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Intersectin Regulates Epidermal Growth Factor Receptor Endocytosis, Ubiquitylation, and Signaling[S]

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ABSTRACT

Receptor tyrosine kinases (RTKs) are critical for normal cell growth, differentiation, and development, but they contribute to various pathological conditions when disrupted. Activation of RTKs stimulates a plethora of pathways, including the ubiquitylation and endocytosis of the receptor itself. Although endocytosis terminates RTK signaling, it has emerged as a requisite step in RTK activation of signaling pathways. We have discovered that the endocytic scaffolding protein intersectin (ITSN) cooperated with epidermal growth factor receptor (EGFR) in the regulation of cell growth and signaling. However, a biochemical link between ITSN and EGFR was not defined. In this study, we demonstrate that ITSN is a scaffold for the E3 ubiquitin ligase Cbl. ITSN forms a complex with Cbl in vivo mediated by the Src homology (SH) 3 domains binding to the Pro-rich COOH terminus of Cbl. This interaction stimulates the ubiquitylation and degradation of the activated EGFR. Furthermore, silencing ITSN by RNA interference attenuated EGFR internalization as well as activation of the extracellular signal-regulated kinase-mitogen-activated protein kinase pathway, thereby demonstrating the importance of ITSN in EGFR function. Given the cooperativity between ITSN and additional RTKs, these results point to an important evolutionarily conserved, regulatory role for ITSN in RTK function that is necessary for both signaling from receptors as well as the ultimate termination of receptor signaling.

RTKs control a plethora of intracellular pathways involved in cell growth, differentiation, development, and apoptosis through translating extracellular stimuli into biochemical signals that alter cellular function. These receptors activate numerous signaling pathways, with the Ras-MAPK pathway as perhaps the most well characterized of these pathways. Upon ligand binding, receptors dimerize and undergo trans-autophosphorylation on tyrosine residues, thereby creating high-affinity binding sites for proteins with Src homology (SH)2 or phosphotyrosine binding domains (Pawson and Nash, 2003). These phosphotyrosine binding proteins recruit components of specific pathways such as Shc, Grb2, Src, phosphoinositide-3 kinase, phospholipase Cγ, or Cbl, thus ultimately affecting cell fate decisions regarding growth, differentiation, and apoptosis.

After activation, a receptor must be inactivated to prevent chronic stimulation of cells. Prolonged activation of RTKs resulting from receptor amplification, chromosomal translocation, or point mutations is associated with development and progression of numerous tumors (Blume-Jensen and Hunter, 2001). However, a number of feedback control mechanisms aid in attenuating activated RTKs, including Ser/Thr phosphorylation of the receptor or its substrates, activation of phosphatases, and endocytosis. Ubiquitylation also plays an important role in trafficking and degradation of RTKs by lysosomes. In the case of EGFR, monoubiquitylation is sufficient for receptor internalization, although more recent studies suggest that EGFR is monoubiquitylated at multiple sites (Haglund et al., 2003). c-Cbl is an E3 ubiquitin ligase responsible for

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ABBREVIATIONS: RTK, receptor tyrosine kinase; MAPK, mitogen-activated protein kinase; SH, Src homology; EGFR, epidermal growth factor receptor; TKB, tyrosine kinase binding; PRD, Pro-rich domain; EH, Eps15 homology; ITSN, intersectin; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; HA, hemagglutinin; GST, glutathione transferase; EGF, epidermal growth factor; PBS, phosphate-buffered saline; siRNA, small interfering RNA; BSA, bovine serum albumin; YFP, yellow fluorescent protein; JNK, c-Jun NH_2-terminal kinase; DS, Down syndrome.

the ubiquitylation of EGFR (Schmidt and Dikic, 2005). The amino-terminal SH2-like tyrosine kinase binding (TKB) domain of Cbl binds phosphotyrosine sites within the activated receptor, thereby allowing the RING finger domain of c-Cbl to catalyze the transfer of ubiquitin from an E2-ubiquitin complex to the EGFR. Ubiquitylation of EGFR results in the recruitment of endocytic proteins containing ubiquitin-interacting motifs such as Eps15 (de Melker et al., 2004). Overexpression of c-Cbl increases the degradation of EGFR, whereas dominant-negative mutants of Cbl with impaired ligase activity (i.e., v-Cbl or 70Z-Cbl) inhibit down-regulation of EGFR (Schmidt and Dikic, 2005). In addition to the TKB and RING finger domains, Cbl also contains a Pro-rich domain (PRD) that recruits a number of SH3-containing proteins such as Grb2, Nck, Src, and CIN85 (Schmidt and Dikic, 2005). Although these interacting proteins are important in Cbl regulation, deletion of the PRD of Cbl has mixed effects on the ability of Cbl to regulate RTKs (Petrelli et al., 2002). Thus, the details of how these interacting proteins regulate Cbl remain unclear.

Endocytosis is also important for the activation of specific signaling pathways although the mechanistic details of this activity remain unclear (for review, see Sorkin and Von Zastrow, 2002). For example, endocytosis is involved in the activation of MAPK by RTKs and G protein-coupled receptors (Ceresa and Schmid, 2000). Furthermore, endocytic vesicles provide a platform for compartmentalized activation of particular pathways (Sorkin and Von Zastrow, 2002). These findings suggest that endocytosis is both a positive mediator of cellular signaling as well as an attenuator of those signals.

We have demonstrated that the endocytic scaffolding protein ITSN stimulates mitogenic signaling pathways (Mohney et al., 2003). Like many signal transduction proteins, ITSN is composed of multiple protein-protein interaction domains, including two amino-terminal Eps15 homology (EH) domains, a central coiled-coil domain, and five tandem SH3 domains (Fig. 3C). Mammalian ITSNs also possess a longer splice product, termed ITSN-long, with an extended carboxyl terminus encoding an exchange factor domain specific for activating Cdc42 (Hussain et al., 2001). The presence of multiple protein-protein interaction domains in ITSN each with distinct ligand specificities suggests that ITSN acts as a scaffolding or adaptor protein that regulates various biochemical pathways. Our work and that of others supports this model (O'Bryan et al., 2001; Irie and Yamaguchi, 2002; Jenna et al., 2002; Mohney et al., 2003; Predescu et al., 2003; Zamanian and Kelly, 2003). However, the elucidation of these pathways and their relationship to endocytosis and signaling remain to be determined.

Although a link between ITSN and EGFR signaling has been described previously (Adams et al., 2000), the role of ITSN in EGFR function has not been defined. Here, we report that ITSN is necessary for internalization of the activated EGFR as well as activation of the ERK-MAPK pathway by the receptor. In addition, we report that ITSN associates with Cbl to coordinate the ubiquitylation and degradation of the EGFR. Finally, we demonstrate that ITSN cooperates with additional growth factors in the stimulation of transcription. These results reveal a prominent role for ITSN in the regulation of the EGFR and potentially other RTKs as well.

Materials and Methods

Cell Lines and Transfection. HEK293T, COS-1, and A431 cells were maintained according to standard protocols. HEK293T cells were transfected by the calcium phosphate precipitation method as described previously (Mohney et al., 2003). COS-1 and A431 were transfected with Lipofectamine (Invitrogen, Carlsbad, CA) and FuGENE6 (Roche Diagnostics, Indianapolis, IN) as recommended by the manufacturers.

DNA Constructs. cDNA constructs encoding the various ITSN proteins were described previously (Mohney et al., 2003). Hemagglutinin (HA) epitope-tagged wild-type human c-Cbl and Cbl mutant constructs were gifts from Drs. Yosef Yarden (Weizmann Institute of Science, Rehovot, Israel) and Ivan Dikic (Johann Wolfgang Goethe University, Frankfurt, Germany) and have been described previously (Levkowitz et al., 1999; Kowanetz et al., 2003) and in Supplemental Materials. Glutathione transferase (GST)-tagged SH3 proteins consisting of the individual ITSN SH3 domains were purified as described previously (Mohney et al., 2003).

In Vitro Binding Assays. Lysates from HEK293T cells either nonstimulated or stimulated with EGF were prepared as described below. Binding to Cbl was determined as described previously (Mohney et al., 2003).

Generation of Anti-ITSN SH3 Antibodies. ITSN antibodies were generated by immunizing rabbits with GST-SH3D derived from *Xenopus laevis* ITSN (Mohney et al., 2003). This antibody recognizes human, mouse, rat, and *X. laevis* ITSN isoforms (both short and long). However, this antibody has weak to no reactivity for ITSN2 proteins.

Immunoprecipitation and Western Blot Analysis. Lysates were prepared as described previously (Oldham et al., 2002). For analysis of ubiquitylation, lysis buffer was supplemented with 5 mM *N*-ethylmaleimide. Unless noted, EGF was used at 100 ng/ml for stimulation of cells. Equal amounts of protein extracts were precleared then incubated with appropriate antisera for 1 h. For immunoprecipitation of HA epitope-tagged proteins, a mouse anti-HA antibody (BAbCO, Richmond, CA) was used. Rabbit anti-Cbl antisera (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), specific for the carboxyl-terminal 15 amino acids of Cbl, was used to immunoprecipitate c-Cbl proteins. Endogenous EGFR was immunoprecipitated using Ab-13 antibody (NeoMarkers, Fremont, CA). Immunopurified proteins were then analyzed by SDS-polyacrylamide gel electrophoresis. Membranes used for detection of ubiquitylated proteins were denatured in 6 M guanidine-HCl, 20 mM Tris-HCl, pH 7.5, and 5 mM β-mercaptoethanol plus protease inhibitor at 4°C for 30 min; washed in PBS; incubated with methanol; and dried before incubation with ubiquitin antibody (Covance, Princeton, NJ). Blots were washed several times with Tris-buffered saline/Tween 20, probed with horseradish peroxidase-linked secondary antibody, and then developed with SuperSignal West Pico chemiluminescence reagents (Pierce Chemical, Rockford, IL).

For Western blotting of GST-pull-downs, 10 μl of in vitro mixes was electrophoresed on NuPAGE gels and then transferred to Immobilon-P membranes (Millipore, Billerica, MA). The upper halves of the membranes were probed with PY20 antibodies and then stripped and reprobed for endogenous Cbl. The lower halves of the membranes were probed with horseradish peroxidase-linked rabbit anti-GST antisera (Santa Cruz Biotechnology, Inc.). Signals were developed as stated above.

Internalization Assays. HEK293T cells were stably transfected with EGFR and maintained in selection media. Cells were transfected with ITSN small interfering RNA (siRNA) or scrambled siRNA as a control (see Supplemental Materials for sequences). Twenty hours after transfection, cells were split into 24-well plates and serum-starved overnight (1 ml of Dulbecco's modified Eagle's

medium per well). Forty-eight hours after-transfection, cells were incubated with ^{125}I-EGF (1 ng/ml) on ice and later moved to 37°C for the times indicated in Fig. 6. Surface-bound and internalized EGFR was determined according to standard protocol (Haglund et al., 2003). In brief, media in each well were replaced with 250 μl of block solution containing Dulbecco's modified Eagle's medium, 25 mM HEPES, and 0.1% BSA, and plates were placed on ice for 30 min. Cell surface receptors were coated with ^{125}I-EGF (1 ng/ml) in block solution (250 μl/well) for 1 h. Duplicate wells received ^{125}I-EGF (1 ng/ml) and unlabeled EGF (100 ng/ml) for later determination of nonspecific binding. Next, cells were either lysed immediately, or the media in each well were replaced with 500 μl of block solution, and cells were incubated at 37°C for appropriate amount of time before being lysed. The total radioactivity in cells that were lysed initially was used as total bound. After incubation at 37°C and endocytosis of radioligand, cells were washed twice with PBS, acid-washed twice (10 min/wash) in 50 mM acetic acid and 150 mM NaCl, and lysed in 0.1% SDS and 0.5 M NaOH. Samples were all prepared in duplicate wells. Nonspecific binding was subtracted for each sample at each time point. The amount of internalized receptor was determined as a ratio of internalized radioactivity compared with total cell-associated radioactivity.

Confocal Microscopy. Transfected cells were plated on glass-bottomed plates and serum-starved overnight. The following day, media were removed, and cells were washed with ice-cold serum-free media containing 20 mM HEPES, pH 7.5, and 1 mg/ml BSA placed on ice for 15 to 30 min to stop endocytosis. Labeled EGF (Alexa Fluor555; Invitrogen) was added to media (C final = 1 μg/ml), and cells remained on ice for an additional hour. For all time points (5, 15, 30, and 60 min) except for the "zero" time point, media were replaced with warm serum-free media, and cells were shifted to 37°C to start endocytosis. Cells were placed on ice after the indicated times and washed two times with ice-cold PBS. For the 5- to 60-min samples, duplicate wells were washed twice with 0.2 M acetic acid and 0.5 M NaCl for 8 min followed by two washes with PBS. Cells were fixed in 4% formaldehyde and permeabilized with PBS + 3% BSA + 0.1% Triton X-100 (block solution) either at room temperature for 1 h or overnight at 4°C. For immunostaining of tagged proteins, primary antibodies (either anti-SH3 for ITSN, monoclonal anti-HA) were added to samples for 1 h at room temperature. Samples were washed with PBS and then incubated with secondary antibody (anti rabbit/mouse-fluorescein isothiocyanate-, tetramethylrhodamine B isothiocyanate-, or AlexaFlour350-conjugated) prepared in block solution and added to each sample for 1 h at room temperature. After three washes with PBS, a Zeiss LSM 510UV (Carl Zeiss, Thornwood, NY) was used for confocal microscopy.

Reporter Assays. HEK293T cells were transfected with reporter plasmids, and luciferase activity was measured as described previously (Mohney et al., 2003). Small interfering RNAs were used as described for internalization assays (see Supplemental Materials for sequences).

Results

ITSN Regulates EGFR Ubiquitylation and Degradation. In addition to its role as an endocytic scaffold, ITSN stimulates specific signaling pathways in cells leading to

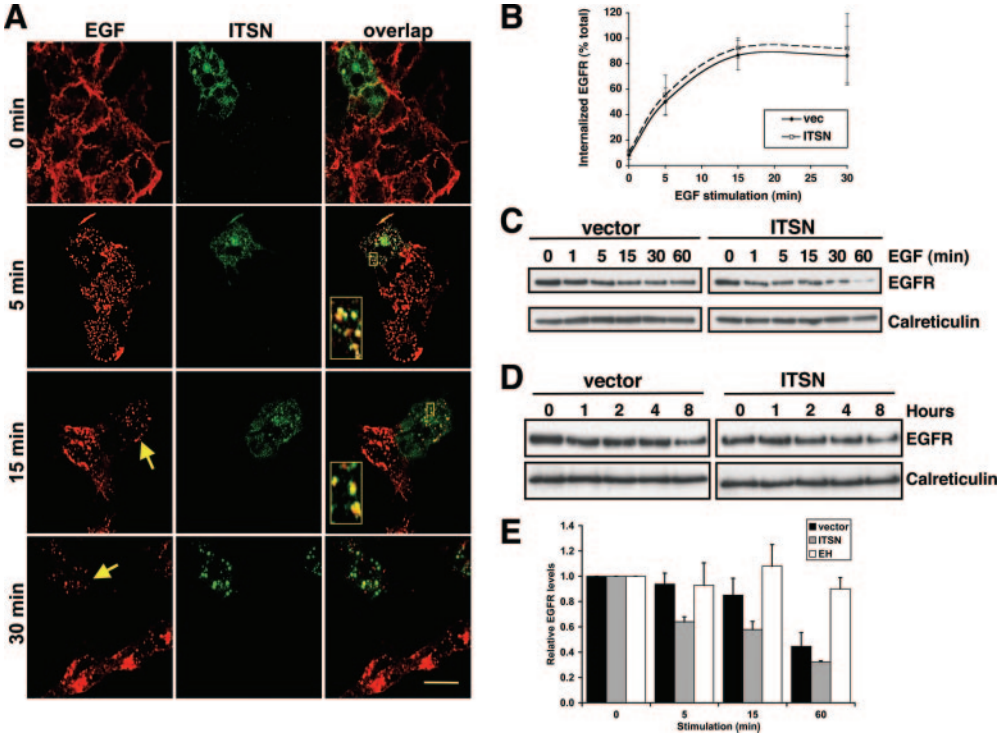

Fig. 1. ITSN enhances EGFR trafficking and degradation. A, transiently transfected COS-1 cells (YFP-ITSN, green) were stimulated with EGF-Alexa555 (red) for the indicated times. For the 5- and 15-min time points, an enlarged area indicating overlap of endogenous EGFR and YFP-ITSN is shown in the inset. The arrows point to YFP-ITSN-expressing cells that have decreased levels of internalized EGF-Alexa555 compared with cells in which YFP-ITSN is not expressed. The results shown are representative images of 100 captured frames from five independent experiments. Scale bar, 20 μm. B, ITSN overexpression does not alter EGFR internalization. Uptake of ^{125}I-EGF was quantitated in cells transfected with vector (vec) or ITSN (ITSN). The graph represents the average of two independent experiments performed in duplicate ± S.E.M. C, EGFR turnover is enhanced by ITSN. Endogenous EGFR levels were measured in 10 μM cycloheximide-treated COS-1 cells transfected with empty vector or ITSN. After stimulation with EGF for the indicated times, EGFR levels were monitored by Western blot analysis of cell lysate. Calreticulin was used as a control for loading. D, same as C except cells were unstimulated and lysed at the indicated times in hours. ITSN expression did not alter EGFR turnover in the absence of stimulation. E, same as C except cells were transfected with either empty vector, ITSN, or an ITSN truncation mutant lacking the coiled-coil and SH3 domains (EH). Changes in EGFR levels were determined by densitometry using NIH Image (http://rsb.info.nih.gov/nih-image/) and normalized to calreticulin levels. The results represent the average of two independent experiments.

oncogenic transformation and cooperates with the EGFR in these events (Adams et al., 2000; Mohney et al., 2003). Given the association of ITSN with numerous components of the endocytic machinery and its role as a stabilizing scaffold in the process of endocytosis (Koh et al., 2004), we postulated that ITSN may cooperate with RTKs through modulating the trafficking and signaling of these receptors. To address this question, we transiently expressed YFP-tagged ITSN in COS cells and then examined the effect of ITSN on trafficking of the EGFR in individual cells. Given the cell-to-cell variation in expression of transfected proteins, we specifically chose cells that expressed low levels of the transfected construct and that were also adjacent to cells not expressing YFP-ITSN. This approach allowed for comparison of EGF internalization in YFP-ITSN-expressing and -nonexpressing cells in the same microscopic field and also decreased the likelihood of inhibitory effects due to vast overexpression of this scaffold protein (Ferrell, 2000). Overexpression of ITSN did not affect binding of Alexa555-EGF (Fig. 1A) or total EGFR levels (Fig. 1, C and D). Shifting cells to 37°C to promote endocytosis resulted in rapid internalization of the Alexa555-EGF into endocytic vesicles as apparent by the disappearance of surface fluorescence and formation of numerous cytoplasmic vesicles. Alexa555-EGF and ITSN colocalization on these vesicles was evident at 5 and 15 min after the shift to 37°C. However, at 15, 30, and 60 min (data not shown), we observed significantly reduced Alexa555-EGF fluorescence in YFP-ITSN cells compared with cells without YFP-ITSN. This result suggested that ITSN expression either blocked internalization of the receptor (and ligand) or promoted more rapid trafficking and degradation of the complex by the lysosome. To distinguish between these possibilities, we compared ^{125}I-EGF internalization in cells overexpressing ITSN versus vector control cells. Overexpression of ITSN had no significant effect on ^{125}I-EGF internalization (Fig. 1B). However, analysis of EGFR turnover revealed that ITSN overexpression enhanced EGF-stimulated disappearance of the receptor compared with vector-transfected cells (Fig. 1C). In the absence of EGF, ITSN overexpression did not affect the steady-state levels of receptor (Fig. 1D). The effect of ITSN on EGF-stimulated receptor turnover was dependent on the full-length protein, because expression of the isolated EH domains did not enhance EGFR disappearance (Fig. 1E). These results indicated that ITSN did not alter the steady-state levels of receptor but rather enhanced ligand-dependent disappearance of the receptor.

To determine whether this disappearance was due to enhance degradation of the receptor in the lysosome, we pre-

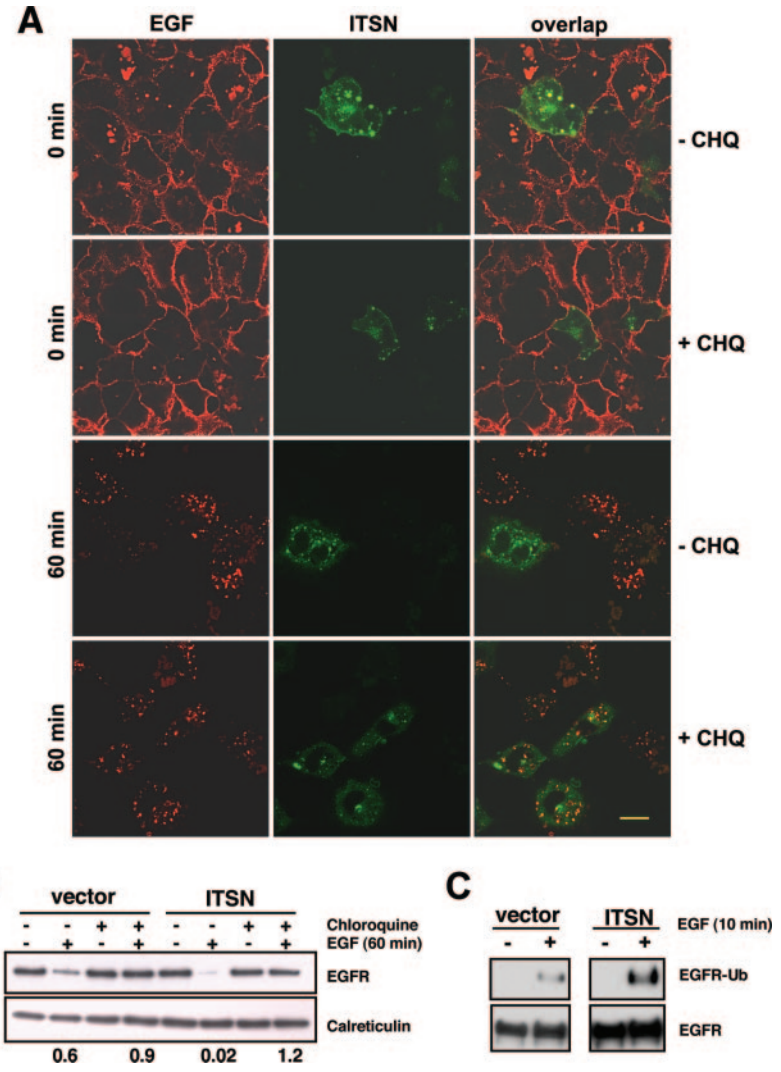

Fig. 2. ITSN-induced loss of EGFR is reversible by chloroquine treatment. A, the internalization assay, as described in Fig. 1, was carried out on cells treated with (+CHQ; 2 h; 500 μM) or without (−CHQ) chloroquine. Inhibition of the lysosome (+CHQ) results in equivalent Alexa555-EGF fluorescence in cells with or without ITSN overexpression. Scale bar, 20 μm. B, ITSN-stimulated turnover of EGFR levels is reversed by inhibition of the lysosome. Cells were treated as in Fig. 1C except chloroquine was added where indicated. Cell lysates were probed with antibodies to EGFR (top) or calreticulin as a normalization control (bottom). Data are representative of three independent experiments yielding the same result. The fraction of EGFR remaining after stimulation is indicated below the blot. Each value is relative to the unstimulated counterpart. C, ITSN enhances EGF-stimulated ubiquitylation of the EGFR. A431 cells stably expressing ITSN or control cells transfected with empty vector were stimulated with (+) or without (−) EGF for 10 min. Western blot of EGFR immunoprecipitates probed with an anti-ubiquitin antibody (top) or an anti-EGFR antibody (bottom). Vector and ITSN samples were analyzed simultaneously on the same gel. Irrelevant samples were cropped from the figure for clarity. Data are representatives of at least three independent experiments yielding the same result.

treated cells with chloroquine (Fig. 2). Inhibition of lysosomal activity resulted in equivalent Alexa555-EGF signals in cells with or without YFP-ITSN expression (Fig. 2A, compare 60-min time points ± chloroquine). In addition, chloroquine treatment reversed the effect of ITSN overexpression on ligand-induced EGFR disappearance (Fig. 2B).

Given that EGFR internalization and trafficking are regulated in part through monoubiquitylation of the receptor (Haglund et al., 2003), we tested whether ITSN enhanced ubiquitylation of the receptor. Due to the low levels of EGFR in many cell types and the inherent difficulty in detecting endogenous ubiquitylation of cellular proteins, we turned to the A431 human carcinoma cell line, which overexpresses the endogenous EGFR because of genomic amplification. Stimulation of these cells with EGF increased ubiquitin conjugation to the activated receptor (Fig. 2C, lane 2). Overexpression of ITSN significantly enhanced EGFR ubiquitylation after stimulation (Fig. 2C, compare lane 2 with lane 4), suggesting that ITSN-regulated trafficking and degradation of activated EGFR through increasing the ubiquitylation of the activated receptor.

ITSN and Cbl Interact on Cytoplasmic Vesicles. Both genetic evidence in *Caenorhabditis elegans* and *Drosophila melanogaster* and biochemical studies in mammalian cells revealed that Cbl negatively regulates RTKs. Because the SH3 domains of ITSN bound Pro-rich peptides in vitro that resembled several of the sequences in Cbl, we examined whether Cbl and ITSN were associated in cells. Endogenous Cbl and ITSN colocalized on vesicles in cells (Fig. 3A) and were coimmunoprecipitated from mouse brain lysates (Fig.

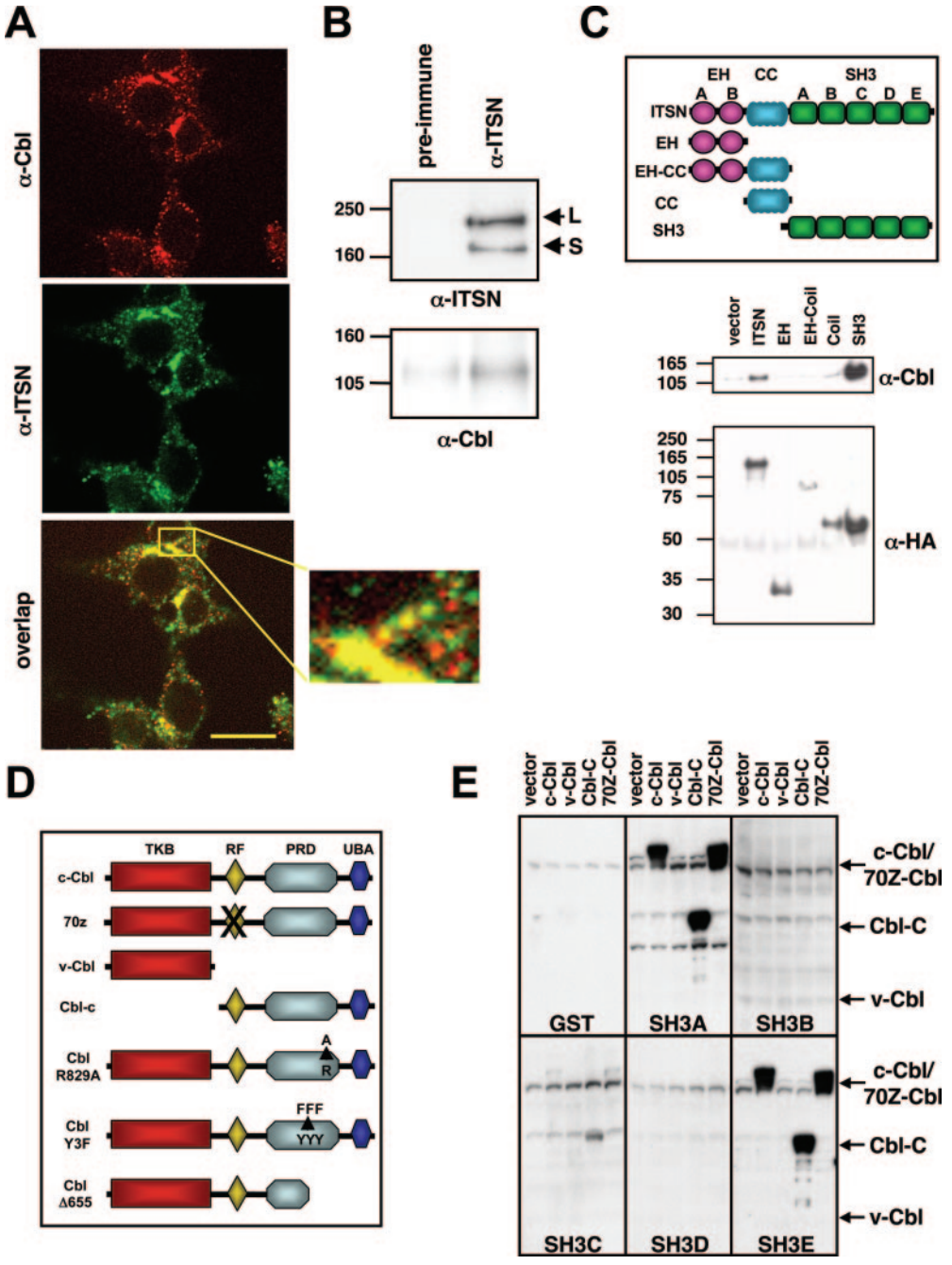

Fig. 3. ITSN colocalizes and coimmunoprecipitates with c-Cbl. A, Endogenous ITSN (green) and c-Cbl (red) colocalize on intracellular vesicles (yellow overlap) in HEK293T cells grown at 37°C. Scale bar, 20 μm. B, endogenous ITSN was immunoprecipitated from mouse brain tissue using antibodies against ITSN or preimmune serum as a control. Immunoprecipitates were probed for ITSN (top) and c-Cbl (bottom) as described under *Materials and Methods*. The two ITSN immunoreactive bands represent the short (S) and long (L) isoforms, which are both present in brain. C, ITSN expression constructs (top). All proteins possess an NH_2-terminal HA epitope tag. Association of endogenous c-Cbl with ITSN (bottom). Full-length or truncation mutants of ITSN were immunoprecipitated with anti-HA antibodies from extracts of HEK293T cells followed by Western blot analysis for endogenous c-Cbl. Expression of the various ITSN proteins is shown (bottom). The results are representative of three independent experiments. Similar results were also obtained with HA-tagged *X. laevis* ITSN (data not shown). D, Cbl expression constructs. All proteins possess an NH_2-terminal HA epitope tag. CblY3F is mutated at Tyr 700, 731, and 774. The Cbl domains are as follows: TKB, SH2-like tyrosine kinase binding domain; RF, RING finger E3 ligase domain; PRD, Pro-rich domain; and UBA, ubiquitin-associated domain. E, Cbl proteins were immunoprecipitated with HA antibody and then analyzed for binding to either GST or GST fusions with the individual SH3 domains of ITSN by Far Western analysis. Arrows indicate the positions of Cbl proteins. The GST probe used is indicated in each box. All the Cbl proteins were expressed equally well (data not shown). These results are representative of three independent experiments.

3B). Using truncation mutants of ITSN, we observed that the region encoding the five SH3 domains was sufficient for coimmunoprecipitation of endogenous Cbl (Fig. 3C). Far Western analysis of cell lysates expressing various truncation mutants of Cbl (Fig. 3D) revealed specificity in the interaction of the individual SH3 domains with Cbl. SH3A, -C, and -E each bind Cbl, although SH3A and -E seem to bind more avidly (Fig. 3E). Similar results were obtained using these same bacterially expressed GST fusions to purify Cbl from cell lysates (Supplemental Fig. 1A). Although the first Pro-rich sequence in Cbl (amino acids 491–504) represents a consensus binding site for SH3A of ITSN (Tong et al., 2000), mutation of the Pro residues in this sequence did not significantly decrease binding of ITSN (data not shown), further highlighting the multivalent nature of this interaction. Using a series of Cbl deletion constructs, we mapped the interaction of ITSN with Cbl to the carboxyl-terminal PRD domain (Fig. 3E; Supplemental Fig. 1B). Full-length ITSN (data not shown) and the isolated SH3 region (i.e., SH3s A–E) specifically coprecipitated c-Cbl, 70Z-Cbl, or Cbl-C (Supplemental Fig. 1B). Another SH3-containing protein, CIN85, also associates with Cbl and endophilin to down-regulate EGFR after ligand binding (Soubeyran et al., 2002). Mutation (CblR829A) or truncation (CblΔ655) of Cbl to remove the CIN85 binding site did not alter interaction of the SH3A domain of ITSN with Cbl (Supplemental Fig. 1C), indicating that ITSN and CIN85 bind distinct sites in Cbl.

EGF stimulation increased association of Cbl and CIN85, and this increase was abrogated by mutation of the Cbl phosphorylation sites (Y700/731/774F; Cbl Y3F) (Soubeyran et al., 2002). However, association of ITSN and Cbl was unaffected by mutation of these tyrosine residues (Cbl Y3F) consistent with the lack of effect of EGF on ITSN-Cbl association (Supplemental Fig. 1C; data not shown). Indeed, Cbl and ITSN were constitutively associated in HEK cells (Fig. 3A), and this interaction was not altered by EGF stimulation (data not shown). Given the rapidity with which clathrin-coated pits are released from the plasma membrane, we examined whether incubation of cells on ice to inhibit endocytosis and trafficking (Stang et al., 2004) might reveal any difference in Cbl localization after EGF stimulation in the presence or absence of ITSN overexpression. In contrast to cells grown at 37°C (Fig. 3A), Cbl was localized in a diffuse pattern in the absence of ITSN overexpression and relocalized to vesicles upon overexpression of ITSN (Fig. 4, top) (Stang et al., 2004). As seen with HEK cells, stimulation with EGF at 4°C did not alter the association of ITSN and Cbl (Fig. 4). However, we observed an increase in the localization of the ITSN-Cbl double-positive vesicles near the plasma membrane upon growth factor stimulation (Fig. 4, bottom). These results suggested that ITSN promoted the accumulation of vesicles at or near the plasma membrane that are Cbl-ITSN-positive.

Next, we investigated whether this change in localization resulted in higher levels of Cbl phosphorylation or extended association with EGFR. It is noteworthy that ITSN had no effect on Cbl association with EGFR or on EGF-induced phosphorylation of Cbl (data not shown). These results demonstrate that ITSN did not alter Cbl association with the receptor.

ITSN Enhances Cbl-Induced Ubiquitylation and Degradation of the EGFR. Cbl-mediated ubiquitylation of the EGFR resulted in trafficking of the activated receptor to the lysosome for degradation (Schmidt and Dikic, 2005). To determine the relevance of ITSN-Cbl interaction, we examined the effect of coexpression of these two proteins on EGFR ubiquitylation and turnover. Immunocytochemical analysis revealed that ITSN, Cbl, and EGFR colocalized on intracellular vesicles (Fig. 5A; Supplemental Fig. 2), although we were unable to immunoprecipitate ITSN with the EGFR (data not shown). These results suggest that this interaction between ITSN, Cbl, and EGFR may not be sufficient to allow for coimmunopurification. Expression of either Cbl or ITSN alone increased ligand-dependent ubiquitylation of EGFR; however, coexpression of ITSN and Cbl resulted in a synergistic enhancement of EGFR ubiquitylation (Fig. 5B). To determine whether ITSN and Cbl acted through the same or separate pathways, we coexpressed ITSN with a Cbl dominant-negative mutant (70Z-Cbl) that also binds ITSN (Fig. 3E). As illustrated in Fig. 5C, expression of 70Z-Cbl blocked the increase in EGFR ubiquitylation by ITSN. Thus, Cbl ligase activity was necessary for the effect of ITSN on EGFR ubiquitylation. Furthermore, coexpression of ITSN and Cbl decreased the half-life of the EGFR after stimulation compared with cells expressing ITSN or Cbl alone (Supplemental Fig. 3A). In contrast, expression of an ITSN mutant incapable of binding Cbl did not enhance EGFR turnover (Fig. 1E). These results indicate that ITSN enhanced EGFR degrada-

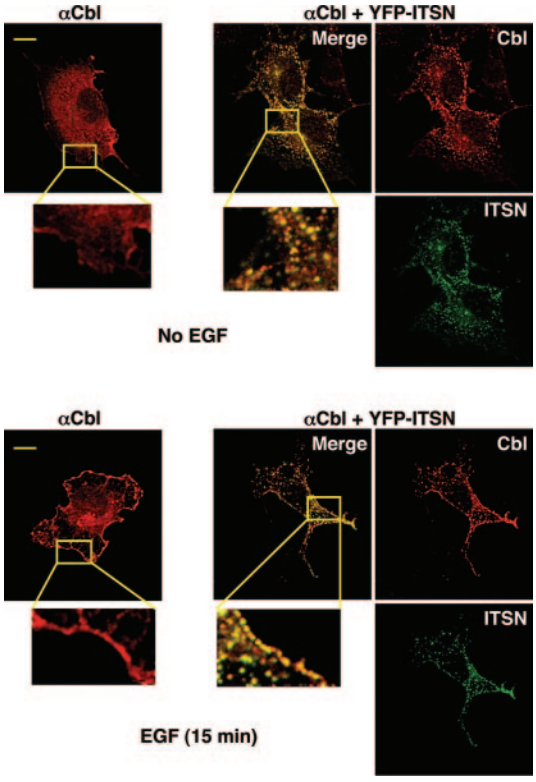

Fig. 4. EGF stimulation promotes relocalization of ITSN-bound c-Cbl proximal to the membrane. COS-1 cells transiently expressing HA-tagged Cbl and/or YFP-ITSN were serum-starved overnight and incubated on ice for 30 min before stimulation with EGF at 4°C (15 min; bottom). After stimulation, cells were fixed and stained as described under Materials and Methods. Cbl is diffusely localized throughout the cytosol in the absence of EGF stimulation (top) and relocalizes to the membrane after 15 min of EGF stimulation (bottom). Overexpression of YFP-ITSN relocalizes Cbl to vesicles (right) and these ITSN-Cbl vesicles accumulate near the plasma membrane after EGF treatment (bottom right), Scale bar, 20 μm.

tion through Cbl-induced ubiquitylation of the activated receptor.

Silencing ITSN Inhibits EGFR Endocytosis and Signaling. Our previous work demonstrated that ITSN cooperated with the EGFR to synergistically enhance transcriptional activation and transformation of cells (Adams et al., 2000). The mechanism for this cooperativity involved the mitogen-activated protein kinase kinase-ERK-MAPK pathway, even though ITSN itself did not activate this pathway (Adams et al., 2000). Endocytosis of the EGFR as well as certain G protein-coupled receptors is important for stimulation of the MAPK pathway (Ceresa and Schmid, 2000). Our data indicate that ITSN overexpression enhanced EGFR trafficking and signaling. To determine whether this result reflected the function of endogenous ITSN in EGFR endocytosis and signal transduction, we used RNA interference to decrease ITSN expression. Transfection of siRNA directed against ITSN resulted in a 50 to 75% reduction in ITSN protein and a corresponding decrease in EGFR internalization as indicated by the decrease in fluorescent EGF levels in cells after acid washing of the cells (Fig. 6, A and B; Supplemental Fig. 3B). This decrease in ITSN resulted in a significant decrease in both the extent and duration of ERK-MAPK activation after EGF stimulation (Fig. 6C) as well as a decrease in EGF-stimulated transcription (Fig. 6D). Vieira et al. (1996) reported a similar effect on EGFR signaling when endocytosis of the EGFR was blocked using a dominant-negative dynamin. These results indicated that ITSN was necessary for the internalization of activated EGFR and signaling from the receptor. This study represents the first demonstration that ITSN is an integral component in the regulation of EGFR endocytosis and signaling.

ITSN Cooperates with Additional Growth Factor Receptors. Given the common regulation of many RTKs and the activation of similar pathways by different RTKs, we tested whether ITSN might function in the regulation of additional RTKs. As observed with EGF, ITSN overexpression enhanced gene expression in response to stimulation with hepatocyte growth factor or basic fibroblast growth factor (Fig. 6E). These finding suggested that ITSN may play a general role in the regulation of a number of RTKs.

Discussion

Endocytosis plays an important role in regulating mitogenic signaling pathways (Sorkin and Von Zastrow, 2002). Indeed, internalized EGFR activates pathways regulating growth and survival, including Ras, ERK-MAPK, and phosphoinositide-3 kinase (Sorkin and Von Zastrow, 2002). Given the differential signaling of Ras at specific cellular compartments (Chiu et al., 2002), internalized receptors may activate distinct signals compared with plasma membrane-bound receptors. We have demonstrated that ITSN activated Ras on a subset of intracellular vesicles and that this pool of Ras did

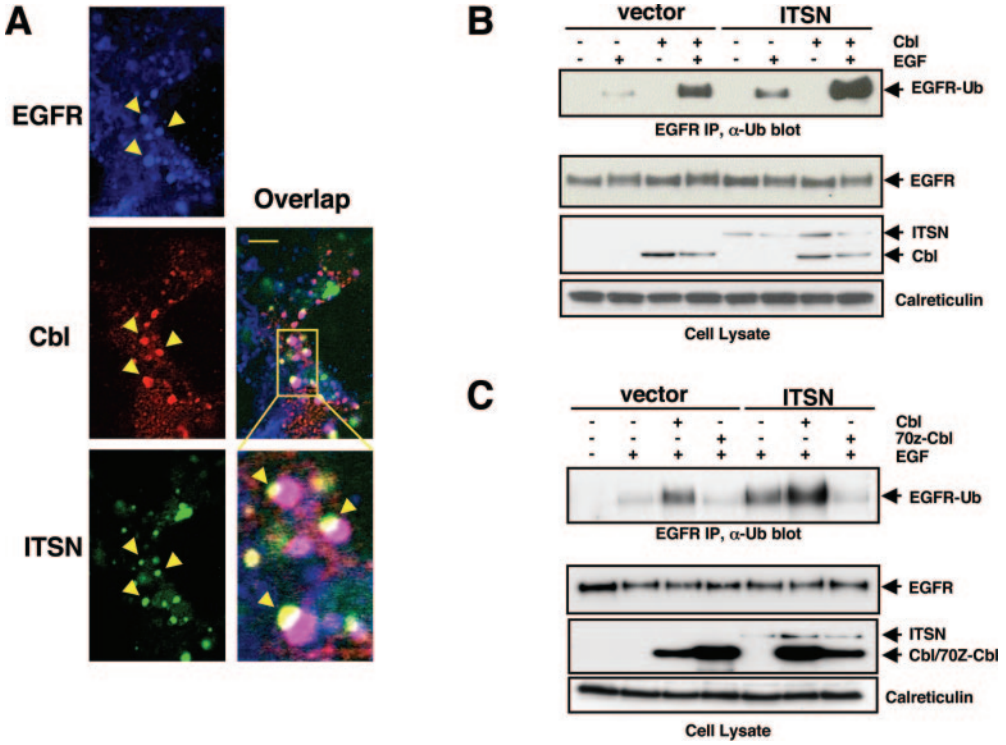

Fig. 5. ITSN stimulates EGFR ubiquitylation through Cbl. A, ITSN, c-Cbl, and EGFR are found in a complex in cells. A431 cells expressing YFP-ITSN (green) and HA-c-Cbl (red) were stimulated with EGF for 10 min, fixed, and stained with antibodies to c-Cbl (α-Cbl) and EGFR (α-EGFR). Colocalization of c-Cbl, ITSN, and EGFR is shown in the middle right panel. The inset has been enlarged (bottom right) to illustrate the overlap of all three proteins on enlarged vesicles. The existence of these vesicles was not due to overexpression of ITSN or Cbl, because they were also present in nontransfected cells (data not shown). Scale bar, 10 μm. Additional images are provided in Supplemental Fig. 2. B, ITSN enhances ubiquitylation of EGFR. A431 cells expressing HA-ITSN, HA-c-Cbl, or both proteins were stimulated with EGF for 10 min. EGFR was immunoprecipitated and analyzed for ubiquitin conjugation (top). Expression levels of EGFR, ITSN, and Cbl in cell lysates are shown in the bottom three panels. C, E3 ligase activity of Cbl is necessary for ITSN-induced EGFR ubiquitylation. EGFR ubiquitylation was measured as described in B in A431 cells transfected with HA-ITSN, HA-Cbl, and/or dominant-negative HA-70Z-Cbl and stimulated with EGF. Expression of ITSN, Cbl, and 70Z-Cbl is shown in the bottom panels. Calreticulin was used as a loading control in B and C.

not activate the ERK- or JNK-MAPK pathways (Mohney et al., 2003). Thus, endocytosis provides a means of compartmentalized activation of signaling pathways.

Our study demonstrates that ITSN associates with c-Cbl, a negative regulator of RTKs, and enhances c-Cbl-mediated ubiquitylation of the EGFR. Our data suggest that ITSN acts

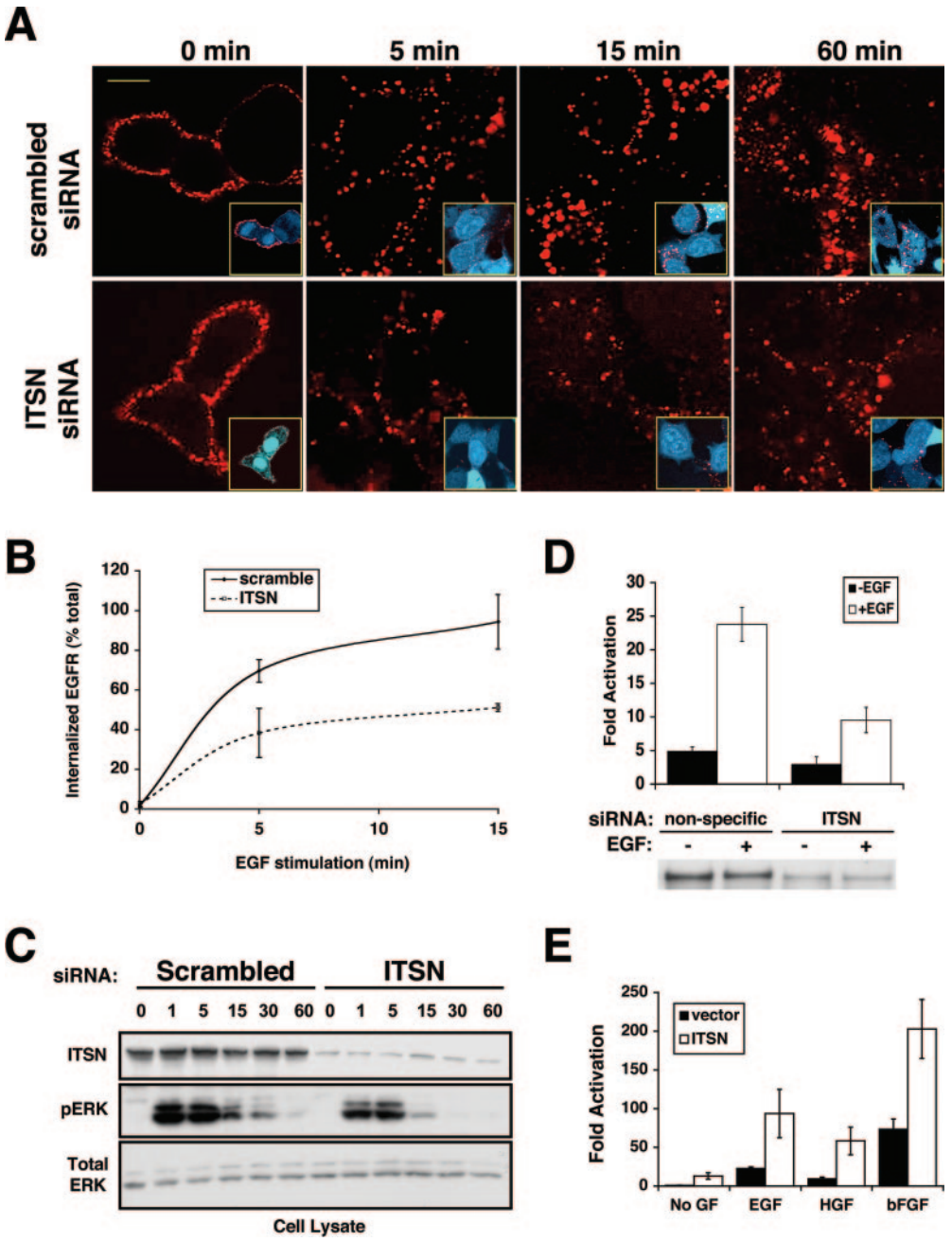

Fig. 6. Silencing of ITSN decreases EGFR trafficking and signaling. A, HEK293T cells stably transfected with EGFR were transiently transfected with either scrambled or ITSN silencing RNAs. Cyan fluorescent protein (inset) was cotransfected with the siRNA to identify transfected cells. Cells were stimulated for varying times (minutes) with EGF-Alexa555 (red), acid-washed (5- to 60-min samples), and then fixed. Pictures are representative of 100 images from three independent experiments. Scale bar, 20 μm. B, quantitation of EGFR internalization. Level of internalized ^{125}I-EGF was monitored as described under *Materials and Methods*. The graph represents the average of two independent experiments performed in duplicate. Similar results were obtained using a second ITSN siRNA targeting a different region of the mRNA (see Supplemental Fig. 3B). The differences in ^{125}I-EGF at the 5- and 15-min time points are statistically significant in a Student's t test ($p < 0.032$ and $p < 0.0011$, respectively). C and D, silencing ITSN decreased EGF stimulation of ERK-MAPK (C) and EGF activation of ELK-1-dependent transcription (D). C, HEK293T cells were transiently transfected with ITSN siRNAs or control scrambled siRNA. Cells were serum-starved overnight, stimulated with EGF for the indicated period of time (minutes), and then lysates were probed for ITSN, phospho-ERK, and total ERK as a control for loading. D, HEK293T cells were transiently transfected with siRNAs to ITSN or control siRNA to green fluorescent protein along with the Gal-Elk reporter constructs. Relative ELK activation was determined as described previously (Mohney et al., 2003). ITSN expression is shown in bottom panel. The results presented are the mean ± S.E.M. from at least three independent experiments performed in duplicate. E, ITSN potentiates ELK activation after growth factor stimulation. Cells were transfected with an expression construct encoding HA-ITSN (ITSN) or empty vector along with the Gal-Elk reporter plasmids as described previously (Mohney et al., 2003). Relative ELK activation in samples stimulated with 100 ng/ml EGF, 100 ng/ml hepatocyte growth factor (HGF), and 10 ng/ml basic fibroblast growth factor (bFGF) were determined as described previously (Mohney et al., 2003). The results presented are the mean ± S.E.M. from at least three independent experiments performed in duplicate.

to prime the cell for internalization of activated EGFR through recruitment of Cbl to vesicles (Fig. 4) where it promotes the ubiquitylation of the activated EGFR. This interaction is provocative given the ability of ITSN to synergize with EGFR in the activation of cellular signaling pathways and oncogenic transformation of cells (Adams et al., 2000). However, our results leave unanswered the question of whether the ITSN-Cbl interaction is necessary for the synergistic activation of signaling between ITSN and EGFR. Although ITSN cooperates with Cbl in enhancing EGFR turnover after stimulation, it is not clear whether this enhancement of EGFR trafficking to the lysosome enhances signaling from the receptor before its degradation. Nevertheless, this study represents the first demonstration that ITSN, through interaction with Cbl, functions in the covalent attachment of ubiquitin to the EGFR. Although the cooperativity of ITSN with multiple growth factors to stimulate transcription suggests a potential role for ITSN-Cbl in mediating this effect (Fig. 6E), additional studies will be needed to address this possibility.

ITSN regulates protein trafficking in part through assembly of the clathrin-coated vesicles (for review, see O'Bryan et al., 2001). However, our data indicate an additional role for ITSN in regulating receptor ubiquitylation. Thus, the decrease in EGFR internalization upon silencing ITSN may be due to a decrease in c-Cbl-directed ubiquitylation of EGFR, a decrease in functional endocytic complexes, or both. We favor the last possibility. ITSN has at least two roles in regulating EGFR trafficking. First, ITSN is necessary for assembly of endocytic complexes by recruiting endocytic accessory factors to the vesicle (Koh et al., 2004). The interaction of ITSN with epsin, endophilin, and arfaptin (data not shown), each of which possess domains that induce membrane curvature (Itoh et al., 2001; Kalthoff et al., 2001; Peter et al., 2004), suggests that ITSN is important for the invagination of the coated pit or in determining the size or shape of endocytic vesicles. Indeed, mutation of *D. melanogaster* ITSN, Dap-160, results in fewer endocytic vesicles, abnormally large vesicles, and an accumulation of endocytic intermediates (Koh et al., 2004). Second, ITSN acts through c-Cbl to regulate EGFR ubiquitylation, which is necessary for sorting of the receptor to the lysosome for degradation, although the mechanism by which ITSN regulates Cbl is not clear. Deletion analysis of Cbl revealed that truncation of sequences carboxyl to amino acid 440 does not impair the ability of Cbl to stimulate EGFR ubiquitylation and internalization (Levkowitz et al., 1999; Petrelli et al., 2002). However, point mutations in the PRD of Cbl (CblR829A) (Kowanetz et al., 2003) or the presence of amino acids 441 to 480 (Petrelli et al., 2002) attenuate Cbl activity, suggesting that the carboxyl terminus of Cbl may negatively regulate its activity. We propose the following model for ITSN in regulating RTK endocytosis (Fig. 7). ITSN binding to Cbl may relieve negative constraints on the ligase, such as those imposed by the Cbl-interacting proteins Sprouty2, Sts1/2, or Cool/Pix (Schmidt and Dikic, 2005). These interactions then allow Cbl to more efficiently ubiquitylate the activated RTKs, resulting in enhanced trafficking and degradation. We are currently investigating the effects of ITSN on the interaction of Cbl with the aforementioned proteins. In addition, ITSN facilitates the recruitment of additional components necessary for clathrin-coated pit formation (e.g., adaptor protein-2, epsin, and dynamin), thereby allowing for coordinate assembly and regulation of the internalization process.

ITSN shares similar activities in regard to Cbl binding and regulation of EGFR ubiquitylation with the SH3 protein CIN85, a regulator of Cbl function (Schmidt and Dikic, 2005). However, several observations suggest that these two proteins are not entirely redundant in function. Cbl possesses distinct binding sites for ITSN and CIN85 (see Supplemental Fig. 1), suggesting that both proteins may be necessary for Cbl regulation. Although ITSN and CIN85 each contain a coiled-coil domain and multiple SH3 domains, ITSN has two more SH3 domains than CIN85, thus allowing ITSN to associate with additional partners. Furthermore, ITSN possesses two N-terminal EH domains, thereby allowing for novel interactions through this domain (O'Bryan et al., 2001). It is noteworthy that we have identified CIN85 as an ITSN binding partner in yeast two-hybrid, suggesting that the interaction of these two proteins may be important for the dynamic regulation of Cbl function.

The importance of our results is underscored by the conservation of both ITSN and Cbl in higher eukaryotes (O'Bryan et al., 2001; Schmidt and Dikic, 2005). Although *D. melanogaster* (D-Cbl) and *C. elegans* (Sli-1) have been widely reported to lack the conserved SH3-binding sites found in mammalian c-Cbl and Cbl-b, these proteins do indeed possess several Pro-rich motifs, although fewer in number (Robertson et al., 2000). Both D-Cbl and Sli-1 possess a PPLPPR sequence that is nearly identical to an ITSN binding site in c-Cbl (PPVPPR). Indeed, D-Cbl interacts with Dap-160 (Robertson et al., 2000), suggesting an important role for this association. Although ITSN binds this Pro-rich sequence in vitro (data not shown), mutation of this site does not impair the interaction of ITSN with Cbl, consistent with the multivalent interaction of Cbl with SH3 domains of ITSN.

Although previous studies suggested that ITSN inhibited

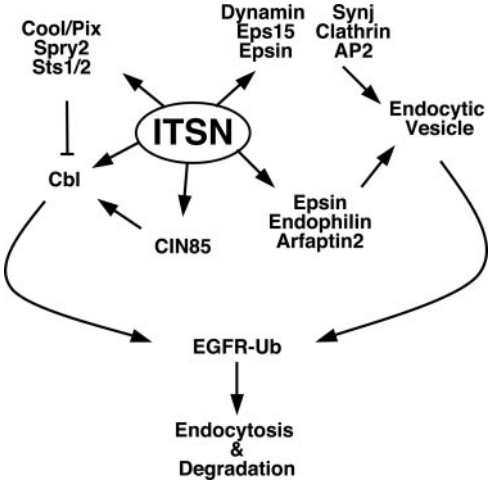

Fig. 7. Model for ITSN regulation of EGFR endocytosis and trafficking. ITSN interacts with Cbl to stimulate EGFR ubiquitylation. This increase in Cbl function may arise through enhancing the interaction of Cbl activators (e.g., CIN85) or blocking the interaction with Cbl inhibitors (e.g., Spry2, Sts1/2, or Cool/Pix). In addition, ITSN aids in assembling clathrin-coated vesicles through association with endocytic accessory proteins (e.g., Dynamin and Eps15) and proteins that induce membrane curvature (e.g., Epsin, Endophilin, and Arfaptin2). Together, these two functions of ITSN facilitate the endocytosis, trafficking, and degradation of the activated EGFR and possibly other RTKs. Arrows denote positive interaction; bars denote inhibitory interactions.

receptor endocytosis (Sengar et al., 1999; Pucharcos et al., 2000; Predescu et al., 2003), our data indicate that ITSN is a positive mediator of receptor endocytosis and trafficking. Silencing ITSN attenuated internalization (Fig. 6, A and B; Supplemental Fig. 3B) and overexpression of ITSN, although not affecting internalization (Fig. 1B), enhanced receptor trafficking and degradation (Figs. 1 and 2). The difference in our results and those of previous studies may be due to two factors. First, previous studies only measured endocytosis at late times after ligand stimulation (>25–60 min), and comparison with our data indicates similar results. However, based on the earlier time points in our endocytosis assays (Fig. 1A) coupled with the receptor half-life experiments (Fig. 1C) and RNAi results (Fig. 6; Supplemental Fig. 3B), we conclude that ITSN overexpression stimulates rather than inhibits receptor trafficking. Second, we observed that transfection of 5-fold more ITSN into cells as used in the analyses in Fig. 1 inhibited ^{125}I-EGF internalization (data not shown). This result is probably due to vast overexpression of ITSN resulting in the binding of limiting amounts of endocytic components, thus titrating these components into nonproductive complexes and inhibiting endocytosis. This biphasic response is a common property of scaffolds (Ferrell, 2000). Thus, the combination of these two factors may account for the observed differences.

Although ITSN aids in the modulation of cargo (i.e., EGFR), it may also modulate the ubiquitylation of additional proteins within the endocytic complex, including epsin, Eps15, and Hrs, all of which are monoubiquitylated (Oldham et al., 2002; Polo et al., 2002). Given the importance of the ubiquitin proteasome system in regulation of both pre- and postsynaptic plasticity (Ehlers, 2003; Speese et al., 2003) coupled with the observed defects in synapse morphology and synaptic transmission in ITSN mutant flies (Koh et al., 2004), ITSN probably plays an integral role in the reorganization of synapses through facilitating the targeted ubiquitylation and degradation of important components in this process.

The predicted role for ITSN in receptor endocytosis and nervous system function is further highlighted by its link to Down syndrome (DS). ITSN is localized to Chr21 in the DS critical region and is overexpressed in the brains of DS patients and in a mouse model for DS (Pucharcos et al., 1999; Gardiner, 2003). This increased ITSN expression may enhance JNK activation and promote increased apoptosis in the brain, thereby contributing to the neurodegeneration seen in DS (Mohney et al., 2003). This study suggests that the increased ITSN in DS may alter RTK trafficking. Indeed, increased nerve growth factor internalization was observed in synaptosomes from the Ts65Dn mouse model for DS (Cooper et al., 2001). Thus, ITSN overexpression in DS may result in both increased activation of the JNK pathway as well as enhanced RTK trafficking. The combined stimulation of these two pathways coupled with increased activation of other ITSN-regulated pathways is probably a contributing factor for the sequelae of DS.

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