) Relative c-Cbl levels (panel B) were quantified, and the values were expressed relative to c-Cbl level in control cells.
(D) SYF cells were transfected with plasmids encoding EGFR and GFP-Cbl, together with a plasmid encoding Y529F-Src or an empty vector. Forty-eight hours post-transfection, cell monolayers were untreated or treated with EGF (100 ng/ml) for the indicated time intervals. Thereafter, cell extracts were immunoblotted with the indicated antibodies.

Fig. 5. The catalytic activity of c-Src is essential for down-regulation of c-Cbl
(A) SYF cells transiently co-expressing c-Cbl and increasing amounts of Y529F-Src were subjected to radioactive metabolic labeling (14 hours). For control, cells were transfected with an empty vector. Anti-HA antibodies were used to immunoprecipitate c-Cbl. Quantification of the resulting autoradiogram is also presented.
(B) SYF cells were co-transfected with a c-Cbl expression vector, along with increasing amounts of a control empty vector or plasmids encoding the indicated Src mutants. Whole cell lysates were probed 48 hours later with antibodies to c-Cbl or to tubulin. Quantification of the c-Cbl's signals is presented in the lower panel.
(C) SYF cells were transfected with a plasmid encoding HA-tagged c-Cbl along with plasmids encoding the indicated mutants of c-Src. The respective empty plasmid was used for control. Cell lysates were analyzed forty-eight hours post-transfection.
(D) SYF cells grown on glass slides were transfected with plasmids encoding Y529F-Src (a-c), HA-tagged c-Cbl (d-f), or their combination (g-i). Twenty-four hours post-transfection, cells were fixed, permeabilized and incubated with murine antibodies to c-Src and a rat antibody to HA. FITC-conjugated antibody specific to mouse immunoglobulins (green), and Cy3-conjugated antibodies to rat immunoglobulins (red) were used for fluorescent confocal microscopy.

Fig. 6. Structural requirements for c-Src-induced down regulation of c-Cbl
(A) A schematic diagram of c-Cbl featuring the phosphotyrosine-binding (PTB) domain, a RING finger (RF), a proline-rich domain (Pro-Rich), and the combined ubiquitin-associated domain and leucine zipper domain (UBA/LZ). Also delineated are the structures of oncogenic forms of c-Cbl: v-Cbl and 70Z-Cbl. The locations of critical amino acids mutated for functional analyses are marked.
(B-F) SYF cells were transfected with plasmids encoding the indicated HA-tagged forms of c-Cbl, together with a plasmid encoding Y529F-Src (2 μg/plate, unless otherwise indicated), or the respective empty plasmid. Forty-eight hours post-transfection, cell extracts were analyzed as indicated.

Fig. 7. c-Src elevates tyrosine phosphorylation, self-ubiquitylation, and proteasomal degradation of c-Cbl
(A) Upper panel: SYF cells were transfected with a plasmid encoding HA-tagged c-Cbl, along with a plasmid driving expression of Y529F-Src from a zinc-inducible promoter. For control we used an empty plasmid (lanes labeled -). Forty-eight hours post-transfection, cells were treated with $ZnCl_2$ (75 μM) for the indicated time intervals and cell extracts analyzed.
Middle panel: SYF cells were transfected with a plasmid encoding HA-tagged c-Cbl, along with a plasmid encoding a zinc-inducible Y529F-Src (2 μg/plate). Forty-eight hours later cells were treated for 4 hours with $ZnCl_2$ (75 μM) together with chloroquine (0.2 and 2 mM) or solvent (dimethylsulfoxide; *Cont*).
Lower panel: Cells were transfected with a plasmid encoding c-Cbl, along with a vector encoding a zinc-inducible Y529F-Src (0.5, 2 and 4 μg/plate), or an empty vector (lanes labeled -). Forty hours later cells were treated for 4 hours with $ZnCl_2$ (75 μM) in the absence or presence of MG132 (10 μM) and cell extracts analyzed by immunoblotting.
(B) SYF cells were co-transfected with plasmids encoding Y529F-Src or the respective empty vector, along with vectors encoding HA-tagged c-Cbl, or 70Z-Cbl, EGFR and Flag-tagged ubiquitin. Forty-eight hours post-transfection, cells were treated with either EGF (100 ng/ml; 10 minutes), or solvent. Ubiquitylated proteins were immunoprecipitated (IP) from cell extracts by using an anti-Flag antibody. Immunoblotting (IB) was carried out with an anti-Cbl antibody. Thereafter, the membrane was stripped and re-blotted with an anti-EGFR antibody. Whole cell extracts were analyzed as indicated.
(C) Schematic representation of the inferred interactions between c-Src and c-Cbl, and their effect on EGFR trafficking. EGF binding promotes endocytosis of the receptor through clathrin-coated areas of the plasma membrane. This process involves recruitment of c-Cbl, receptor ubiquitylation, and consequent receptor sorting to

lysosomal degradation. In the presence of an oncogenic mutant of Src, both phosphorylation and ubiquitylation of c-Cbl are enhanced, and the protein is subsequently degraded in the 26S proteasome. Due to Src-induced elimination of c-Cbl, sorting of EGFR to lysosomal degradation is reduced and the receptor is presumably diverted to the recycling pathway. This regulatory loop may explain the association between Src activation and EGFR over-expression in tumor cells.

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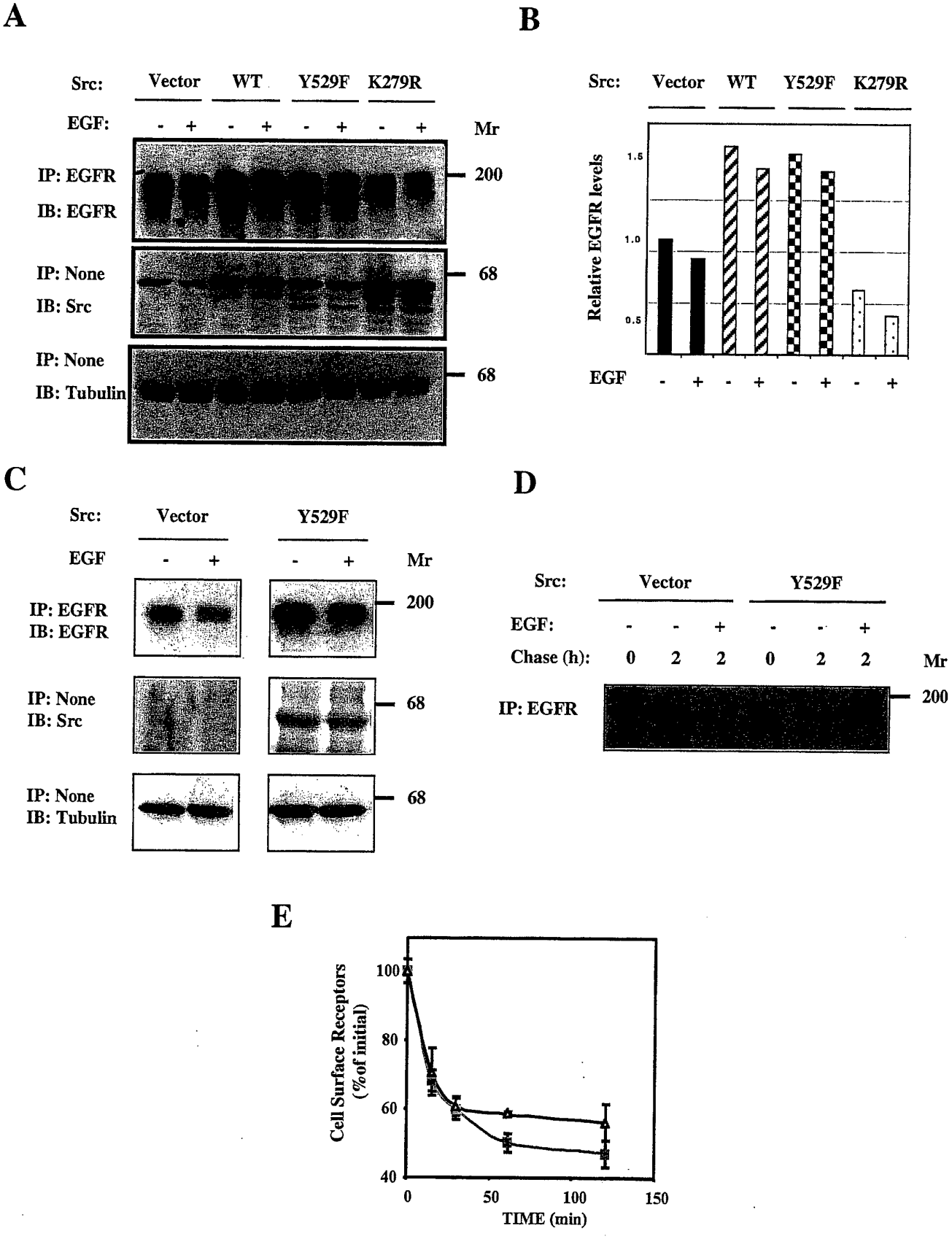
Figure 1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68
B
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Relative EGFR levels
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1.0
0.5
EGF - + - + - + - +
C
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EGF - + - + Mr
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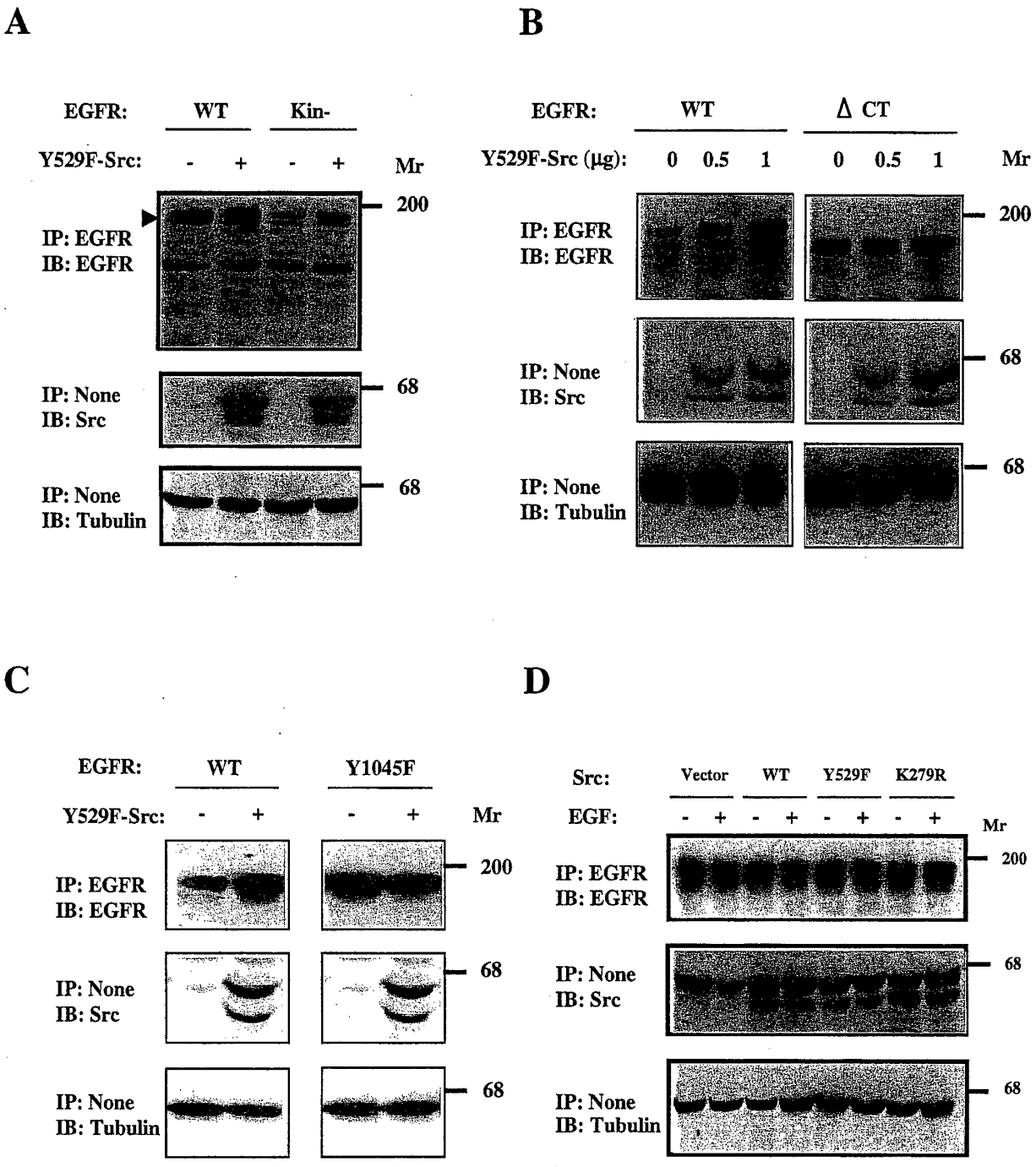
200
IP: None
IB: Src
68
IP: None
IB: Tubulin
68
D
Src: Vector Y529F
EGF: - - + - - +
Chase (h): 0 2 2 0 2 2 Mr
IP: EGFR
200
E
Cell Surface Receptors (%of initial)
100
80
60
40
0 50 100 150
TIME (min)

Figure 2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Mr
200
IP: EGFR
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IB: Src
68
IP: None
IB: Tubulin
B
EGFR: WT Δ CT
Y529F-Src (μg): 0 0.5 1 0 0.5 1
Mr
200
IP: EGFR
IB: EGFR
68
IP: None
IB: Src
68
IP: None
IB: Tubulin
C
EGFR: WT Y1045F
Y529F-Src: - + - +
Mr
200
IP: EGFR
IB: EGFR
68
IP: None
IB: Src
68
IP: None
IB: Tubulin
D
Src: Vector WT Y529F K279R
EGF: - + - + - + - +
Mr
200
IP: EGFR
IB: EGFR
68
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IB: Src
68
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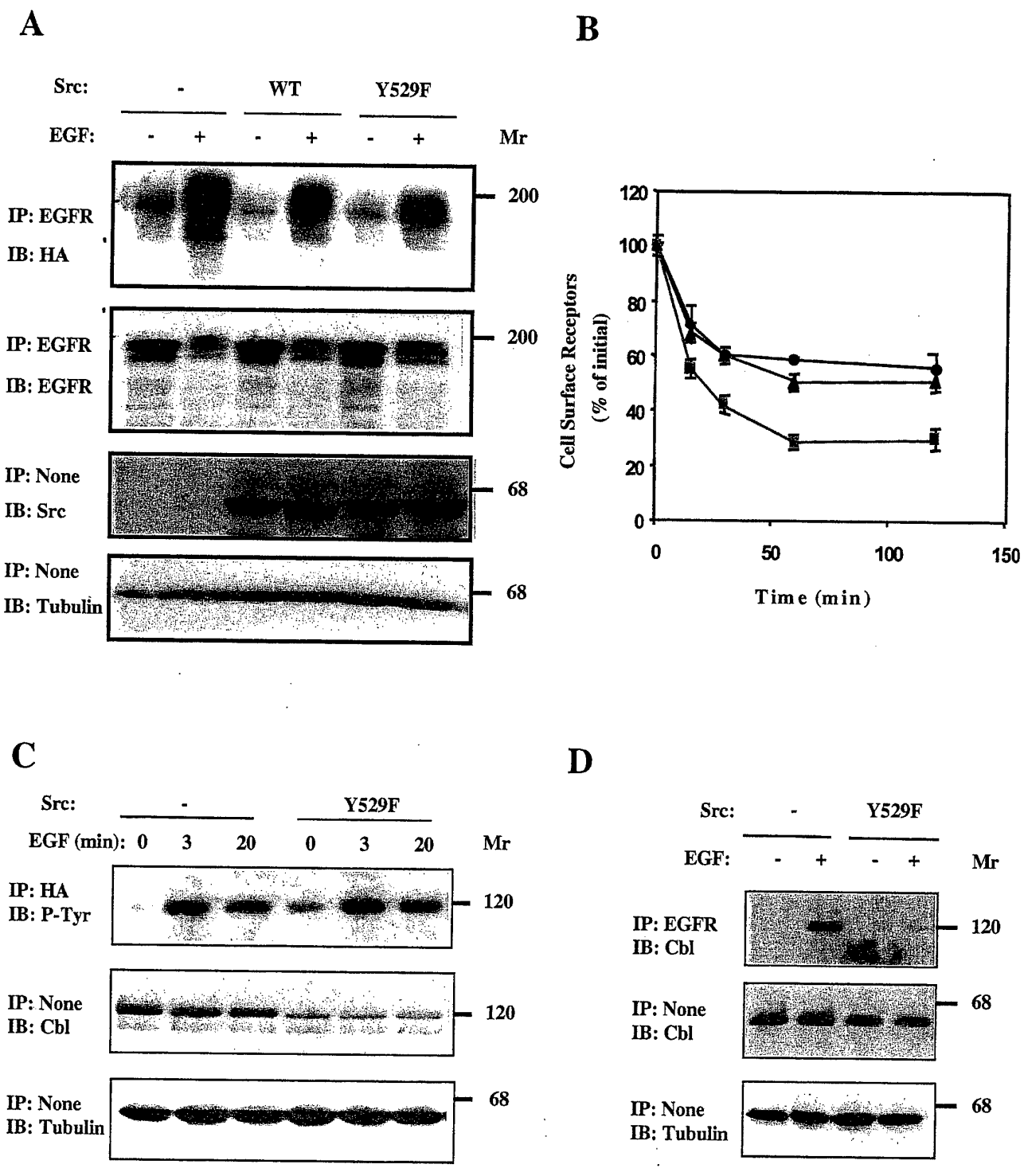
IB: Tubulin

Figure 3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Mr
IP: EGFR
IB: HA
200
IP: EGFR
IB: EGFR
200
IP: None
IB: Src
68
IP: None
IB: Tubulin
68
B
Cell Surface Receptors (% of initial)
120
100
80
60
40
20
0
0
50
100
150
Time (min)
C
Src: - Y529F
EGF (min): 0 3 20 0 3 20
Mr
IP: HA
IB: P-Tyr
120
IP: None
IB: Cbl
120
IP: None
IB: Tubulin
68
D
Src: - Y529F
EGF: - + - +
Mr
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IB: Cbl
120
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IB: Cbl
68
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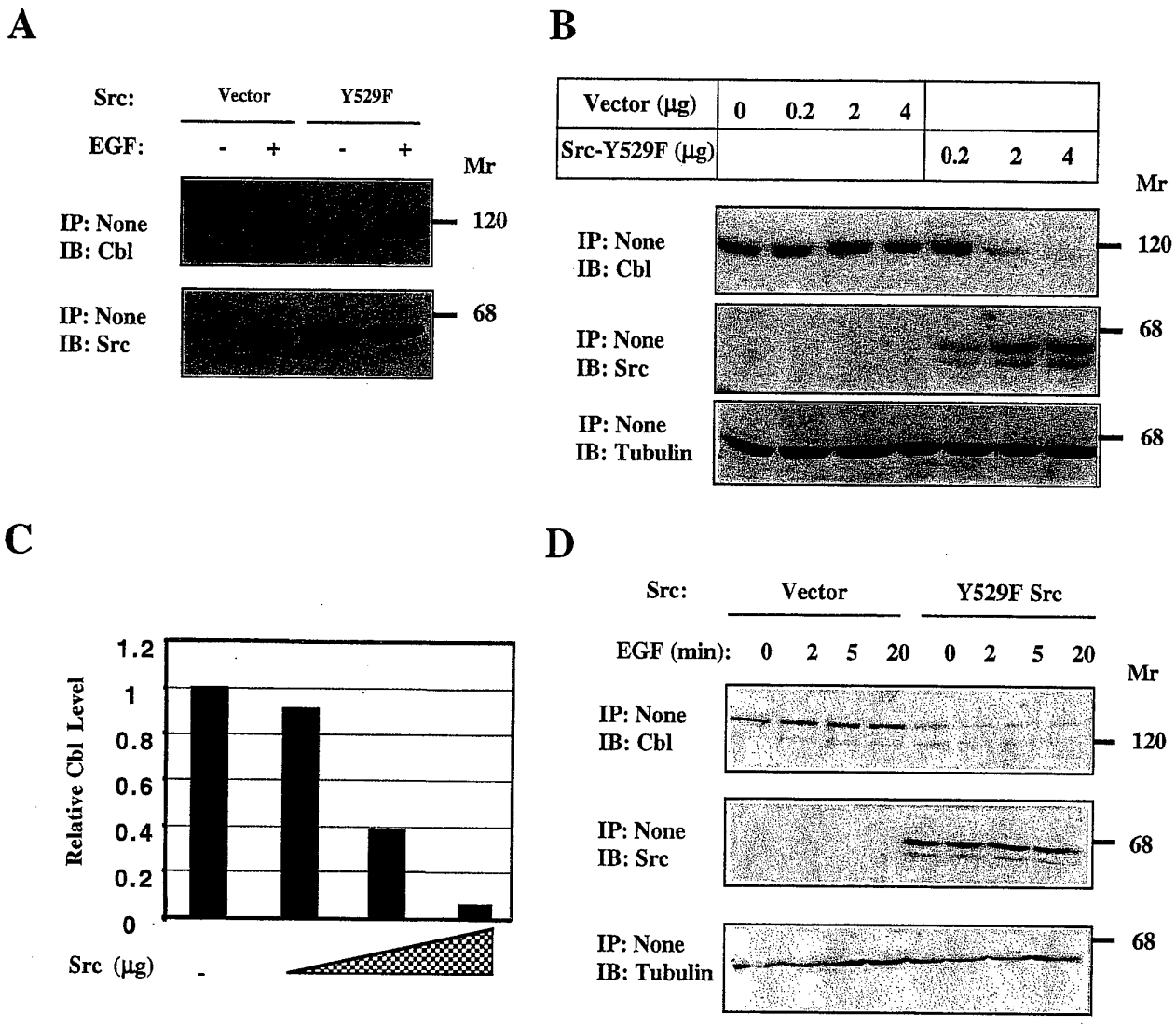
IB: Tubulin
68

Figure 4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Y529F
EGF:
- + - +
Mr
IP: None
IB: Cbl
120
IP: None
IB: Src
68
B
Vector (μg)
0 0.2 2 4
Src-Y529F (μg)
0.2 2 4
Mr
IP: None
IB: Cbl
120
IP: None
IB: Src
68
IP: None
IB: Tubulin
68
C
Relative Cbl Level
1.2
1
0.8
0.6
0.4
0.2
0
Src (μg)
-
D
Src:
Vector
Y529F Src
EGF (min):
0 2 5 20 0 2 5 20
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IP: None
IB: Cbl
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68
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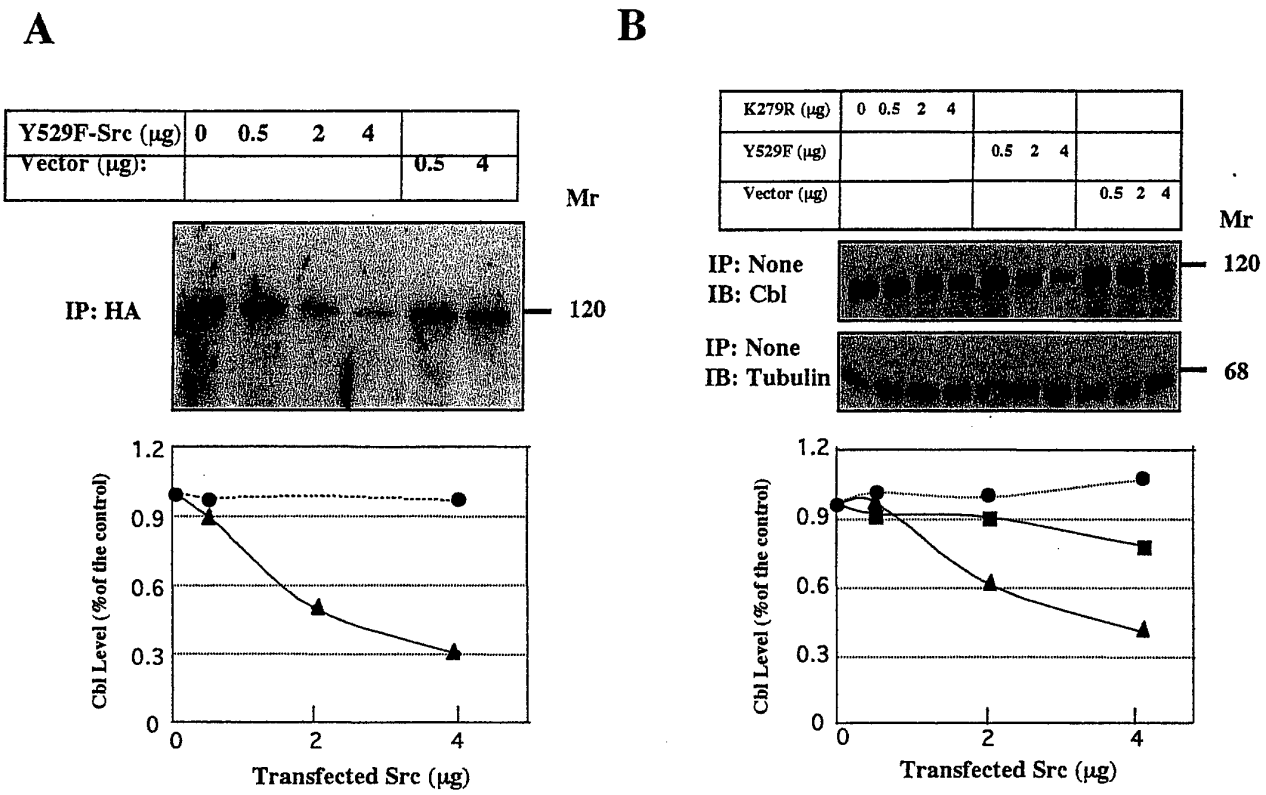
IB: Tubulin
68

Figure 5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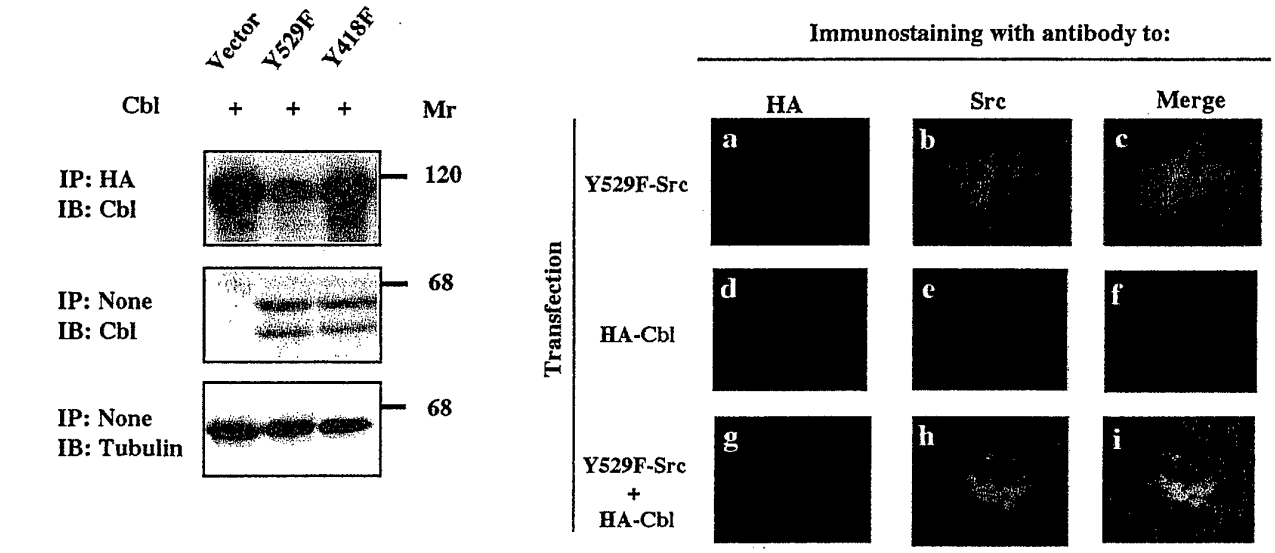
Vector (μg): 0.5 4
Mr
IP: HA
120
Cbl Level (%of the control)
1.2
0.9
0.6
0.3
0
0 2 4
Transfected Src (μg)
B
K279R (μg) 0 0.5 2 4
Y529F (μg) 0.5 2 4
Vector (μg) 0.5 2 4
Mr
IP: None
IB: Cbl
120
IP: None
IB: Tubulin
68
Cbl Level (%of the control)
1.2
0.9
0.6
0.3
0
0 2 4
Transfected Src (μg)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Vector
Y529F
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Cbl + + +
Mr
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120
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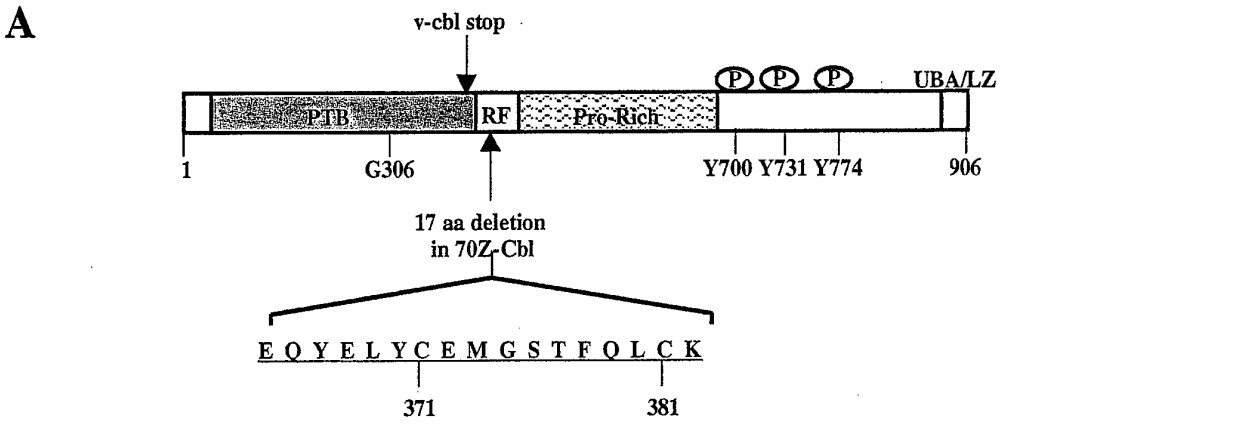
IB: Cbl
68
IP: None
IB: Tubulin
68
D
Immunostaining with antibody to:
HA
Src
Merge
Transfection
Y529F-Src
HA-Cbl
Y529F-Src
+
HA-Cbl
a
b
c
d
e
f
g
h
i

Figure 6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PTB
RF
Pro-Rich
P P P
UBA/LZ
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Y700 Y731 Y774
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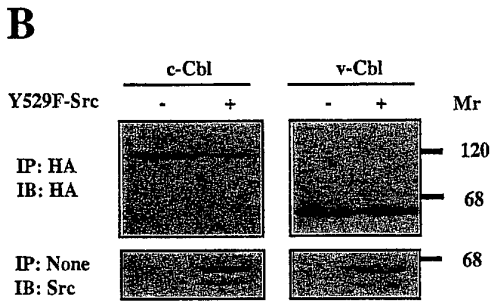
17 aa deletion
in 70Z-Cbl
E Q Y E L Y C E M G S T F Q L C K
371
381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- + - +
Mr
IP: HA
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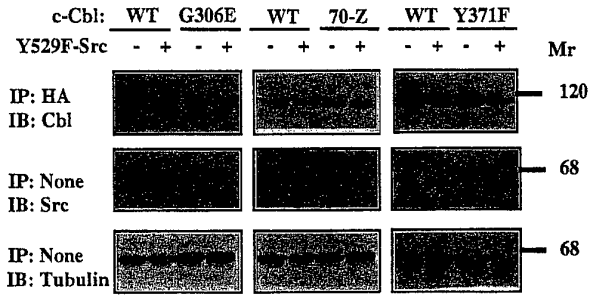
120
68
IP: None
IB: Src
68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IB: Cbl
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IB: Src
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IP: None
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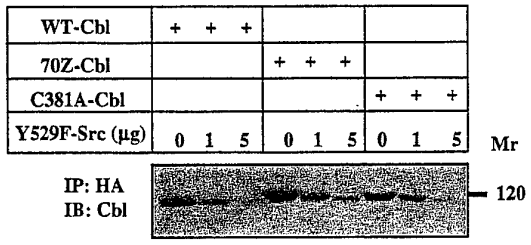
68


D
WT-Cbl + + +
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C381A-Cbl + + +
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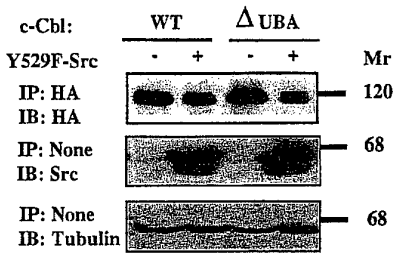
Mr
IP: HA
IB: Cbl
120


E
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Y529F-Src - + - +
Mr
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120
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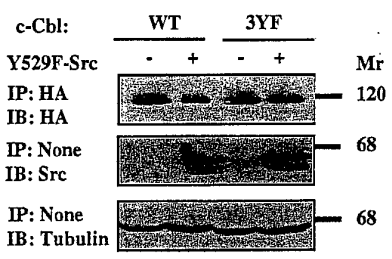
IB: Src
68
IP: None
IB: Tubulin
68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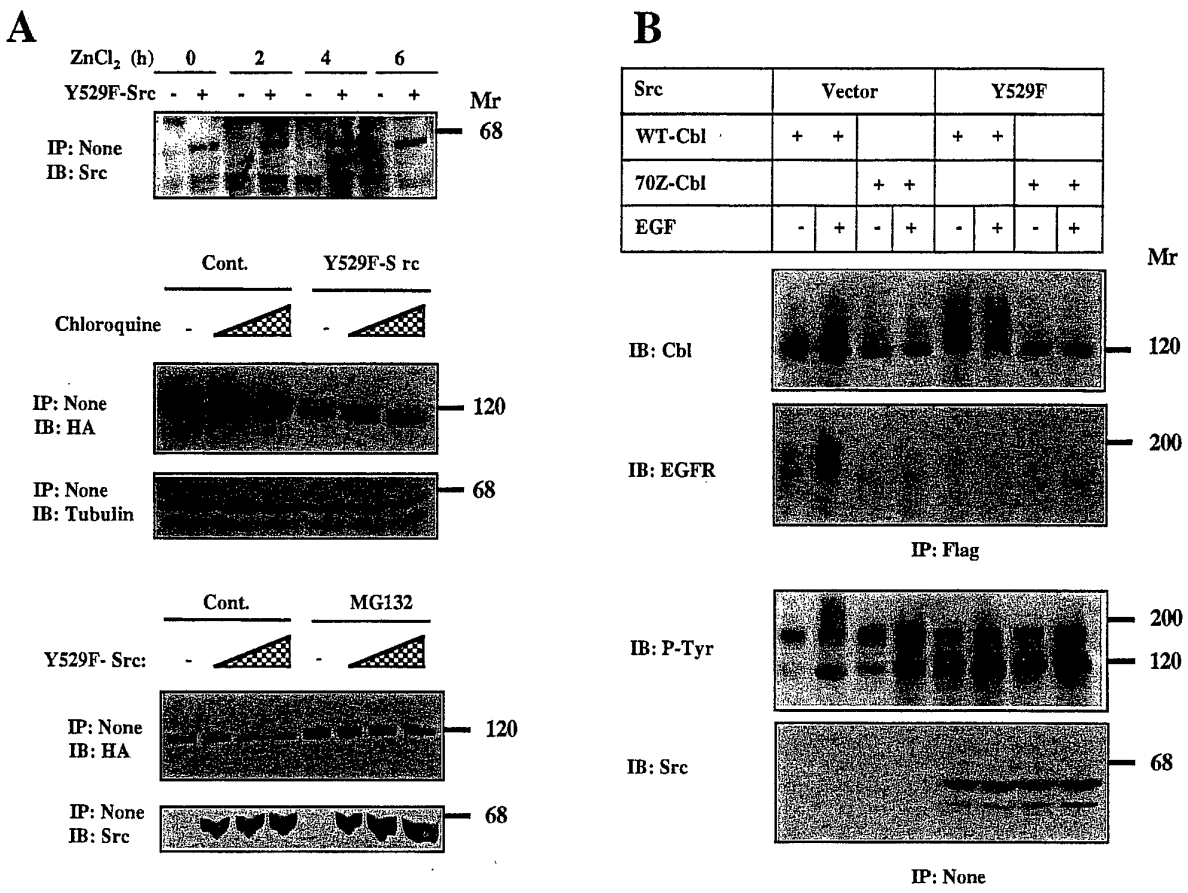
IB: Tubulin
68

Figure 7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Mr
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Cont.
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Chloroquine
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Cont.
MG132
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68
B
Src
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WT-Cbl
70Z-Cbl
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C
Normal Src
Oncogenic Src
EGF
EGF
Cbl
Cbl
Src
Ub
Cbl
P
26S
Cbl
Ub
?

Ligand-independent endocytosis involves receptor ubiquitylation and Hgs, an adaptor whose UIM domain targets self-ubiquitylation by Nedd4

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Running title: Regulation of EGFR endocytosis by Hgs

Keywords: epidermal growth factor, endocytosis, Hgs/Hrs, Nedd4, signal transduction, tyrosine kinase, ubiquitin

The abbreviations used are: CHO, Chinese hamster ovary; EGFR, epidermal growth factor receptor; GFP, green fluorescence protein; GST, glutathione S-transferase; HA, hemagglutinin; mAb, monoclonal antibody; PI3P, phosphatidyl inositol 3 phosphate; UIM, ubiquitin-interaction motif.

*The contribution of these authors was equal

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Abstract

Ligand-dependent endocytosis of the epidermal growth factor receptor (EGFR) involves recruitment of an ubiquitin ligase, and sorting of ubiquitylated receptors to lysosomal degradation. By studying Hgs, a mammalian homologue of a yeast vacuolar-sorting adaptor, we provide information on the less understood, ligand-independent pathway of receptor endocytosis. Constitutive endocytosis involves receptor ubiquitylation and translocation to Hgs-containing endosomes. Whereas the lipid binding motif of Hgs is necessary for receptor endocytosis, the ubiquitin-interacting motif (UIM) negatively regulates receptor degradation. We demonstrate that the UIM is endowed with two functions: it binds ubiquitylated proteins and it targets self-ubiquitylation by recruiting Nedd4, an ubiquitin ligase previously implicated in endocytosis. Based upon the dual function of the UIM and its wide occurrence in endocytic adaptors, we propose a UIM network that relays ubiquitylated membrane receptors to lysosomal degradation through successive budding events.

Introduction

Desensitization of ligand-activated receptors for growth factors plays a pivotal role in signal transduction, and its deregulation may lead to neoplastic transformation [reviewed in (1, 2)]. Regulated endocytosis through clathrin-coated areas of the plasma membrane is the primary process underlying the desensitization mechanism. Lessons learned from yeast membrane proteins suggest that sorting of receptors to endocytosis and to subsequent degradation in the vacuole, the yeast counterpart of the lysosome, is controlled by ubiquitylation of both the internalizing receptors and components of the endocytic machinery (3, 4). In the case of the epidermal growth factor receptor (EGFR), ligand-induced sorting to lysosomal degradation involves recruitment of a ubiquitin ligase, c-Cbl, to a specific tyrosine residue located at the carboxyl terminal tail of EGFR, and concurrent assembly of a complex comprising a ubiquitin-loaded E2 enzyme (5-8). Like in yeast, hydrophobic signals intrinsic to the appended ubiquitin molecule may be sufficient for sorting of receptors to degradation in mammalian cells (9). In addition, a juxtamembrane localized leucine-based motif has been implicated in internalization of EGFR (10), but the relationships between intrinsic internalization motifs (e.g., di-leucine) and inducible sites (i.e., a specific phosphotyrosine) that mediate endocytosis of EGFR are currently unclear.

Along with rapid ligand-induced endocytosis, a simultaneous constitutive process of slow internalization and subsequent degradation of EGFR takes place in living cells. This process may be exemplified by the internalization capability of a kinase- defective mutant of EGFR (11-13), and it seems to be shared by cargo receptors for vitamins and nutrients. The mechanisms underlying constitutive endocytosis, and the role, if any, of ubiquitylation in this process are currently unknown. It is relevant, however, that Eps15, an endocytic adaptor that binds to the clathrin complex, and regulates EGFR endocytosis (14), undergoes ligand-induced ubiquitylation (15). Several other adaptors, like Hgs (formerly called Hrs) and members of the STAM family [reviewed in (16)] share a coiled coil domain, which allows Hgs-STAM interactions. By binding to clathrin (17), PI3P (18) and Eps15 (19), Hgs recruits clathrin to early endosomes and disrupts the association of AP-2, the di-leucine binding component of the clathrin-coated pit, with Eps15. Thus, a multi-molecular complex comprising Eps15, Hgs and STAM may exist in cells and control receptor endocytosis through regulated interactions with the AP-2/clathrin machinery.

Several structural and functional lines of evidence provide further evidence for the participation of Hgs in receptor endocytosis. The early endosomal antigen 1, which is involved in endocytic membrane fusion, shares with Hgs a PI3P-binding FYVE domain (18), and localization to endosomes (20, 21). Moreover, the probable yeast orthologue of Hgs, Vps27p, belongs to the class E vacuolar protein sorting group, which is involved in membrane traffic through the pre-vacuolar/endosomal compartment (22). Accumulation of endocytosed proteins, similar to the class E phenotype of yeast, was observed in mammalian cells lacking Hgs expression (23). Interestingly, over-expression of Hgs leads to similar endosomal accumulation of cargo proteins (17, 21, 24, 25). However, it is currently unclear how Hgs is involved in EGFR endocytosis.

To address the endocytic function of the Hgs-coordinated multi-protein complex, we made use of a series of Hgs mutants. Our results attribute to Hgs a role in both the inducible and the ligand-independent pathways of receptor endocytosis. Interestingly, we find that the constitutive pathway is associated with receptor poly-ubiquitylation and translocation to Hgs-containing endosomes. Unlike receptor ubiquitylation, which positively drives endocytosis, we report that ubiquitylation of Hgs negatively modulates this process. Ubiquitylation of Hgs depends on its UIM, an ubiquitin binding motif that enables recruitment of Nedd4, an E3 ubiquitin ligase whose yeast homologue is widely implicated in endocytosis of membrane proteins. Because UIMs are present in several other endosomal sorting molecules (26), our results attribute to this domain a general role in regulating endocytosis of cargo receptors.

Results

An FYVE mutant of Hgs unveils a function in the ligand-induced pathway of receptor endocytosis

To analyze the role of Hgs in endocytosis of EGFR, we attempted to generate an interfering mutant. Figure 1A presents the domain structure of Hgs. Because impairment of the FYVE domain resulted in loss of function of Vps27p, the yeast homologue of Hgs (22), we generated three mutants in the FYVE domain (27). Mutations were introduced at two of the conserved cysteines of the domain (mutants denoted C166A and C215A), and in the basic motif surrounding cysteine^{182}, a putative PI3P binding pocket (mutant denoted C-3). In addition, we used the dC2 mutant (28), which is mis-localized (24). When co-expressed with EGFR in receptor-negative CHO cells, dC2 exerted no significant effect on the rate of EGF internalization (Fig. 1B). In contrast, each of the three FYVE-mutant Hgs proteins inhibited internalization of EGF. For example, the results we obtained with C166A are presented in Figure 1B. The observed impairment of EGFR internalization suggested inhibition of a normal, Hgs-mediated pathway of receptor trafficking. Alternatively, it may reflect sequestration of one of the many Hgs-binding partners.

Because ligand-induced ubiquitylation sorts EGFRs to endocytosis and degradation (29, 30), we tested the effect of C166A on EGF-induced ubiquitylation and degradation of EGFR. As demonstrated in Figure 1C, C166 significantly inhibited the rate of receptor degradation and also reduced the extent of ligand-induced receptor ubiquitylation, a modification initiated by c-Cbl at the cell surface and progressively enhanced en route to the multivesicular body (31, 32). Conceivably, in addition to its recently identified role in endosome membrane invagination (33), Hgs may regulate, either directly or indirectly through one of its partners, ligand-induced sorting of EGFRs to invaginated regions of the plasma membrane.

Hgs induces ligand-independent ubiquitylation and degradation of EGFR

Endocytosis of membranal yeast and mammalian receptors involves ubiquitylation of the cargo proteins (4). Hence, if wild type Hgs is involved in receptor endocytosis, an associated effect on receptor ubiquitylation and degradation is expected to accompany overexpression of Hgs. To test this prediction we analyzed receptor s ubiquitylation and degradation following short exposure to EGF of CHO cells expressing wild type or mutant forms of Hgs. Over-expression of Hgs moderately decreased receptor levels in a ligand-independent manner, and the remaining receptors displayed extensive and constitutive poly-ubiquitylation (Fig. 2A). These ligand-independent activities require PI3P binding, because FYVE mutants displayed impaired ability to induce receptor degradation, and their effect on basal ubiquitylation of EGFR was smaller than the effect of wild type Hgs (Figs. 2A and 2B). In contrast with the FYVE domain, deletion of the coiled coil and part of the proline- and glutamine-rich region enhanced EGFR ubiquitylation and degradation (dM mutant; Fig. 2B). However, more extensive deletion of the whole carboxyl terminal half of Hgs almost completely abolished the effects observed with wild type Hgs (dC2 mutant).

Because mutant Hgs proteins inhibit EGFR degradation through the ligand-induced, c-Cbl-regulated pathway (Fig. 1C), as well as through a ligand-independent mechanism (Fig.2A), it is worthwhile to compare the effect of c-Cbl to those of wild type Hgs. Prolonged overexpression of c-Cbl dramatically reduced EGFR expression, even in the absence of EGF (Fig. 2A), in line with our previous report (6). Nonetheless, upon stimulation by EGF, c-Cbl rapidly enhanced receptor ubiquitylation, and the pattern of this modification displayed higher stoichiometry relative to the effect of Hgs (compare receptor levels and ubiquitin signals in Fig. 2A). The relatively slow, ligand-independent effects of Hgs on receptor stability were reflected by two other lines of evidence: metabolic labeling of EGFR followed by a variable chase (in the absence of EGF) revealed that ectopic Hgs shortens the half life of EGFR from approximately 9.5 hours to 6 hours (Fig. 2C). The alternative approach utilized a hormone-inducible expression system. Transfection of ecdysone receptor-expressing HEK-293 cells with a plasmid driving Hgs expression from a Muristerone A responsive promoter was followed by selection of several cell clones. Treatment of two independent clones with the inducing agent led to Hgs accumulation, and a delayed reduction in EGFR levels (Fig. 2D), consistent with activation of a slow receptor degradation pathway.

Probing whole cell extracts with a rabbit anti-Hgs antibody detected the endogenous 110-kilodalton Hgs protein, and also confirmed ectopic expression of Hgs (Fig. 2A, lower panel). Interestingly, a ladder of reactive protein bands was detectable when using a polyclonal antibody, whereas a mAb to Hgs detected smeary bands. These observations suggested that Hgs undergoes ligand-independent ubiquitylation, an issue addressed by our later experiments. In summary, whereas the FYVE domain seems essential for enhanced receptor ubiquitylation by Hgs (probably due to mis-localization of mutant proteins), the coiled coil domain and the flanking regions (mutant denoted dM), which serve as binding sites for STAM, Eps15, Hbp and SNAP-25 [reviewed in (16)], seem inhibitory, probably because one of their ligands limits receptor ubiquitylation.

Hgs controls the ligand-independent pathway of EGFR internalization

To understand the mechanism by which FYVE mutants inhibit endocytosis of EGFR, we compared their cellular locations with that of EGFR and an ectopically expressed wild type Hgs. These experiments made use of a previously described chimeric EGFR fused to GFP (34). When singly expressed in CHO cells, GFP-EGFR displayed primarily cell surface localization (data not shown). Consistent with previous reports (25), over-expression of the wild type form of Hgs induced the appearance of large, Hgs-containing vesicular structures (Fig. 3A), formerly identified as early endosomes [(20) and references therein]. In more than 85% of wild type Hgs-overexpressing cells a large fraction of GFP-EGFR translocated to Hgs-containing endosomes, independently of EGF binding. In contrast, we observed that dC2 was mis-localized to the periphery of the cell, probably due to the defective proline- and glutamine-rich domain (24), and the distribution of EGFR in dC2-overexpressors was indistinguishable from cells transfected with an empty vector. Receptor translocation requires an intact FYVE, because none of the three FYVE mutants could effectively translocate EGFR (see for example the results obtained with C-3, Fig. 3A). Unlike wild type Hgs, these mutants were confined to peripheral aggregates that were largely devoid of EGFR. We concluded that the FYVE domain of Hgs is necessary for translocation of EGFR from the

plasma membrane to an endosomal localization. The inability of the FYVE mutants to induce the type E-like phenotype and translocate EGFR is consistent with their inhibitory effect on EGF internalization (Fig. 1B).
Several previous studies that tested both structural (35) and functional (36, 37) aspects of EGFR internalization concluded that the slower, ligand-independent pathway is undertaken by kinase-defective mutants of EGFR. To relate the Hgs-regulated mechanism, we utilized an EGFR mutant (Kin^-) whose ATP-binding site is inactive due to a mutation at lysine 721. Despite lack of catalytic activity, this form of EGFR underwent enhanced ubiquitylation in cells expressing an ectopic Hgs, or the more potent dM mutant (Fig. 3B). Consistent with these biochemical lines of evidence, morphological studies indicated that wild type Hgs, unlike the dC2 mutant, retained the ability to translocate Kin^- to large Hgs-containing vesicular structures (Fig. 3C). Taken together with other lines of evidence, these observations unveiled the ability of Hgs to channel EGFR to endocytosis down the ligand-dependent route, as well as along the ligand-independent pathway.

The UIM of Hgs autonomously mediates self- ubiquitylation

The ladder—like appearance of Hgs in immunoblots (Fig. 2A) suggested that this endosomal adaptor undergoes ubiquitylation, reminiscent of the modification of c-Cbl (38) and Eps15 (15). To test this possibility, we co-expressed in CHO cells Flag-tagged ubiquitin and HA-tagged forms of Hgs. The results of this experiment confirmed the existence in living cells of poly-ubiquitylated forms of Hgs (Fig. 4A). Moreover, we demonstrated that Hgs ubiquitylation is independent of the FYVE, coiled coil or the glutamine- and proline-rich domains. However, deletion of the UIM led to complete abolishment of Hgs ubiquitylation. Effects due to expression levels, or to specific peptide tags, were excluded by transfecting increasing quantities of the Hgs plasmid and by swapping the peptide tags between ubiquitin and Hgs (Fig. 4B). The existence of a UIM in STAM, a tight partner of Hgs, prompted us to examine whether STAM undergoes ubiquitylation. Co-expression of STAM and a peptide-tagged ubiquitin detected an apparently mono-ubiquitylated form of STAM, but a mutant lacking the UIM displayed low, if any, modification (Fig. 4C). Thus, ubiquitylated forms of Hgs and STAM exist in vivo, and the corresponding UIMs are necessary for this protein modification.

The amino acid sequence alignment shown in Figure 4D compares the UIM of Hgs to this motif present in other proteins, including two direct binding partners of Hgs, namely STAM and Eps15. Originally identified in S5a, a poly-ubiquitin binding subunit of the proteasome, the core of the UIM consists of eighteen amino acids: an amino-terminal acidic box flanks a stretch of alternating long and short hydrophobic amino acids that terminate at a conserved serine residue (39). Because the UIM of Hgs contains no lysine residue, we inferred that this domain targets the ubiquitylation process rather than serving as an ubiquitylation site. To test this hypothesis we individually fused the UIMs of Hgs and STAM, along with short flanking regions, to a modified GST protein (mGST). When expressed in CHO cells, the mGST-UIM^{Hgs} and mGST-UIM^{STAM} fusion proteins respectively underwent poly- and mono- ubiquitylation (Fig. 4E), in line with the corresponding ubiquitylation patterns of Hgs and STAM in living cells. Moreover, mutagenesis of the conserved UIM s serine (Ser^{270} of Hgs) severely reduced ubiquitylation (Fig. 4F), indicating the importance of this residue. Three control mGST proteins containing either no added sequence, or long inserts derived from non-relevant proteins (full-length CDC37 or the kinase domain of ErbB-2), displayed no ubiquitylation signals (Fig. 4F, and data not shown). Hence, the results obtained ascribe a novel function to UIMs of endosomal adaptors: these relatively short amino acid sequences autonomously target the machinery responsible for attachment of ubiquitin to substrate adaptor molecules.

The UIM of Hgs binds ubiquitylated proteins and enables Nedd4-mediated ubiquitylation in vitro and in living cells

The tandem UIM of S5a binds poly-ubiquitylated lysozyme and free poly-ubiquitin chains (40). To test possible binding of poly-ubiquitylated proteins to the UIM of Hgs we used bacterial GST fusion proteins (see scheme in Fig. 5A) and a pull-down assay. Extracts were prepared from CHO cells expressing HA-ubiquitin-tagged proteins, whose binding to recombinant UIMs was tested. As expected, recombinant GST-S5a bound many high molecular weight ubiquitylated proteins (Fig. 5B), and no ubiquitylated substrates bound to the isolated GST portion. Remarkably, the UIM of Hgs conferred binding to a relatively restricted set of ubiquitylated proteins. The specificity of these interactions was confirmed by lack of binding to the FYVE domain of Hgs or to the SH2 domain of Grb2 (Fig. 5B). Taken together with the results presented in Figure 4, these observations suggested that the UIM of Hgs is associated with a dual, possibly integrated, function: along with recruiting a ubiquitylation machinery, this domain can bind ubiquitylated substrates. The dual activity of UIM^{Hgs} was reflected in another in vitro assay that made use of radiolabeled ubiquitin (Fig. 5C). When incubated with radiolabeled ubiquitin and whole cell extracts, GST fusion proteins containing the UIM of Hgs, but not GST-FYVE or GST alone, mediated two functions: they underwent self-ubiquityaltion and they enabled binding of monomeric ubiquitin and ubiquitylated proteins. S5a similarly bound

high molecular weight ubiquitylated proteins, but unlike the UIM of Hgs it bound little or no monomeric free ubiquitin, in line with its reported ability to discriminate between mono- and poly-ubiquitin (40). Notably, washing the GST-UIM beads with a solution containing high salt concentration removed both ubiquitin and ubiquitylated high molecular weight proteins (data not shown).

Next, we addressed the mechanism that enables an intrinsic UIM to direct protein self ubiquitylation. By testing two promiscuous E2 ubiquitin-conjugating enzymes, namely UBC-H5C and UBC-H7, we found that the UIM cannot recruit these E2 molecules (data not shown), and therefore it does not qualify as an E3 ligase. Alternatively, the UIM may target an E3 ubiquitin ligase to UIM-containing proteins. Studies in yeast and mammalian cells implicated Nedd4, as well as the yeast homologue, Rsp5p, in a number of endocytic and exocytic processes [reviewed in (41)]. When tested in vitro in the presence of isolated E1 and E2 (UBC-H5C) enzymes, a recombinant bacteria-derived Nedd4 enhanced the ubiquitylation of not only UIM-containing fusion proteins, but also of high molecular weight aggregates whose identity remains unknown (Fig. 5D). Significantly weaker signals were observed in the absence of Nedd4, or when the E3 ligase was incubated with control fusion proteins lacking the UIM. To complement this in vitro line of evidence, we constructed a catalytically inactive mutant of Nedd4, in which the HECT domain cysteine capable of forming a thiolester bond with ubiquitin (42) was replaced by a serine (C853S-Nedd4). When introduced into cells expressing the mGST-UIM^{Hgs}, the catalytically inactive Nedd4 almost abolished poly-ubiquitylation of the UIM-containing protein (Fig. 6A), implying that Nedd4 is a major ubiquitin ligase recruited by the UIM^{Hgs}. In line with this conclusion, an ectopically introduced wild type Nedd4 moderately increased ubiquitylation of mGST-UIM^{Hgs} (Fig. 6A), suggesting that Nedd4 is constitutively active in living cells.
To verify relevance of these findings to full length Hgs, we examined the effect of ectopic Nedd4 on the level of Hgs ubiquitylation in living cells, and observed increased poly-ubiquitylation (Fig. 6B). Consistent with an essential role for the UIM, Nedd4 exerted minimal or no effect on the state of ubiquitylation of Hgs proteins, which are either devoid of the UIM (dU mutant), or carrying an inactivating mutation (S270D; Fig. 6B). While verifying expression of the exogenously introduced Nedd4 and Hgs proteins, we noted differences in levels of expression, but more experiments are needed to test the possibility that the HECT and UIM regions of Nedd4 and Hgs respectively modulate protein stability. Regardless of this possibility, the results presented in Figures 5C and 6 establish functional interactions between a HECT-domain ubiquitin ligase, namely Nedd4, and the UIM of Hgs. Conceivably, when present on a substrate protein like Hgs, the UIM specifically directs the catalytic activity of Nedd4, resulting in ubiquitylation of the substrate.

Ubiquitylation of Hgs may inactivate its endocytic function

To test the role of Hgs ubiquitylation in endocytosis and degradation of EGFR, we compared the effects of wild type Hgs and a mutant lacking the UIM (denoted dU). When co-expressed with EGFR in CHO cells, both forms of Hgs enhanced disappearance of the receptor, but dU was reproducibly associated with a more extensive effect and with the appearance of a presumed degradation product (p150; Fig. 7A). A similar protein band appeared upon expression of another active mutant of Hgs, namely dM, which is devoid of the coiled coil and flanking regions (Fig. 7A; see also Figs. 2B and 3B). Interestingly, in the presence of dM or wild type Hgs, the residual receptor displayed more extensive poly-ubiquitylation than the modification we observed with dU, probably because most ubiquitylated receptors available for endocytosis are degraded in dU-expressing cells. Consistent with this scenario, in three out of twelve experiments we observed enhanced ubiquitylation of EGFR in cells expressing dU, and in all experiments receptor degradation was significantly more extensive than in cells expressing similar levels of ectopic wild type Hgs. Morphological analyses confirmed that dU retained the ability to enhance endocytosis of EGFR (Fig. 7B). This mutant, like wild type Hgs (see Figs. 3A and 3B), led to translocation of EGFR from the cell surface to vesicular structures, some of which contained Hgs (Fig. 7B). Hence, deletion of the UIM does not impair localization of Hgs to endocytic vesicles, but it seems to promote degradation of EGFR. Because the UIM is absolutely necessary for ubiquitylation of Hgs (Fig. 6B) and this modification is mediated primarily by Nedd4 (Fig. 6A), we propose that Nedd4 inactivates the endocytic function of adaptors by elevating their ubiquitylation. Possible functional links between ubiquitin binding to the UIM, consequent targeting of ubiquitin ligases to substrate proteins, and the concerted activities leading to endocytosis of cargo receptors are discussed below.

Discussion

Observations made in yeast established the notion that endocytosis of membrane proteins (e.g., Ste2p) is associated with ubiquitylation of the internalizing protein [reviewed in (4)]. However, because endocytosis of a Ste2p-ubiquitin chimera, a receptor whose internalization does not require ubiquitylation, is defective in cells expressing a mutant Rsp5p/Nedd4, it has been concluded that ubiquitylation of a component of the endocytic machinery is required for receptor endocytosis (3). In analogy, endocytosis of the growth hormone receptor depends on intact ubiquitylation

machinery, even when the cytoplasmic domain contains no ubiquitylation site (43). Thus, ubiquitylation of both the cargo receptor and a trans-acting target seems involved in endocytosis. Unlike Ste2p and the growth hormone receptor, at least two distinct endocytic pathways are accessible to growth factor receptors harboring an intrinsic tyrosine kinase [reviewed in (1)]: The ligand-induced pathway, which involves receptor auto-phosphorylation, and recruitment of the c-Cbl ubiquitin ligase, has been extensively studied. In the case of EGFR, the much less understood alternative route is often studied by following the internalization of a kinase-dead receptor (13, 36, 37). Understanding this slow, ligand- independent pathway relies on identification of its molecular players. The results we obtained with Hgs identify this endosomal adaptor as one regulator of the constitutive pathway. Along with regulating ubiquitylation of the cargo receptor in a process involving the FYVE domain, the UIM of Hgs autonomously mediates self-ubiquitylation. However, whereas receptor ubiquitylation positively drives endocytosis, UIM-dependent and Nedd4-mediated ubiquitylation of Hgs may inactivate its endocytic action.

The role of Hgs in receptor endocytosis

Several previous reports attributed to Hgs a mostly inhibitory action in ligand-induced endocytosis of different receptors (17, 21, 28, 33, 44, 45). However, most previous studies were based upon overexpression, which in the case of Hgs yields a phenotype similar to nullifying Hgs expression (23). In contrast, utilizing three mutants we provide evidence for a positive role of Hgs in both constitutive (Fig. 3) and ligand-induced endocytosis (Figs. 1B and 1C). Consistent with involvement in the constitutive pathway, it has been reported that Hgs regulates uptake of a fluid phase marker (17), as well as transferrin (19), two well-characterized cargo proteins of the constitutive pathway. Moreover, our evidence implicates receptor ubiquitylation in the Hgs-associated sorting of EGFRs. Thus, mutant Hgs proteins incapable of elevating receptor ubiquitylation (e.g., dC2 and C-3) were unable to translocate EGFR from the plasma membrane to endocytic vesicles (Fig. 3), whereas expression of a relatively potent mutant of Hgs (i.e., dM) was associated with extensive ubiquitylation, endocytosis and degradation of EGFR (Figs. 2B and 3B). It is therefore plausible that receptor ubiquitylation is a shared pre-requisite for endocytosis via the two pathways of EGFR internalization.

How exactly Hgs is involved in endocytosis of EGFR remains incompletely understood. This adaptor directly binds to clathrin (17), which may explain why overexpression of wild type Hgs enhanced ubiquitylation, endocytosis and degradation of EGFR (Figs. 2 and 3). Likewise, binding of Hrs-2 (a neural homologue of Hgs) to Eps15 disrupts AP2-Eps15 complexes (19), which may underlie the internalization-inhibitory function of mutant Hgs proteins (Fig. 1). Moreover, Hrs-2 binds to a plasma membrane protein called SNAP-25 (46), suggesting that a fraction of cellular Hgs may transiently translocate to the plasma membrane. However, most morphological analyses localized Hgs to early endosomes [see for example (20)], and genetic studies ascribed to the yeast homologue, Vps27p, a function in a pre-lysosomal compartment (22). Hence, the observed effects on internalization of wild type and mutant forms of Hgs may be interpreted in two ways: either direct binding of Hgs to components of the clathrin coat, or sequestration of molecules essential for receptor sorting at the membrane.

A network of UIM-containing adaptors that relays ubiquitylated membrane receptors to lysosomal degradation

Our finding that both Hgs and STAM undergo ubiquitylation in living cells (Fig. 4) implicates ubiquitylation of an endocytic machinery encompassing at least the two adaptors. A key issue, however, is the relationship between receptor endocytosis and covalent modification of the endocytic machinery. Unlike Ste2p and the growth hormone receptor, whose endocytosis depends on ubiquitylation of the endocytic machinery (3, 43), blocking ubiquitylation of and ubiquitin binding to Hgs enhanced endocytic degradation of EGFR (Fig. 7). Hence, UIM-mediated ubiquitylation of Hgs may inhibit, rather than enhance, the function of Hgs in endocytosis. De-ubiquitylation of c-Cbl seems to recycle the ligase following dissociation from an endocytosed receptor (38). In addition, genetic analyses identified functional interactions between epsin, a UIM-containing endosomal protein, and a de-ubiquitylating enzyme required for normal eye development in flies (47). In support of a positive endocytic role of de-ubiquitylating enzymes, genetic and biochemical evidence from yeast imply that another de-ubiquitylating enzyme, Doa4p, is necessary for sorting membrane proteins and lysosomal hydrolases to the vacuole (48, 49).

By concentrating on the UIM of Hgs and STAM, we show that this relatively short domain is endowed with two autonomous activities: not only it can bind free mono-ubiquitin and poly-ubiquitylated proteins (Fig. 5), grafting a UIM onto a protein targets the fusion chimera for ubiquitylation by Nedd4 (Figs. 5D and 6). Importantly, ubiquitylation by Nedd4 modifies regions outside of the UIM, and in the context of Hgs, this protein modification may restrict, rather than enhance degradation of internalized receptors (Fig. 7). Are these seemingly distinct activities of the modular UIM domains linked in some way? Further, what common roles are played by the broad spectrum of UIM-containing proteins in vesicular sorting? Ubiquitylation of Hgs and other adaptors may alter protein conformation, much like phosphorylation events. Another mechanism that may be shared by

phosphorylation and ubiquitylation is the creation of new docking sites, which will lead in the case of UIM-containing adaptors to auto-catalyzed nucleation of large multi-protein complexes.

One alternative mechanism integrating the dual functions of UIM-containing adaptors into the two pathways of EGFR endocytosis is presented in Figure 7C. According to this model, UIM-containing adaptors are active in their non-ubiquitylated form, namely: they can bind a mono-ubiquitylated receptor either at the cell surface (e.g., the UIM of epsin) or at endosomal compartments (e.g., the UIM of Hgs). We propose that the other intrinsic activity of the UIM, its ability to target Nedd4-mediated ubiquitylation, promotes recycling of the adaptor: ubiquitin appended by Nedd4 folds over the UIM, thereby unloading the ubiquitylated receptor, which is then relayed to the next UIM-containing adaptor. This mechanism is analogous to inactivation of pp60-Src by binding of a carboxyl terminal phospho-tyrosine to an intrinsic Src homology domain 2 [SH2; (50)].

The data we presented on Hgs (Fig. 1C) and previous data on Eps15 (14) indicate that UIM-containing proteins control both the rapid, ligand-induced pathway of EGFR endocytosis, as well as the tonic endocytosis taking place in the absence of EGF. Hence, one shared rate-determining step for endocytic pathways may be the speed of receptor ubiquitylation at the plasma membrane. Whereas c-Cbl is the ligase responsible for ubiquitylation of active EGFR molecules, the identity of the putative E3 ligase initiating endocytosis of unoccupied receptors is still unknown. Another open question relates to EGF-inducible phosphorylation of UIM-containing adaptors like Hgs, STAM and Eps15. These and other aspects of the proposed mode of action of the UIM require additional studies. It is notable, however, that while this manuscript was under revision, a study of Eps15 and Eps15R performed by Di Fiore and colleagues reported dual activity of UIM^{Eps15} (51) similar to the function we characterized for the UIM^{Hgs}. Despite similarities, the UIMs of Hgs, STAM and Eps15 seem to differ in the patterns of associated ubiquitylation. In addition, each adaptor is endowed with a distinct set of ligands, primarily phospholipids and proteins harboring coiled coil and Eps15 homology domains. Hence, the UIM network of adaptors may regulate not only endocytic pathways, but also other routes of vesicular transport, including exocytosis and biosynthesis-associated protein delivery.

MATERIALS AND METHODS

Reagents and antibodies

Unless indicated, materials were purchased from Sigma (St. Louis, MO). Na^{125}I and ^{35}S-labeled cysteine and methionine were purchased from Amersham Pharmacia Biotech (Buckinghamshire. UK) and IODOGEN from Pierce (Rochford, IL). Lipofectamine was supplied by Gibco BRL (Grand Island, N.Y.). G418 was purchased from Calbiochem (San Diego, CA). An anti-HA rat mAb was purchased from Roche Molecular Biochemicals (Mannheim, Germany). mAbs SG565 and 111.6 to EGFR were generated in our lab. A mAb and a rabbit polyclonal antibody to Hgs were previously described (28). An anti V-5 mouse mAb was purchased from Invitrogen (Groningen, Netherland). Antibodies to GST and EGFR were from Santa Cruz Biotechnology (Santa Cruz, CA). Peroxidase-conjugated and fluorescently labeled antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). GST-S5a and ubiquitin activating enzyme E1 were from Affiniti (Mumhead, Exeter, UK). Peroxidase-conjugated protein-A was from ICN (Costa Mesa, CA). A chemiluminescence kit for immunoblotting was obtained from Amersham (Buckinghamshire, UK). Protein G Sepharose beads was from Pharmacia Biotech (Buckinghamshire, UK). Muristerone A was from Alexis (Lausen, Swizerland). Zeocin was from Invitrogen (Groningen, Netherland).

Buffers

The following buffers were used: Binding buffer: Dulbecco's modified Eagle's medium supplemented with 0.5% bovine serum albumin and 20 mM HEPES (pH 7.5). Lysis buffer: 0.1N NaOH and 0.1% SDS. HNTG: 20mM HEPES (pH 7.5), 150mM NaCl, 0.1% Triton X-100, and 10% glycerol. TBST: 20mM Tris-Hcl (pH 7.5), 0.15M NaCl, and 0.05% Tween 20. Solubilization buffer: 50mM HEPES (pH 7.5), 150mM NaCl, 10% glycerol, 1% Triton X-100, 1mM EDTA, 1mM EGTA, 10mM NaF, 30mM β-glycerol phosphate, 0.2mM Na_3VO_4 and a protease inhibitor cocktail (diluted at 1:1000).

Construction of expression vectors

The pcDNA3 plasmid (Invitrogen) was used for expression in mammalian cells. Plasmids encoding Hgs (wild type) and two mutants (dC2 and dM) have been previously described (28). Additional mutations were generated in pcDNA3-Hgs by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). The C-3 mutant, containing a substitution of the protein motif RKHHCR flanking cysteine^{182} to AAAACA, was generated by overlap extension PCR (27). The UIM deletion mutant (dU, containing a deletion of 35 amino-acids) was similarly

constructed. HA-Hgs was subcloned into pIND expression vector utilizing the *XhoI* and *XbaI* sites, to generate an ecdysone inducible Hgs. An expression vector (pcDNA3) containing a V5-tagged STAM cDNA has been described (28). The dU mutant of STAM (carrying a deletion of 35 amino-acids in between lysine^{162} and tyrosine^{198}) was generated in this vector. A plasmid encoding a flag-tagged ubiquitin (pEF-Ubiquitin-flag) was generated by overlap extension PCR. A vector encoding a fusion protein comprised of a full-length EGFR fused to the green fluorescence protein (GFP) has been described (34). Flag-Nedd4 expression vector was provided by Marius Sudol (Rockefeller University, New York). The ubiquitin-HA expression vector was a gift from Dirk Bohmann (EBI, Heidelberg, Germany). All other expression vectors have been described (6).

Generation of GST fusion proteins

The GST-FYVE-UIM encoding plasmid (18) was provided by Harald Stenmark (Norwegian Radium Hospital, Oslo, Norway). A stop codon was introduced to replace amino acid 231 generating GST-FYVE. The UIM domain of Hgs and its flanking region (amino acids 225-285) was cloned by PCR into pGEX-4T1 for expression in bacteria. Likewise, we cloned the region corresponding to amino acids 225-284 into a modified pEF-BOS vector for expression in mammalian cells. A similar approach was used to generate mGST-UIM-STAM (amino acids 138-197). GST-Nedd4 bacterial expression vector was provided by Allan Weissman (National Institutes of Health, Bethesda). GST-Grb2-SH2 was obtained from Jan Sap (New York University).

Cell culture and transfection

CHO cells were cultured in DMEM:F12 medium supplemented with antibiotics, glutamine and heat-inactivated fetal calf serum (10%). For transfection, cells were grown to 80% confluence in 100mm plates. Transfection was performed using the Lipofectamine method. The following amounts of DNA were used: EGFR and its mutants, 1μg; HA-ubiquitin, 1μg; c-Cbl, 3μg; and Hgs and its mutants, 5μg. The total amount of DNA in each transfection was normalized with the pcDNA3 plasmid. Cells were assayed 48 hours after transfection. COS-7 cells stably expressing HA-Hgs were cultured in G418 (700μg/ml) containing medium. EcR-293 (HEK293 cells constitutively expressing the subunits of the ecdysone receptor from Drosophila) stably transfected with pIND-HA-Hgs, were cultured in medium supplemented with G418 (700μg/ml) and Zeocin (200 μg/ml).

Immunoprecipitation, pull-down and immunoblotting analyses

Transfected cells were washed briefly with ice-cold saline, scrapped in solubilization buffer and incubated on ice for 20 min. Lysates were cleared by centrifugation (10,000 X g, 20 min). In experiments where EGF was introduced, the transfected cells were washed in serum-free medium, and incubated for 10 min at 37°C with EGF (100 ng/ml). For direct electrophoretic analysis, gel sample buffer was added to cell lysates. For equal gel loading, protein concentrations were determined prior to immunoprecipitation by using the Bradford technique. For immunoprecipitation, lysates were incubated for 1-2 hrs at 4°C with antibodies pre-coupled to anti-mouse IgG-agarose beads, or to protein G-agarose beads. The immunoprecipitates were washed thrice with HNTG solution, resolved by gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked in TBST buffer containing 10% low fat milk, blotted with a primary antibody for 1 hour, washed with TBST and incubated for 30 min with a secondary antibody linked to horseradish peroxidase. Immuno-reactive bands were detected using chemiluminescence. For pull-down assays, cell lysates were incubated for 2 hrs at 4°C with glutathione- agarose beads, and tightly bound proteins separated by gel elctrophoresis.

Immunofluorescence

Transfected cells grown on cover-slips were fixed for 15 min with 3% paraformaldehyde in PBS. Fixed cells were washed in PBS and permeabilized for 10 min at 22°C with PBS containing 1% albumin and 0.2% Triton X-100. For labeling, cover-slips were incubated for 1 hour at room temperature with an anti-HA antibody, either alone or in combination with the anti-EGFR mAb 111.6. After extensive washing in PBS, the cover-slips were incubated for an additional hour with a Cy3-conjugated donkey anti-rat F(ab)$_2$ alone, or in combination with a Cy2-conjugated donkey anti-mouse F(ab)$_2$. The cover-slips were mounted in mowiol (Calbiochem). Confocal microscopy was performed using a Zeiss Axiovert 100 TV microscope (Oberkochen, Germany) with a 63X/1.4 plan-Apochromat objective, attached to the Bio-Rad Radiance 2000 laser scanning system, operated by LaserSharp software.

Radiolabeling of EGF, ligand internalization and degradation assays

Human recombinant EGF was labeled with ^{125}I and IODOGEN. For ligand internalization assay, cells were split into a 24-well dish one day after transfection. Twenty-four hours later, triplicate monolayers were washed with cold binding buffer and incubated at 4°C for 90 min in the presence of a radiolabeled EGF (3 ng/ml). Thereafter, the cells

were washed for three times with cold binding buffer, and then incubated at 37ßC for various time intervals. Surface-associated ^{125}I-EGF was collected by using a five minute-long incubation in ice-cold stripping buffer (0.15M NaCl, 0.15M acetic acid). Thereafter, the cells were solubilized in lysis buffer, and radioactivity determined in both fractions.

In-vitro ubiquitylation assay

GST fusion proteins (4 µg per reaction) were immobilized on glutathione- agarose beads, washed and incubated in buffer containing 25mM Tris HCl, 6mM $MgCl_2$, 100mM NaCl, 2µM DTT, 0.2mM ATP and ^{125}I-Ubiquitin (0.5 µg per reaction). Whole cell extracts (100 µg) or purified E1 (160 ng), E2 (UBC-H5C; 7µl of crude bacterial extract) and Nedd4 (100 ng; produced in bacteria as a GST fusion protein and cleaved with thrombin to release GST) were added as indicated. Reaction mixtures were incubated for 1 hour at 37ßC. The beads were then extensively washed, GST-fusion proteins were eluted with gel sample buffer and resolved by gel electrophoresis.

Metabolic labeling of cultured cells

COS-7 cells were incubated for 14 hours in cysteine- and methionine-free medium supplemented by 0.1 mCi/ml ^{35}S-labeled amino acids. Cells were then washed thoroughly, and incubated in media containing non-labeled cysteine and methionine for the indicated time intervals (chase). This was followed by cell lysis, immunoprecipitation, electrophoresis and autoradiography.

Legends to Figures

Figure 1: An FYVE mutant of Hgs inhibits ligand-induced endocytosis and degradation of EGFR. *A*, The domain structure of Hgs mutants used in this study is schematically depicted. The following domains are indicated: an N-terminal VHS, a zinc finger FYVE, a UIM, a proline-rich region (*Pro*), a coiled coil domain (*CC*), and a carboxyl terminal proline- and glutamine- rich region (*Pro/Glu*). *B*, CHO cells were co-transfected with plasmids encoding EGFR, along with vectors encoding Hgs mutants dC2 (squares) and C166A (open triangles). For control, cells were transfected with an empty expression vector (diamonds). Forty-eight hours later cells were transferred to 4ßC and incubated for 90 minutes in the presence of a radiolabeled EGF (3 ng/ml). Thereafter, unbound ligand was removed, cells transferred to 37ßC, and incubated for the indicated time intervals. Surface-bound ^{125}I-EGF was then removed at 4ßC by using a mild acid wash. Thereafter the cells were solubilized, and radioactivity determined in both fractions (surface-bound and intracellular). The results (means of triplicates –S.D.) represent the fraction of internalized ligand as a percentage of total cell-associated radioactivity. The experiment was repeated three times. *C*, CHO cells were co-transfected with an EGFR expression vector and a plasmid encoding HA-tagged ubiquitin, along with a vector encoding a mutant of Hgs (C166A). For control, we used an empty expression vector (lanes labeled -). Forty-eight hours after transfection, the cells were incubated at 37ßC for the indicated time intervals with EGF (100 ng/ml). Whole cell extracts were prepared and subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with a rat mAb to HA, or a rabbit anti-EGFR antibody.

Figure 2: Hgs increases constitutive ubiquitylation and degradation of EGFR in an FYVE domain-dependent manner. *A*, CHO cells were co-transfected with an EGFR expression vector and a plasmid encoding HA-tagged ubiquitin, along with plasmids encoding the indicated Hgs proteins or a c-Cbl protein. For control, we used an empty expression vector (-). Forty-eight hours after transfection, cells were incubated at 37ßC for 10 minutes without or with EGF (100 ng/ml). Cell extracts were subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with a rat mAb to HA or a rabbit anti-EGFR antibody. The lower panel shows immunoblotting of whole cell lysates with a rabbit anti-Hgs antibody. Note the endogenous p110 Hgs. *B*, CHO cells were transfected as in *A* with plasmids encoding the indicated Hgs proteins. The cells were incubated at 37ßC for 48 hours and cleared cell extracts were subjected to immunoprecipitation (IP) with an anti-EGFR antibody. This was followed by immunoblotting (IB) with a rat mAb to HA, or a rabbit anti-EGFR antibody. The lower panel shows immunoblotting of whole cell extracts with a mAb to Hgs. *C*, COS-7 cells constitutively expressing HA-Hgs (squares) or untransfected COS-7 cells (control; open circles) were subjected to metabolic labeling with ^{35}S-labeled amino acids for 12 hours. Following washes, cells were treated in media containing no radioactivity. Following the indicated time intervals, cells were extracted and the endogenous EGFR immunoprecipitated. Shown are autoradiograms and the corresponding decay curves. *D*, HEK-293 cells constitutively expressing the subunits of the ecdysone receptor (from *Drosophila*) were transfected with a plasmid driving expression of HA-Hgs from an ecdysone inducible promoter. Stable clones were selected by incubation with G418. Two clones of cells were

incubated at 37ßC without or with Muristerone A (2μM) for the indicated time intervals. Whole cell extracts were prepared and directly analyzed by immunoblotting (lower panel), or they were first subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with a mAb to EGFR. The levels of EGFR in both clones were quantified and presented (lower panel).

Figure 3: Hgs translocates surface receptors to large cytoplasmic vesicles independently of the tyrosine kinase activity of EGFR. *A,* CHO cells were transfected with expression vectors encoding a full-length EGFR fused to the green fluorescence protein (GFP-EGFR), along with plasmids encoding wild type (WT) or mutant HA-tagged Hgs proteins (dC2 or C-3). Cells were grown for 2 days on cover slips, fixed, permeabilized, and incubated with a rat anti-HA mAb. The primary antibody was followed by a Cy3-conjugated anti-rat immunoglobulin G antibody. The fluorescent signal of GFP-EGFR (green) is shown on the left column and the middle column presents the location of Hgs proteins (red). An overlay of GFP and Cy3 fluorescence, generating yellow color in areas of co-localization, is shown in the right column. All panels show middle confocal sections of the cells. *B*, CHO cells were co-transfected with vectors encoding a kinase-defective EGFR (K721A; Kin^-), along with the indicated Hgs proteins and HA-ubiquitin. As a control, we used an empty expression vector (-). The cells were incubated at 37ßC for 48 hours and their whole extracts were subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. *C*, CHO cells transiently expressing a kinase-defective EGFR (EGFR Kin^-) along with the indicated Hgs proteins were grown on cover slips for two days. The cells were then fixed, permeabilized, and incubated with antibodies that detect Hgs and EGFR. Cover slips were then incubated with a Cy3-conjugated anti-rat and Cy2-conjugated anti-mouse immunoglobulin G antibodies. The left column presents EGFR staining (green) and the middle column shows Hgs localization (red). An overlay of the two fluorescent signals, generating a yellow color in areas of co-localization, is shown in the right column.

Figure 4: Hgs and STAM are ubiquitylated proteins whose UIM domains autonomously drive self ubiquitylation. *A*, CHO cells were co-transfected with an expression vector encoding flag-tagged ubiquitin, along with plasmids encoding the indicated HA-tagged Hgs proteins (see Fig. 1A) or an empty expression vector (-). Cell extracts were prepared two days after transfection, cleared, and subjected to immunoprecipitation (IP) with an anti-Hgs antibody, followed by immunoblotting (IB) with a mouse anti-Flag mAb or a rat anti-HA mAb, as indicated. Arrows mark the locations of the indicated Hgs protein bands, and a non-specific band at the 110 kilodalton region. *B*, CHO cells were co-transfected with an HA-tagged ubiquitin expression vector along with increasing amounts of expression vectors encoding either flag-tagged wild type Hgs or the dU mutant (1, 2, and 5 μg DNA). As a control, we used an empty expression vector (-). Following transfection, cells were incubated for 48 hours at 37ßC and cell lysates subjected to immunoprecipitation (IP) with an anti-Hgs antibody, followed by immunoblotting (IB) with a rat anti-HA mAb. The lower panel shows immunoblotting of cleared whole cell extracts with a rabbit anti-Hgs antibody recognizing both endogenous and ectopic Hgs proteins. *C*, CHO cells were co-transfected with plasmids encoding the indicated V5-tagged STAM proteins and HA-ubiquitin. As a control, we used an empty expression vector (-). Cell extracts were prepared two days after transfection and subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Filled arrows mark the STAM protein band and an open arrow indicates the ubiquitylated form of STAM. *D,* The UIM of Hgs (residues 257-274) is aligned with the corresponding regions of the indicated human proteins (with the exception of yeast Vps27p). Sequence identities are shaded and underlined with a star, whereas conserved alignments are displayed with a colon. *E,* CHO cells were co-transfected with plasmids encoding HA-tagged ubiquitin, along with vectors driving expression of the UIM domains of STAM or Hgs fused to mGST. For control we used a plasmid encoding the mGST alone (+). Forty eight hours post transfection, whole cell lysates were subjected to a pull - down assay utilizing GSH- agarose beads. Proteins were then separated by gel electrophoresis and immunoblotted with the indicated antibodies. *F,* HEK-293 cells were co-transfected with plasmids encoding HA-tagged ubiquitin and either mGST alone, or mGST fused to either the wild-type UIM domain of Hgs or a domain mutated at the conserved serine residue (see *D*). Forty eight hours post transfection, whole cell lysates were subjected to a pull - down assay utilizing GSH- agarose beads. Proteins were then separated by gel electrophoresis and immunoblotted with the indicated antibodies. Note retarded electrophoretic mobility of ubiquitylated proteins.

Figure 5: The UIM of Hgs binds poly-ubiquitylated proteins and enhances Nedd4-induced ubiquitylation in vitro. *A*, Schematic representation of bacterially expressed GST-Hgs fusion proteins. *B*, CHO cells were transfected with a vector encoding for HA-ubiquitin. Following 48 hours, cell extracts were directly resolved by gel electrophoresis (*Input*; 6% of extract applied to each mixture), or they were subjected to a pull-down assay using the indicated bacterial proteins (approximately 50 μg each, except for GST-S5a: 10μg; see lower panel). Proteins

strongly adhering to the immobilized fusion proteins were electrophoretically separated and immunoblotted with anti-HA antibodies. GST fusion proteins were detected by blotting with GST-specific antibodies. *C,* The indicated GST fusion proteins immobilized on GSH agarose beads, were incubated for an hour at 37ßC with cleared whole cell extracts (from CHO cells) in the presence of radiolabeled ubiquitin. Thereafter, the reaction mixture was collected and directly resolved by gel electrophoresis (*input*). Proteins immobilized on the beads were washed four times in Triton X-100-containing buffer (HNTG) and resolved by gel electrophoresis and autoradiography. Free radiolabeled ubiquitin is indicated, as well as the ubiquitylated fusion proteins. A filled arrow marks ubiquitylated high molecular weight proteins. *D,* In vitro ubiquitylation was preformed as in *C,* except that whole cell extracts were replaced by a mixture of a purified E1 enzyme and a recombinant E2 (UBC-H5C) in the absence or presence of an E3 enzyme (recombinant Nedd4 from bacteria). The lower panel shows a stained gel with the respective GST fusion proteins. Note a non-specific protein band co-migrating with ubiquitylated FYVE-UIM.

Figure 6: Nedd4 mediates UIM-directed ubiquitylation of Hgs in living cells. *A*, HEK-293 cells were co-transfected with a plasmid encoding for mGST-Hgs-UIM and HA-tagged ubiquitin, along with increasing amounts of expression vectors encoding either a Flag-tagged wild type (*WT*) Nedd4 or a protein mutated at the catalytic cysteine (*C853S*; 0.2, 0.4, and 1 µg DNA). As a control, we used an empty expression vector (-). Whole cell extracts were subjected to a pull-down assay utilizing GSH-agarose beads, followed by immunoblotting (IB) with the indicated antibodies. The lowest panel shows immunoblotting of whole cell extracts with an anti-Flag antibody. Note the apparent increase in stability of the catalytically inactive mutant of Nedd4. *B*, HEK-293 cells were co-transfected with plasmids encoding HA-tagged ubiquitin, the indicated Flag-tagged Hgs proteins, and increasing amounts of an expression vector encoding Nedd4 (1 and 3 µg DNA). As a control, we used an empty expression vector (-). Whole cell extracts were prepared, cleared and subjected to immunoprecipitation (IP) with an anti-Hgs antibody, followed by immunoblotting (IB) with the indicated antibodies.

Figure 7: Deletion of the UIM enhances Hgs-induced endocytic degradation of EGFR. *A*, CHO cells were co-transfected with an EGFR expression vector and a plasmid encoding for HA-tagged ubiquitin, along with plasmids driving expression of the indicated Hgs proteins. As a control, we used an empty expression vector (-). Whole cell extracts were prepared and subjected to immunoprecipitation (IP) with an anti-EGFR antibody, followed by immunoblotting (IB) with the indicated antibodies. The lower panel shows immunoblotting of whole cell lysates with an anti-Hgs antibody. *B,* CHO cells were transfected with expression vectors encoding a full-length EGFR fused to the green fluorescence protein (GFP-EGFR), along with a plasmid encoding for an UIM-deleted, HA-tagged Hgs protein (dU). Cells were grown for 2 days on cover slips, fixed, permeabilized, and incubated with a rat anti-HA mAb. The primary antibody was followed by a Cy3-conjugated anti-rat immunoglobulin G antibody. The fluorescent signal of GFP-EGFR (green) is shown on the left column and the middle column presents the location of Hgs proteins (red). An overlay of GFP and Cy3 fluorescence, generating yellow color in areas of co-localization, is shown in the right column. All panels show middle confocal sections of the cells. *C*, Proposed mechanism of action of UIM-containing endocytic adaptors. According to the model, both ligand-dependent endocytosis of EGFR and the ligand-independent pathway are preceded by mono-ubiquitylation at the plasma membrane. Unlike the rapid, phosphorylation-dependent route of ligand-dependent endocytosis, which is initiated by recruitment of the c-Cbl ubiquitin ligase, the constitutive pathway is slow and the E3 ligase involved is still unknown. Once ubiquitylated, the receptor is sorted to the clathrin-coated pit by binding to adaptin-binding UIM-containing adaptors like Eps15 and Hgs. We assume that the ubiquitylated receptor is subsequently relayed to other UIM-containing adaptors, including endosomal adaptors like Hgs and STAM, which control budding into the lumen of late endosomal compartments. Recycling of UIM-containing adaptors is presumably mediated by Nedd4, which ubiquitylates the adaptors outside of the UIM. This allows ubiquitin s folding over and displacement of the ubiquitylated receptor from the UIM. Whereas the receptor is relayed to the next UIM-adaptor, and eventually to lysosomal degradation, de-ubiquitylating enzymes (UBPs) may replenish individual pools of active adaptors ready to handle the next ubiquitylated receptor.

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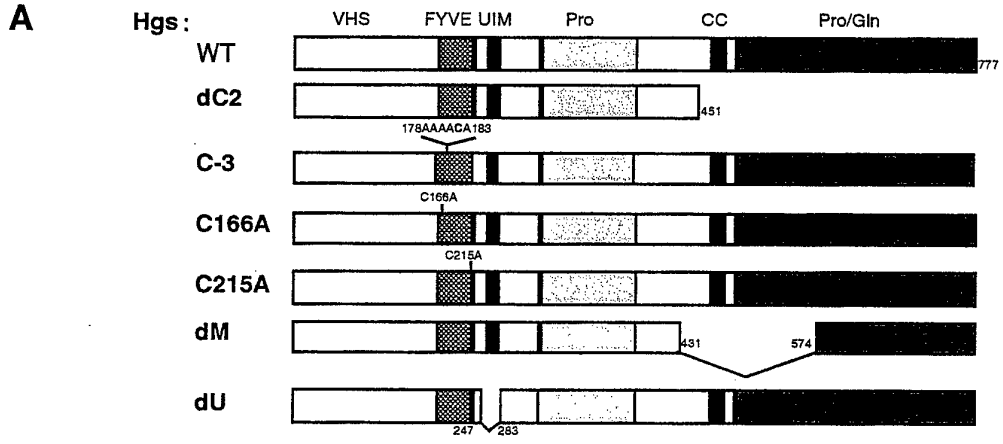
Figure 1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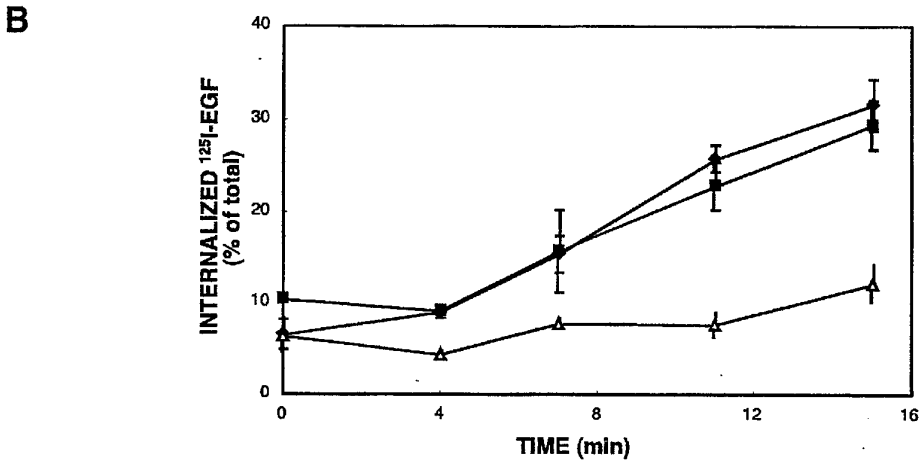
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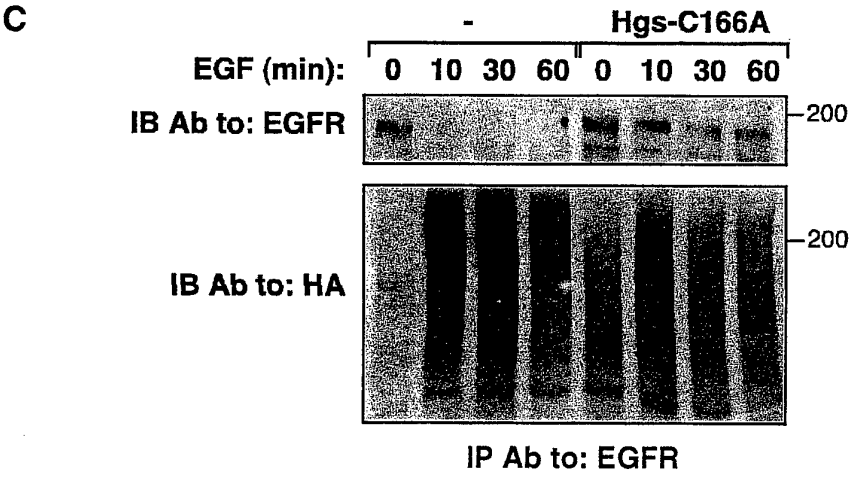
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Figure 2

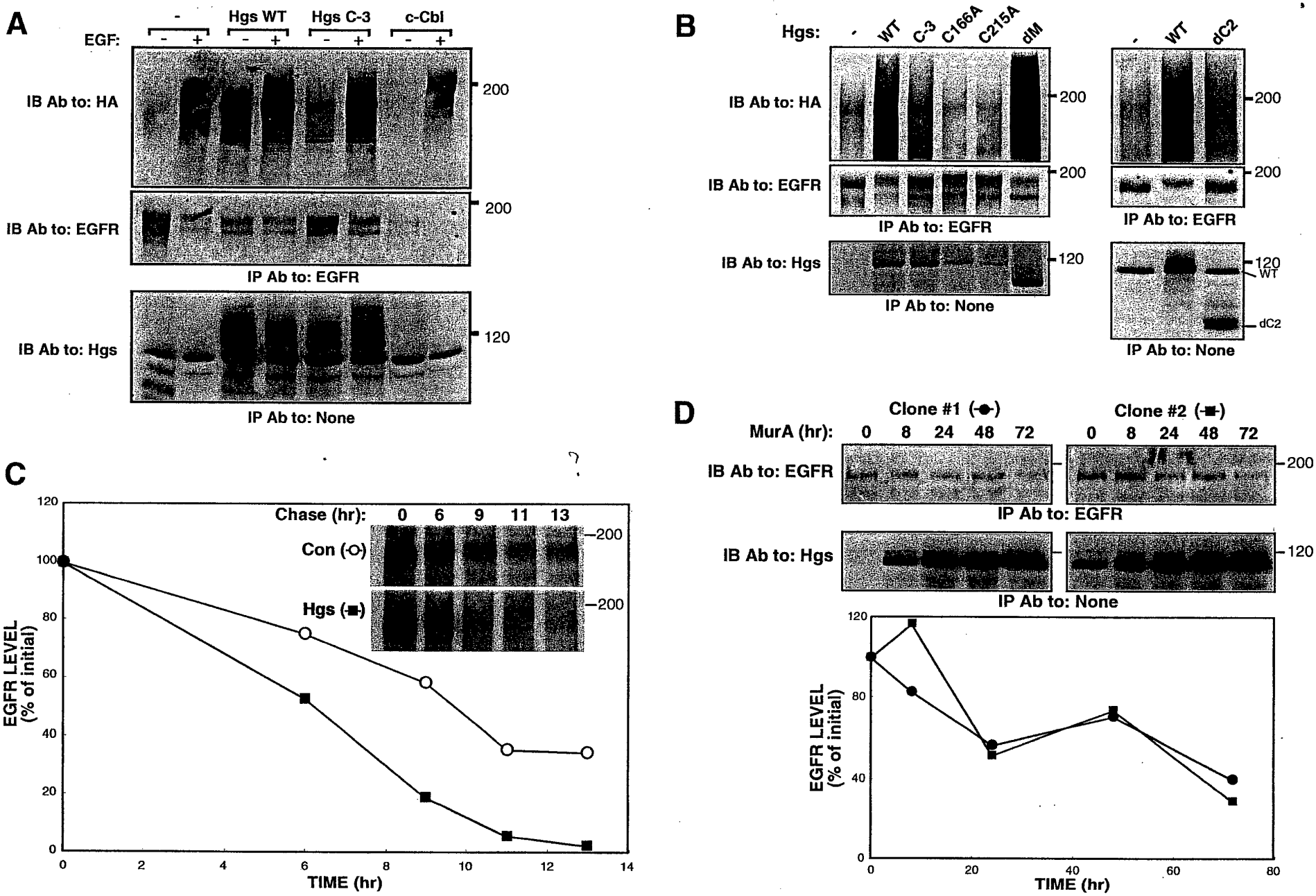
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Figure 3

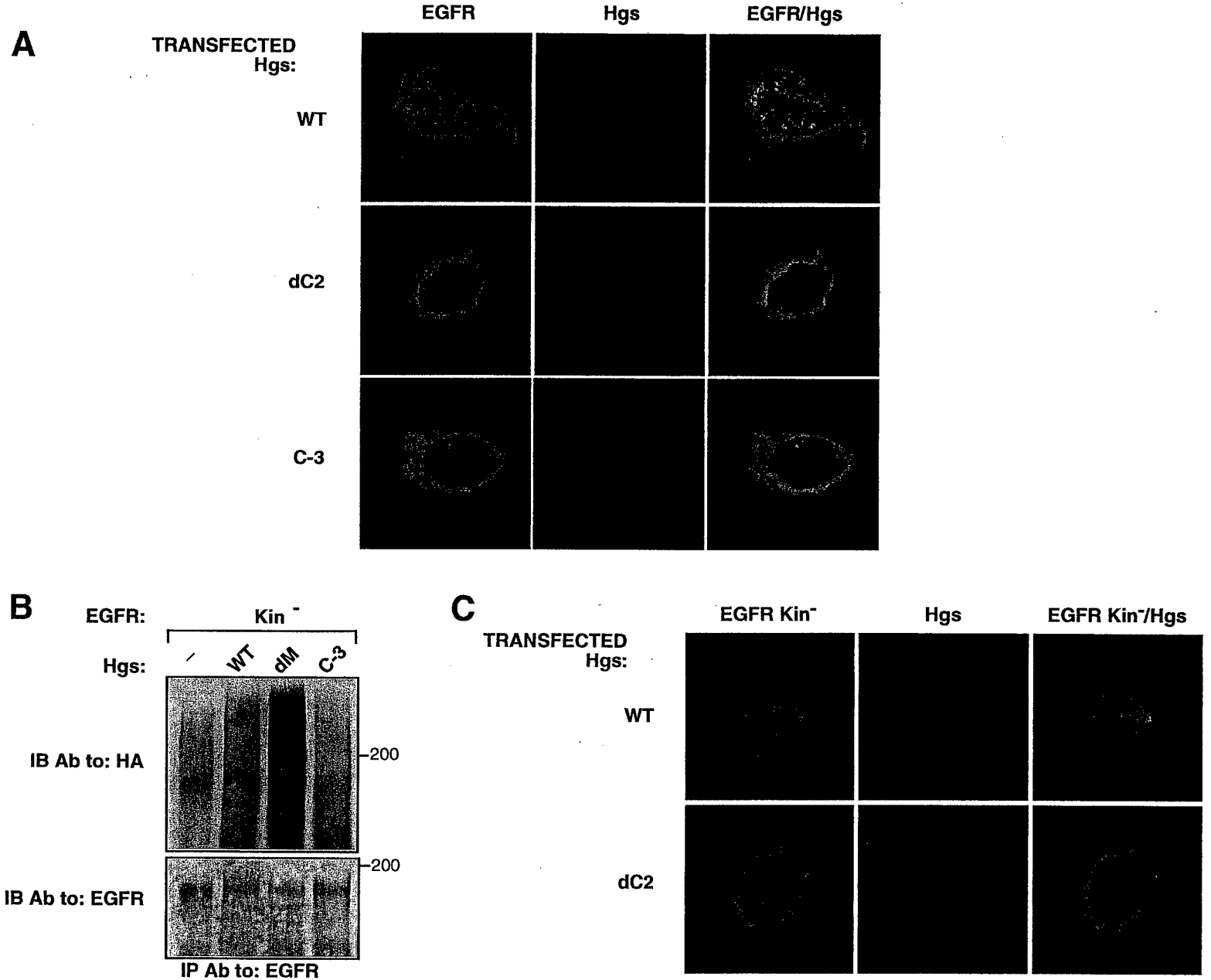
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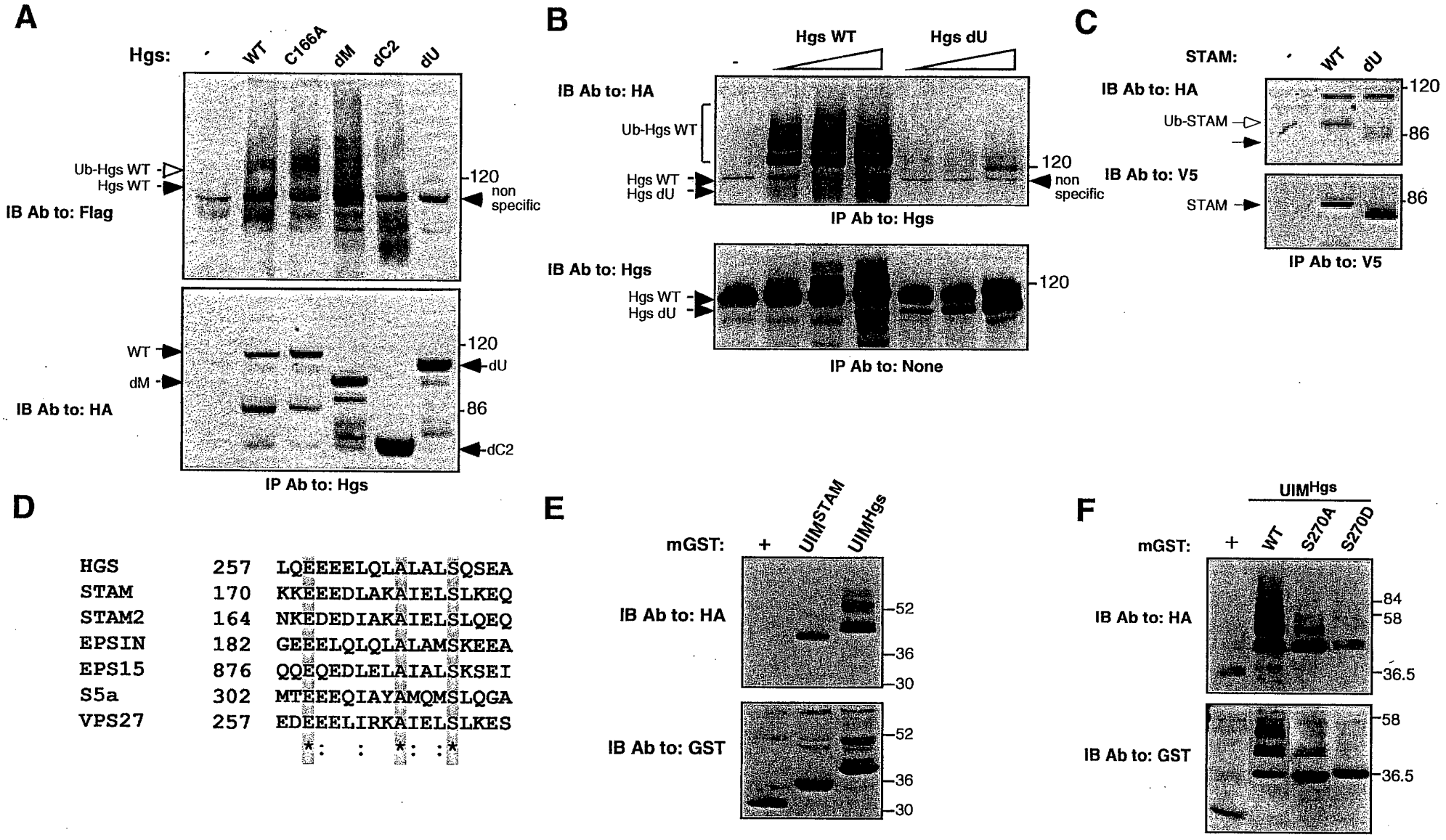
Figure 4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mGST:
+
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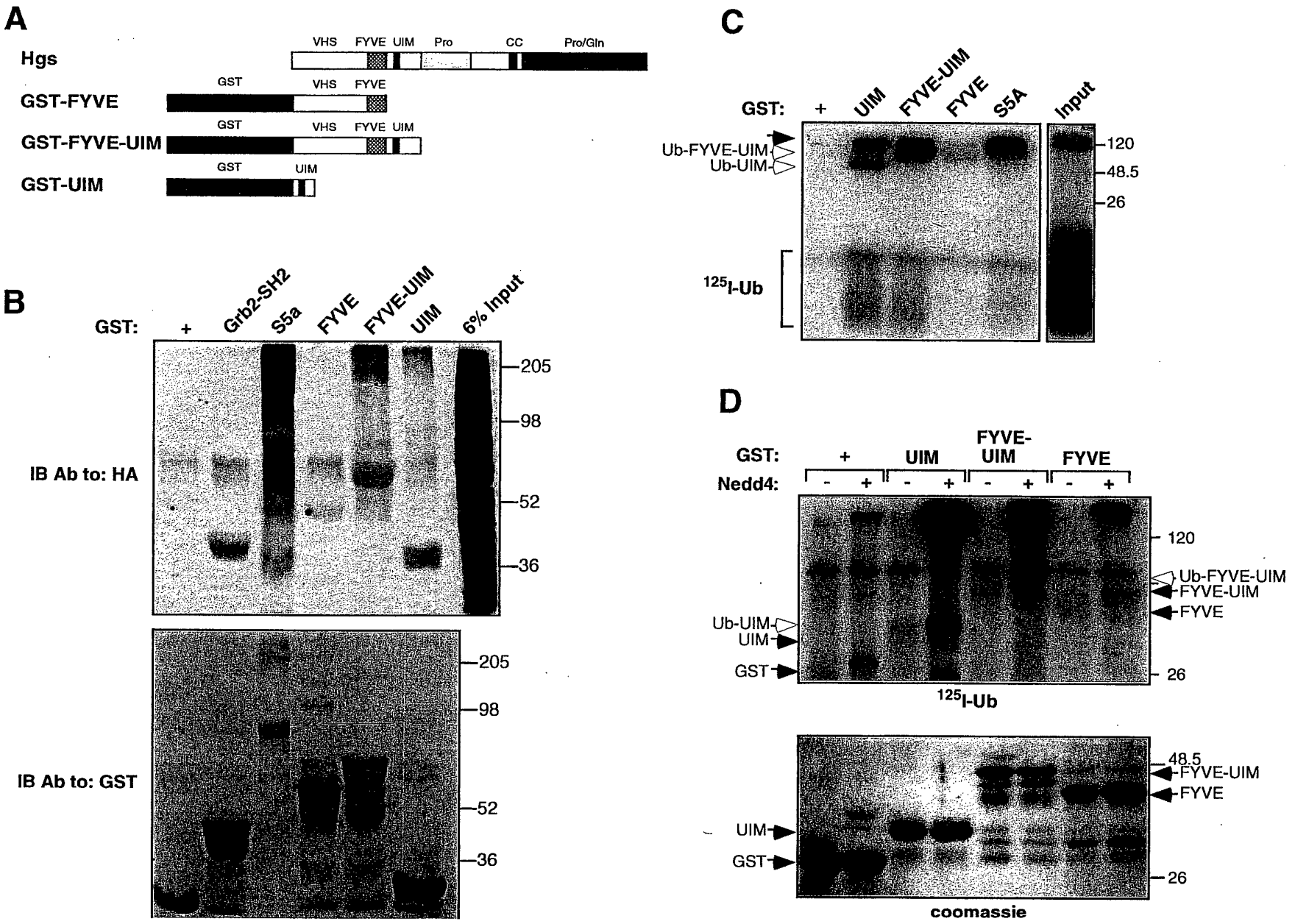
Figure 5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98
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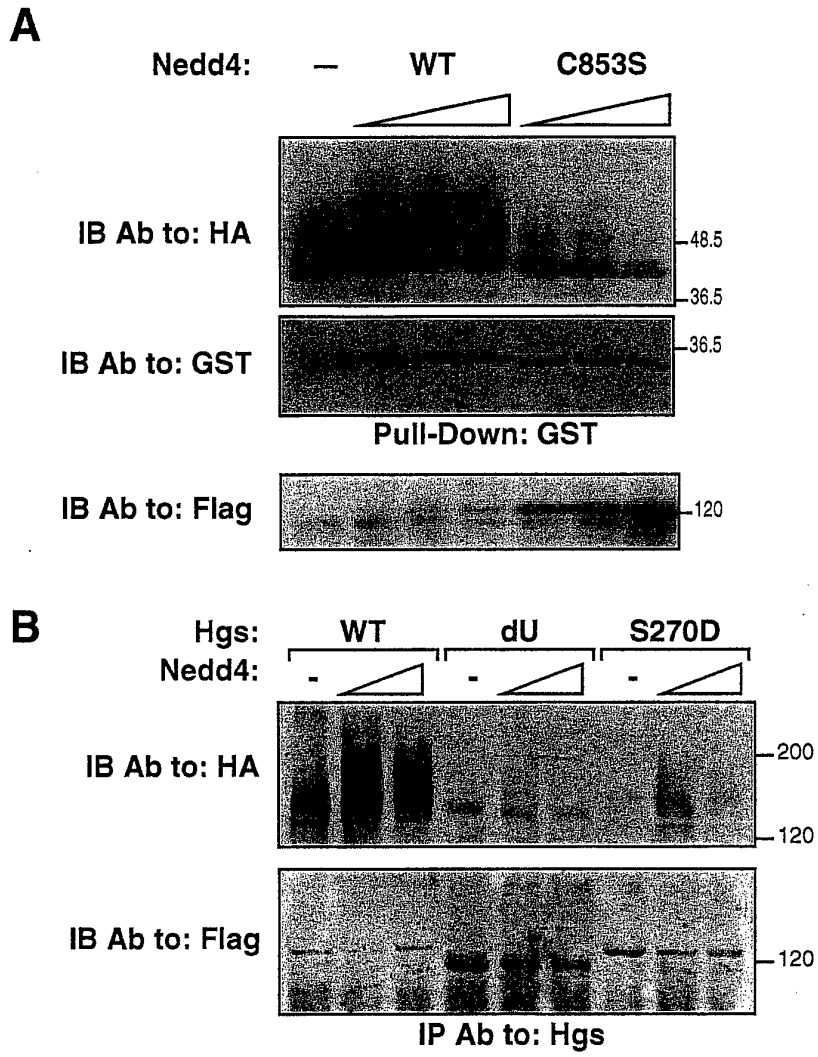
Figure 6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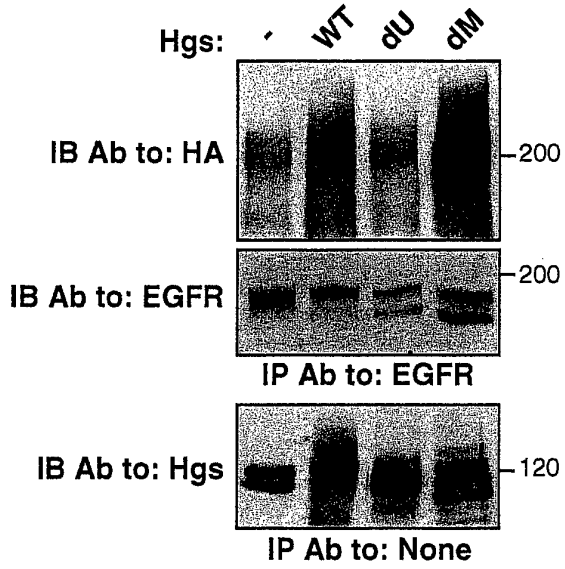
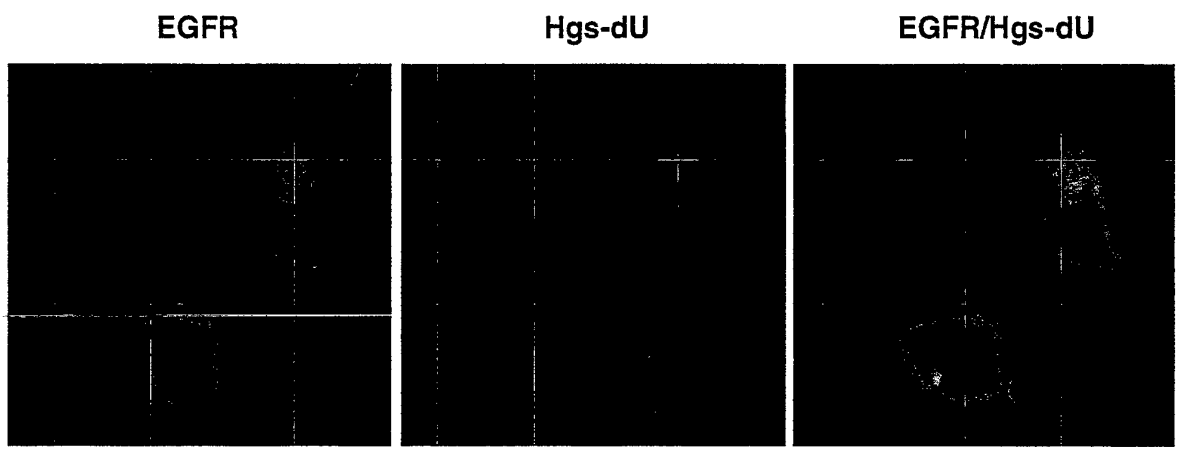
48.5
36.5
IB Ab to: GST
36.5
Pull-Down: GST
IB Ab to: Flag
120
B
Hgs:
WT
dU
S270D
Nedd4:
-
-
-
IB Ab to: HA
200
120
IB Ab to: Flag
120
IP Ab to: Hgs

Figure7

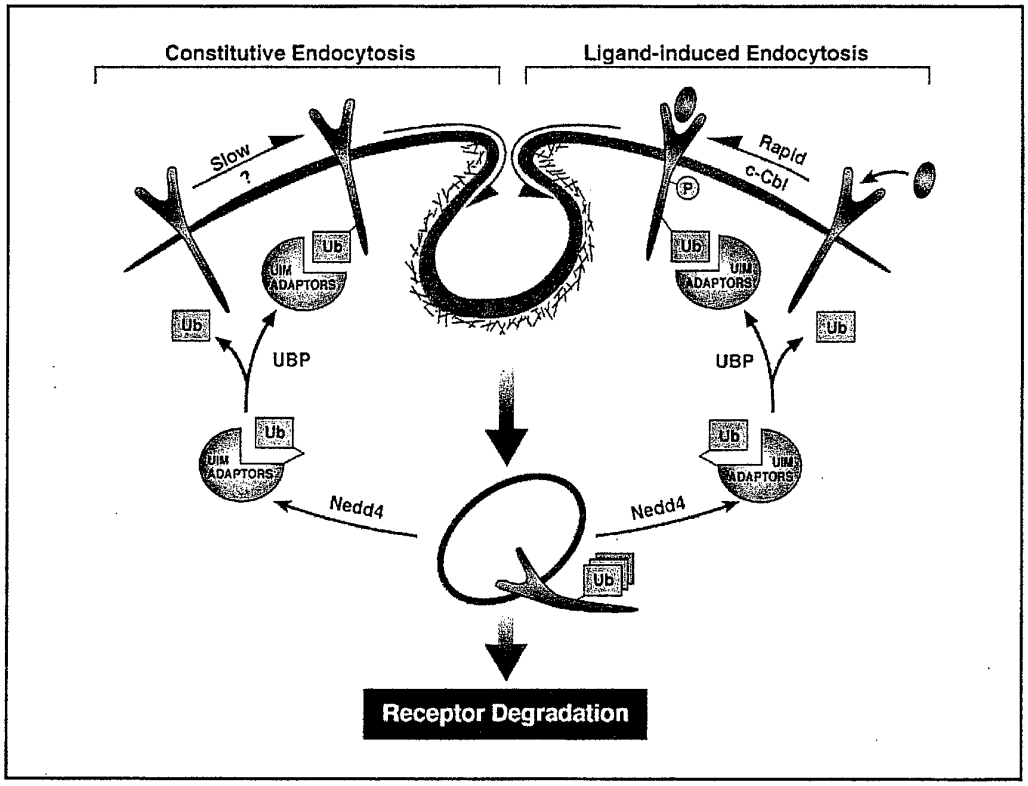
A


Hgs:
-
WT
dU
dM
IB Ab to: HA
200
IB Ab to: EGFR
200
IP Ab to: EGFR
IB Ab to: Hgs
120
IP Ab to: None

B


EGFR
Hgs-dU
EGFR/Hgs-dU

C


Constitutive Endocytosis
Ligand-induced Endocytosis
Slow
?
Rapid
c-Cbl
P
Ub
UIM ADAPTORS
UBP
Nedd4
Receptor Degradation