GRB10/NEDD4-MEDIATED MULTIUBIQUITINATION OF THE INSULIN-LIKE GROWTH FACTOR RECEPTOR REGULATES RECEPTOR INTERNALIZATION.

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Running Title: Grb10/Nedd4-mediated multiubiquitination and IGF-IR internalization.

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ABSTRACT

The adaptor protein Grb10 is an interacting partner of the IGF-I receptor (IGF-IR) and the insulin receptor (IR). Previous work from our laboratory has established a role of Grb10 as a negative regulator of IGF-IR-dependent cell proliferation. We have shown that Grb10 binds the E3 ubiquitin ligase Nedd4 and promotes IGF-I-stimulated ubiquitination, internalization and degradation of the IGF-IR, thereby giving rise to long-term attenuation of signaling.

Recent biochemical evidence suggests that tyrosine-kinase receptors (RTK) may not be polyubiquitinated but monoubiquitinated at multiple sites (multiubiquitinated). However, the type of ubiquitination of the IGF-IR is still not defined.

Here we show that the Grb10/Nedd4 complex upon ligand-stimulation mediates multiubiquitination of the IGF-IR, which is required for receptor internalization. Moreover, Nedd4 by promoting IGF-IR ubiquitination and internalization contributes with Grb10 to negatively regulate IGF-IR-dependent cell proliferation.

We also demonstrate that the IGF-IR is internalized through clathrin-dependent and independent pathways. Grb10 and Nedd4 remain associated with the IGF-IR in early endosomes and caveosomes, where they may participate in sorting internalized receptors. Grb10 and Nedd4, unlike the IGF-IR, which is targeted for lysosomal degradation are not degraded and likely directed into recycling endosomes. These results indicate that Grb10 and Nedd4 play a critical role in mediating IGF-IR down-regulation by promoting ligand-dependent multiubiquitination of the IGF-IR, which is required for receptor internalization and regulates mitogenesis.
INTRODUCTION

Grb10, originally isolated by expression screening using the epidermal growth factor receptor (EGFR) (Ooi et al., 1995), is a member of a family of adaptor proteins that includes at least seven isoforms in human and mouse. Grb10 was later identified as a ligand-dependent interacting partner of the IGF-IR and the IR (Dey et al., 1996; Dong et al., 1997; Frantz et al., 1997; Hansen et al., 1996; Laviola et al., 1997; Liu and Roth, 1995; Morrione et al., 1996; O'Neill et al., 1996).

The members of the Grb10 family of adaptor proteins share a conserved molecular architecture. They contain an SH2 (Src Homology 2) domain at the carboxy terminus, PH (Pleckstrin-Homology) and RA (Ras Association) domains in the central region, a less conserved amino terminus that contains proline-rich sequences (Dong et al., 1997; Stein et al., 2001). More recently, an additional domain has been identified and called BPS (Between the PH and the SH2) domain, which is the binding region for the IGF-IR (He et al., 1998; Stein et al., 2001).

We have shown that mouse Grb10 over-expression inhibits IGF-IR-mediated cell proliferation of mouse embryo fibroblast (MEF) cells over-expressing the IGF-IR (Morrione et al., 1997). Additional studies have pointed out either a negative (Dufresne and Smith, 2005; Langlais et al., 2003; Liu and Roth, 1995; Stein et al., 2001; Wick et al., 2003) or positive role of Grb10 in the regulation of IGF-IR and IR signaling (O'Neill et al., 1996; Wang et al., 1999).

Recently, the Grb10 gene was disrupted by a gene-trap insertion (Charalambous et al., 2003). Mutant mice are 30% larger than normal, suggesting that Grb10 is a potent growth inhibitor (Charalambous et al., 2003). Furthermore, Grb10 transgenic mice showed growth retardation (Shiura et al., 2005), confirming the negative role of Grb10 in cell growth.
The Grb10 SH2 domain has been shown to interact with MEK1 and Raf1 kinases in a phosphotyrosine-independent manner (Nantel et al., 1998). Grb10 SH2 domain has also been reported to interact with the Growth Hormone receptor (Moutoussamy et al., 1998), the PDGF receptor (10), the ELK receptor (Stein et al., 1996) and with BCR-ABL tyrosine kinase (Bai et al., 1998). The N-terminus of Grb10 has been shown to interact with GIGYF1 (Grb10 interacting GYF protein 1) and GIGYF2 (Giovannone et al., 2003). Collectively, these results suggest that Grb10 may play a broad role in regulating RTK-mediated signal transduction pathways.

Using the yeast two-hybrid system we isolated Nedd4 (Morrione et al., 1999) as a novel Grb10-interacting protein.

Nedd4 (mNedd4-1) (Kumar et al., 1997; Kumar et al., 1992) is an ubiquitin protein ligase (E3) containing a C2 domain, 3 WW domains, and a hect (homologous to the E6-AP carboxy-terminal) domain (Bork and Sudol, 1994; Huijbroktse et al., 1995).

The WW domains of Nedd4 have been shown to interact with the epithelial sodium channel (ENaC), recognizing proline-rich PY motifs (Staub et al., 1996) and this interaction promotes ubiquitination of the channel (Staub et al., 1997). Additional substrates of Nedd4-mediated ubiquitination include CNras-GEF (Pham and Rotin, 2001), LAPTM5 (Pak et al., 2006) and PTEN (Wang et al., 2007).

The C2 domain of Nedd4 binds phospholipids, and mediates Ca\(^{2+}\)-dependent translocation to the plasma membrane (Dunn et al., 2004; Plant et al., 1997) through an interaction with Annexin XIIIb (Plant et al., 2000).

Using deletion mutants of Grb10 we showed that it is the SH2 domain of Grb10 that binds the C2 domain of Nedd4. Grb10 is not ubiquitinated by Nedd4 (Morrione et al., 1999) but instead Grb10 acts as a bridge between Nedd4 and the IGF-IR, thereby promoting ligand-
induced ubiquitination of the IGF-IR (Vecchione et al., 2003). Ubiquitination of the IGF-IR targets the receptor for degradation (Vecchione et al., 2003).

Recent biochemical evidence suggests that the epidermal growth factor and the platelet growth factor receptors (EGFR and PDGFR) may be not only polyubiquitinated but monoubiquitinated at multiple sites (multiubiquitinated) (Haglund et al., 2003a; Mosesson et al., 2003). However, the type of ubiquitin modification on the IGF-IR is still not defined.

In this study, using different biochemical approaches we established that the Grb10/Nedd4 complex upon ligand stimulation promotes multiubiquitination of the IGF-IR, which is required for efficient receptor internalization. We also show that Nedd4 plays a role in regulating IGF-I-dependent cell proliferation.

Grb10 and Nedd4 remain associated with the internalized IGF-IR in clathrin-dependent and –independent pathways, but unlike the receptor which is down-regulated (Vecchione et al., 2003) in the lysosomal compartment Grb10 and Nedd4 are not degraded and likely sorted into recycling endosomes.

MATERIALS AND METHODS

Cell Lines. p/6 and p6/Grb10 cells were previously described (Morrione et al., 1999; Morrione et al., 1997): they are mouse embryo fibroblasts over-expressing the IGF-IR or the IGF-IR and myc-tagged mouse Grb10α.

Generation of the 4KR ubiquitin construct. The pcDNA3.1/HA-Ub (wild type ubiquitin) and pcDNA3.1/HA-UbK29,48,63R mutant expression vectors were a kind gift from Dr. Ivan Dikic. We generated the pcDNA3.1/HA-Ub4KR mutant, which bears K to R mutations at lysine 11, 29, 48 and 63 of ubiquitin, by PCR using the Quick-Change XL Site-Directed Mutagenesis Kit.
The mutation was verified by sequencing at the Kimmel Cancer Center Nucleic Acid Facility.

**Ubiquitination assays in cells.** Transient ubiquitination assays in p6 and p6/Grb10 cells were performed as previously described (Vecchione et al., 2003). Cells lysates were pooled and 1 mg (p6/Grb10) or 1.5 mg (p6) of proteins were immunoprecipitated in HNTG buffer (20mM Hepes (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.2 mM sodium orthovanadate, protease inhibitors mix supplemented with MG132 and chloroquine with anti-IGF-IR monoclonal antibodies (Calbiochem EMD, San Diego, CA). To detect ubiquitinated proteins filters were immunoblotted with anti-HA antibodies and after stripping first with anti-ubiquitin FK1 (Affinity Research, Golden, CO) and later with anti-ubiquitin P4D1monoclonal antibodies (Covance, Berkeley, CA). The IGF-IR was detected using polyclonal antibodies (C-20) from Santa Cruz Biotechnology (Santa Cruz, CA). β-catenin ubiquitination was evaluated in p6/Grb10-HA-Ub-transfected cells in the presence of 20 µM MG132 after immunoprecipitation with anti-β-catenin monoclonal antibodies (BDPharmingen, San Diego, CA).

P6/Grb10 cells were also transiently co-transfected with the HA-Ub4KR plasmid in combination with either empty vectors or the pRC-CMV-Nedd4(CS) (a kind gift from Dr. Daniela Rotin) The rat Nedd4 mutant carries a point mutation in the conserved Cys (Cys to Ser) of the hect domain and is unable to transfer ubiquitin and hence catalytically inactive (Pham and Rotin, 2001). The expression of tagged mutant Nedd4 was detected using anti T7 tag monoclonal antibodies (Novagen).

**Internalization assay.** Cell surface receptors were assessed by enzyme-linked immunosorbent assay (ELISA), as previously described (Orsini et al., 1999, Vecchione et al., 2003). p6/Grb10
cells were transfected with pcDNA3.1/Grb10ΔSH2 mutant (Vecchione et al., 2003). The expression of the myc-tagged Grb10ΔSH2 mutant previously described (Vecchione et al., 2003) was detected using anti-myc polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

**Targeting of Nedd4-1, clathrin and caveolin-1 by siRNA.** Gene silencing of mouse Nedd4-1, clathrin, and caveolin-1 was obtained by RNA interference using small interfering RNA (siRNA). P6/Grb10 cells were transfected with vehicle (DEPC-treated water), control siRNA (scrambled), or siRNA directed against clathrin heavy chain, caveolin-1 or Nedd4-1 (200 pmol) using the TransIT-siTKO reagents (Mirus Corporation, Milwaukee, WI) according to the manufacturer’s instructions. siRNA oligos specific for mouse clathrin heavy chain, caveolin-1, Nedd4-1 and controls were from Dharmaco (Lafayette, CO).

24 hr after transfection, p6/Grb10 cells were starved in SFM for 48 hr and then stimulated with IGF-I. Ligand-dependent internalization of the IGF-IR was measured by ELISA assay. The expression of clathrin and caveolin-1 proteins was detected by immunoblot using anti-clathrin polyclonal antibodies (BD Biosciences, San Diego, CA) or anti-caveolin-1 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-Nedd4-1 monoclonal antibodies are from Transduction laboratories (EMD, San Diego, CA).

**Proliferation assays.** To establish the role of Nedd4-1 in IGF-I-induced proliferation of p6/Grb10 cells we either transiently transfected p6/Grb10 cell with a plasmid expressing T7-tagged rat Nedd4-1(a kind gift from Daniela Rotin) or targeted endogenous Nedd4-1 by siRNA approaches. Transfected and Nedd4-1-depleted p6/Grb10 cells were plated in triplicate at a density of 5 x 10^4 cells/35 mm plates. After 24 hr cells were washed 3 times in DMEM and transferred to SFM supplemented with 50 ng/ml of IGF-I. Cells were counted after 48 hr with a
hemocytometer.

**Analysis of IGF-IR, Grb10 and Nedd4 levels.** p6/Grb10 cells were starved in SFM for 48 hr, and then stimulated with 50 ng/ml of IGF-I (Invitrogen, San Diego, CA) for different time points (0, 8, 20 and 24 hr) (Vecchione et al., 2003). Cells were lysed and the level of Grb10 was determined by immunoblot using anti-Grb10 polyclonal antibodies (K20, Santa Cruz Biotechnology, Santa Cruz, CA). The IGF-IR was detected using polyclonal antibodies against the β subunit of the IGF-IR (C-20, Santa Cruz Biotechnology, Santa Cruz, CA). Nedd4 was detected using anti-Nedd4 monoclonal antibodies (Transduction Laboratories, EMD, San Diego, CA). Normalization of proteins was done incubating the same filter with anti β-actin polyclonal antibodies (Sigma-Aldrich, ST Louis, MO).

**Confocal Microscopy analysis.** P6/10 cells were plated onto cover slips, serum-starved for 24 hr and then stimulated with 200 ng/ml of IGF-I for 10 and 30 min at 37 C°. After rinsing twice with PBS, cells were fixed with 3.7% paraformaldehyde for 10 min and permeabilized with 0.5% TRITON 100X for 10 min. Cells were then blocked for 30 min at 37 C° with 0.5% triton X-100, 1% BSA in PBS (blocking buffer). Cover slips were incubated for 30 min at 37 C° with primary antibody in blocking solution. After washing 5 times with the same solution and blocked again for 30 min at 37 C°, samples were incubated for 30 min at 37 C° with secondary antibodies used at 1:100 dilution in blocking solution. The cover slips were washed twice with PBS and mounted using Slow-Fade mounting medium (Molecular Probes, Invitrogen, San Diego, CA). The third additional staining implies a repetition of the protocol after the double staining. The antibodies used are: anti-IGF-IR polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Nedd4 monoclonal antibodies (Transduction Laboratories, EMD, San Diego, CA).
Diego, CA); anti-clathrin heavy chain monoclonal antibodies (Affinity Bioreagents, Golden, CO); anti-caveolin-1 polyclonal antibodies, anti-EEA1 monoclonal antibodies, anti-LAMP1 monoclonal antibodies (BDPharmingen, San Diego, CA); myc-tagged Grb10 was detected with an anti-Myc monoclonal antibody (Calbiochem EMD, San Diego, CA). Secondary antibodies are Alexa-Fluor 488 (green), Alexa-Fluor 594 (red) and Alexa-Fluor 647 (blue) (Molecular Probes, Invitrogen, San Diego, CA). Cover slips were analyzed and photographed at the Kimmel Cancer Center Confocal Microscopy Core Facility with LSM 510 Meta Confocal Microscope (63X objective) using the ip-lab v3.0 software (BioVisions Technologies, Exton, PA). The images were merged using Photoshop 6.

Statistics. Experiments were carried out in triplicate and repeated at least three times. Results are expressed as mean ± SD.

RESULTS

The IGF-IR is monoubiquitinated at multiple sites in p6/Grb10 cells. We have previously shown that Grb10 in complex with the ubiquitin ligase Nedd4 promoted ligand-mediated ubiquitination of the IGF-IR (Vecchione et al., 2003). The ubiquitinated receptor exhibited a smeary SDS-PAGE pattern (Vecchione et al., 2003) that could be attributed to conjugation of either a ubiquitin polymer (hereafter referred to as polyubiquitination), monomeric ubiquitins attached to multiple lysines on the IGF-IR (hereafter referred to as multiubiquitination) or a combination of both (Haglund et al., 2003b; Huang et al., 2006; Mosesson et al., 2003).

In order to determine whether the IGF-IR is polyubiquitinated or multiubiquitinated we performed transient ubiquitination assays in p6/Grb10 cells and determined the ability of
different anti-ubiquitin antibodies to discriminate between poly and monoubiquitinated IGF-IR. The P4D1 antibody against ubiquitin recognizes monoubiquitinated and polyubiquitinated proteins, while the FK1 antibody reacts only with polyubiquitin chains but not single ubiquitin moieties (Haglund et al., 2003b; Wegierski et al., 2006). Ubiquitinated IGF-IR from IGF-I-stimulated p6/Grb10 cells were immunoreactive with the P4D1 antibodies but not with the FK1 antibodies (Fig. 1A) suggesting that the IGF-IR is predominantly multiubiquitinated and not polyubiquitinated. As control, we determined in exponentially growing p6/Grb10 cells polyubiquitination of β-catenin, which was equally detected by both P4D1 and FK1 anti-ubiquitin antibodies (Fig. 1B).

Formation of polyubiquitin chains utilizes predominantly lysine 11, 29, 48 and 63 on ubiquitin (Weissman, 2001): an ubiquitin molecule that bears K11, 29, 48, 63R (4KR) mutations is deficient in forming polymeric chains (Haglund et al., 2003b; Mosesson et al., 2003). We therefore determined the capacity of wild type ubiquitin and ubiquitin 4KR mutant to mediate ubiquitination of the IGF-IR. Transient expression in p6/Grb10 cells of HA-tagged Ub or 4KR Ub mutant reproduced the same smeary pattern of IGF-IR ubiquitination after ligand stimulation (Fig. 1C). Significantly, the ubiquitinated receptors were detected by the P4D1 antibody that recognized monoubiquitination, but not by the FK1 antibody specific for polyubiquitination (Fig. 1C).

In order to confirm that the multiubiquitination of the IGF-IR was detectable in parental p6 cells at endogenous Grb10 level, we transiently co-transfected the ΔSH2 Grb10 mutant into parental p6 cells with the HA-Ub 4KR plasmid, and assessed the level of ubiquitination of the IGF-IR. This Grb10 mutant is unable to bind Nedd4, but still retains the ability to bind the IGF-IR and acts as dominant negative toward ubiquitination of the IGF-IR (Vecchione et al., 2003). As seen in Fig. 1D, there was multiubiquitination of the IGF-IR in p6 cells after stimulation with
IGF-I. In contrast, over-expression of the ΔSH2 Grb10 mutant severely reduced this ubiquitination (Fig. 1D), suggesting that endogenous Grb10 is necessary for multiubiquitination of the IGF-IR in parental p6 cells. Collectively, these results indicate that in p6 and p6/Grb10 cells upon ligand stimulation the IGF-IR is predominantly multiubiquitinated.

*Grb10-mediated multiubiquitination of the IGF-IR is required for efficient receptor internalization.* In order to establish whether multiubiquitination of the IGF-IR is sufficient to drive receptor internalization, we transiently expressed in p6/Grb10 cells HA-tagged wild type ubiquitin and the 4KR ubiquitin mutant to increase receptor ubiquitination (Vecchione et al., 2003) (and Fig. 1). Ligand-dependent internalization of the IGF-IR was enhanced in cells expressing exogenous ubiquitin (Fig. 2A) compared to V-transfected cells, confirming that ubiquitination of the IGF-IR increases receptor internalization (Vecchione et al., 2003). We confirmed that wild type ubiquitin and monoubiquitin mutant were expressed at comparable levels by immunoblot with anti-HA and anti-ubiquitin antibodies on lysates from transfected cells (Fig. 3B). Significantly, expression of exogenous monoubiquitin molecules was as efficient as wild type ubiquitin in promoting ligand-dependent IGF-IR internalization (Fig. 2A), suggesting that multiubiquitination of the IGF-IR is sufficient to ensure receptor internalization.

To further confirm that Grb10-mediated multiubiquitination of the IGF-IR is required for receptor internalization, we transiently transfected dominant negative Grb10 (Grb10ΔSH2) into p6/Grb10 cells and assessed IGF-IR internalization. Consequently, exogenous expression of Grb10 ΔSH2 (Fig. 2B) protein diminished the level of IGF-IR internalization as compared to V-transfected cells. The level of expression of Grb10 mutant protein was determined by immunoblot using anti-Myc (Fig. 2B) antibodies.
These results suggest therefore that Grb10 by promoting IGF-IR multiubiquitination regulates receptor internalization.

*Nedd4-mediated multiubiquitination of the IGF-IR is required for receptor internalization.* To confirm whether endogenous Nedd4 was the E3 responsible for multiubiquitination of the IGF-IR, we transiently co-transfected p6/Grb10 cells with a catalytically inactive Nedd4 (Nedd4(CS)) mutant (Pham and Rotin, 2001) and HA-Ub 4KR, and assessed the level of ubiquitination of the IGF-IR. There was a marked increase of multiubiquitination of the IGF-IR in vector-transfected p6/Grb10 cells after stimulation with IGF-I (Fig. 3A). In contrast, over-expression of Nedd4(CS) mutant diminished multiubiquitination of the IGF-IR (Fig. 3A), suggesting that Nedd4(CS) is acting in a dominant negative fashion to block multiubiquitination of the IGF-IR mediated by endogenous Nedd4.

We further demonstrated that Nedd4-mediated multiubiquitination of the IGF-IR is required for receptor internalization: it is in fact diminished by the transiently transfected Nedd4(CS) mutant (Fig. 3B).

In addition, we confirmed the role of Nedd4 in regulating IGF-IR internalization by depleting endogenous Nedd4-1 by siRNA strategies. Our approach yielded a considerable suppression of endogenous Nedd4-1 protein expression as compared to vehicle treated or control treated cells (Fig. 3C) and a concurrent inhibition of ligand-mediated internalization of the IGF-IR.

Collectively, these results indicate that in p6/Grb10 cells endogenous Nedd4 has intrinsic monoubiquitinating activity toward the IGF-IR, which is critical to direct receptor internalization.
Nedd4 regulates IGF-I-induced proliferation of p6/Grb10 cells. Our previous work (Morrione et al., 1997) has demonstrated that Grb10 acts as a negative regulator of IGF-I-dependent cell proliferation in p6/Grb10 cells. To determine whether Nedd4 by promoting IGF-IR ubiquitination and internalization may contribute with Grb10 in negatively regulating IGF-I-mediated cell proliferation, we tested the level of IGF-I-induced mitogenesis of p6/Grb10 cells that were either transiently transfected with an expression plasmid expressing rat Nedd4-1 or depleted of endogenous Nedd4-1 by siRNA strategies. As shown in figure 4A, transient overexpression of Nedd4-1 severely reduced the ability of p6/Grb10 cells to proliferate in response to IGF-I stimulation compared to V-transfected cells (Fig. 4A). On the contrary, in p6/Grb10 cells depletion of endogenous Nedd4-1 by siRNA approaches determined an increase in mitogenesis in response to IGF-I as compared to vehicle treated or control treated cells (Fig. 4C). The level of overexpressed (Fig. 4B) or depleted (fig. 4D) Nedd4-1 protein in p6/Grb10 cells was determined by immunoblot with anti-Nedd4-1 specific antibodies. All together, these results demonstrate that Nedd4 acts with Grb10 in negatively regulating IGF-I-mediated cell proliferation.

Grb10 and Nedd4 associate with the internalized IGF-IR in clathrin-dependent and independent pathways. The internalization pathway of the IGF-IR is poorly characterized and the data available remain controversial (Lin et al., 1998, Maggi et al., 2002, Huoet al., 2003).

In order to investigate the pathway that mediates internalization of the IGF-IR and to determine whether Grb10 remains associated with the internalized receptor, we analyzed by confocal microscopy whether the IGF-IR and Grb10 co-localize in p6/Grb10 cells with clathrin, a marker for clathrin-coated pits, or caveolin-1, a marker for caveolae. In unstimulated cells, surface IGF-IR was predominantly diffuse without obvious colocalization with either Grb10,
clathrin (Fig. 5A) or caveolin-1 (Fig. 5C). After 10 min of IGF-I stimulation, surface IGF-IR became strongly punctate and showed colocalization with Grb10 and both clathrin (Fig. 5 B) and caveolin-1 (Fig. 5D).

Our earlier work has shown that upon ligand stimulation Nedd4 was recruited by Grb10 to the IGF-IR at the plasma membrane where it promoted receptor ubiquitination (Vecchione et al., 2003). To examine whether Nedd4 remains associated with the internalized IGF-IR we assessed in p6/Grb10 cells Nedd4 localization by confocal microscopy analysis. As seen in Fig. 6 in unstimulated cells there was very limited colocalization between Nedd4, the IGF-IR and clathrin (6A) or caveolin-1 (6C). Upon ligand stimulation Nedd4 colocalized with the IGF-IR and both clathrin (fig. 6B) and caveolin-1 (Fig. 6D).

Together, these results indicate that at early stages of endocytosis Grb10 and Nedd4 remain associated with the internalized IGF-IR which can be detected in both clathrin-coated pits and caveolar compartments.

**Targeting of endogenous clathrin and caveolin-1 by siRNA strategies inhibits IGF-IR internalization.** To provide stronger evidence that clathrin and caveolin-1 play an important role in mediating ligand-dependent internalization of the IGF-IR we utilized clathrin and caveolin-1-specific small interference RNA (siRNA). Our approach yielded a substantial suppression of endogenous clathrin protein expression as compared to vehicle treated or control treated cells (Fig. 7A) and a concurrent inhibition of IGF-I-mediated internalization of the IGF-IR (Fig. 7A). Suppression of endogenous caveolin-1 expression (fig. 7B) determined as well a significant reduction in the level of ligand-dependent internalization of the IGF-IR (fig. 7B). We could not assess the effect of the double depletion of clathrin and caveolin-1 due to an excessive cell death of p6/Grb10 cells.
Together, our findings reveal an essential role for clathrin and caveolin-1 in mediating ligand-stimulated internalization of the IGF-IR.

**Grb10 and Nedd4 associate with the IGF-IR in EEA1-positive early endosomes.** After the initial steps of endocytosis ubiquitinated cargos are sorted into early endosomes en route for degradation into proteosomal/lysosomal compartments (Le Roy and Wrana, 2005). We therefore determined by confocal microscopy analysis whether Grb10, Nedd4 and the IGF-IR colocalized with EEA1, a marker of early endosomes. In unstimulated p6/Grb10 cells there was very limited colocalization between the IGF-IR, Grb10, Nedd4 and EEA1 (Fig. 8A and C). After 30 min of IGF-I stimulation the IGF-IR, Grb10 (Fig. 8B) and Nedd4 (Fig. 8D) colocalized in EEA1-positive endosomes indicating that upon ligand stimulation Grb10 and Nedd4 are sorted with the internalized IGF-IR into early endosomes. At 30 min of IGF-I stimulation Grb10, Nedd4 the IGF-IR could be also detected in caveolar compartments (data not shown) although to a significant lesser extent.

**Grb10 uncouples from the IGF-IR before sorting of the receptor to the lysosomal compartment.** Ubiquitinated RTKs are degraded by the lysosomes as shown for the EGFR (Haglund et al., 2003b; Huang et al., 2006; Mosesson et al., 2003). However, published experiments with the use of proteosomal and lysosomal inhibitors to block IGF-IR down-regulation suggest that the ubiquitinated receptor may be degraded by the lysosomes and/or the proteasome depending on cancer cell type and different ubiquitin ligases involved (Carelli et al., 2006; Girnita et al., 2003; Vecchione et al., 2003). In order to determine whether in p6/Grb10 cells we could detect the IGFIR, Grb10 and Nedd4 in the lysosomal compartment we assessed by confocal microscopy
whether the IGF-IR, Grb10 or Nedd4 colocalized with LAMP-1, a marker of lysosomes. Unstimulated cells showed a diffuse staining for the IGF-IR (Fig. 9A) and lack of colocalization with Grb10 or Nedd4 (Fig. 9 C) and LAMP-1. After 6 hr of IGF-I stimulation, the IGF-IR became punctate and showed significant colocalization with LAMP-1 (Fig. 9B, D). Significantly, both Grb10 (Fig. 9, B) and Nedd4 (Fig. 9, D) showed no colocalization with the IGF-IR and LAMP-1, while Grb10 and Nedd4 were in complex in both unstimulated and IGF-I-stimulated cells (not shown). Prolonged IGF-I stimulation of p6/Grb10 cells promoted IGF-IR degradation (Fig. 9E) while Grb10 and Nedd4 proteins were stable up to 24 hr of ligand stimulation (Fig. 8E) as determined by immunoblot analysis.

Collectively, these results indicate that the Grb10/Nedd4 complex dissociate in late endosomes from the IGF-IR pool en route for lysosomal degradation and is sorted into recycling endosomes.

**DISCUSSION**

Our results can be summarized as follow: a) In p6 and p6/Grb10 mouse embryo fibroblast cells upon receptor activation the Grb10/Nedd4 complex promotes multiubiquitination of the IGF-IR; b) Multiubiquitination of the IGFIR is required for receptor internalization; c) Nedd4 contributes to negatively regulate IGF-I-induced mitogenesis; d) Upon ligand-stimulation Grb10 and Nedd4 internalize with the IGF-IR in both clathrin-dependent and caveolar pathways; e) Depletion of endogenous clathrin and caveolin-1 by siRNA strategies severely reduced ligand-dependent internalization of the IGF-IR; f) Grb10 and Nedd4 are associated with the IGFI-R in early endosomes; g) The Grb10/Nedd4 complex, unlike the IGF-IR which is degraded in the lysosomes, is not targeted for degradation but is sorted into recycling endosomes.
Ubiquitin is a highly conserved 76 amino acid polypeptide that is covalently attached to target proteins through a three step process involving ubiquitin activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes (Hochstrasser, 2006). Proteins can be modified by a single ubiquitin moiety or by polymeric ubiquitin chains, referred to as monoubiquitination and polyubiquitination, respectively. Monoubiquitination serves as a signal for internalization and sorting of trans-membrane proteins in yeast cells (Hicke, 2001; Shih et al., 2000), while polyubiquitination regulates numerous proteins by sorting them for degradation by the 26S proteasome (Hicke and Dunn, 2003; Schnell and Hicke, 2003).

Ligand-mediated polyubiquitination of RTK targets them for degradation to the lysosomal pathway, to mediate receptor down-regulation (Di Fiore and De Camilli, 2001). Recent reports have suggested that the EGFR and the PDGFR may not be polyubiquitinated but rather monoubiquitinated at multiple sites (multiubiquitination), and this modification is sufficient to ensure receptor sorting and degradation (Haglund et al., 2003a; Haglund et al., 2003b; Mosesson et al., 2003).

P6/Grb10 cells are severely impaired in IGF-I-stimulated cell proliferation compared with parental p6 cells (Morrione et al., 1997), implicating Grb10 in this impairment. Accordingly, our results here suggest that Nedd4 associating with Grb10 (Morrione et al., 1999), promotes multiubiquitination, internalization and degradation of the IGF-IR in p6/Grb10 cells, thereby negatively regulating IGF-IR downstream signaling leading to cell growth.

Our previous work (Vecchione et al., 2003) and the present results indicate that Nedd4 is the E3 ligase involved in the regulation of ubiquitination, sorting and stability of the IGF-IR in p6/Grb10 cells. However, additional ubiquitin ligases may play a role in the ubiquitination of the IGF-IR in different cellular systems, utilizing mechanisms independent of Grb10. Girnita et al. (2003) (Girnita et al., 2003) have demonstrated that the ubiquitin ligase Mdm2 promotes
ubiquitination of the IGF-IR (Girnita et al., 2003) via the adaptor function of β-arrestin1 protein (Girnita et al., 2005). Mdm2 is a ring-finger ubiquitin ligase critically involved in the regulation of p53 ubiquitination and stability (Haupt et al., 1997; Kubbutat et al., 1997): the role of Mdm2 in promoting ubiquitination of the IGF-IR is likely more relevant in cellular background where decreased levels of p53 may favor the availability of Mdm2 outside the nucleus. However, whether the β-arrestin1/Mdm2 complex promotes polyubiquitination or multiubiquitination of the IGF-IR remains to be determined.

Although our results are in agreement with recently published data on the EGFR and PDGFR (Haglund et al., 2003b; Mosesson et al., 2003) which support the evidence that the EGFR and the PDGFR are preferentially multiubiquitinated, a more recent report suggests instead that a consistent fraction of the EGFR-associated ubiquitin is in the form of poluybiquitin chains linked through K63 (Huang et al., 2006). Taking into account the different experimental approaches used and different cellular background, these results may collective suggest that the EGFR and possibly other RTK, including the IGF-IR, may be modified by both multiubiquitin and polyubiquitin chains. The mechanisms that determine the prevalence of one modification to the other remain to be elucidated.

Because different ubiquitin ligase proteins have been implicated in mediating the Ubiquitination of the IGF-IR (Girnita et al., 2003; Vecchione et al., 2003) it is tempting to speculate that different complexes may have different abilities in promoting either polyubiquitination or multiubiquitination of the receptor with possibly different effects on the receptor sorting.

The present work provide the first evidence that upon ligand stimulation the internalized IGF-IR is directed into lysosomes for degradation suggesting that Grb10/Nedd4-dependent multiubiquitination targets the receptor for lysosomal degradation.
The mechanisms that determine the segregation of Grb10/Nedd4/IGF-IR complexes into different endocytic compartments (clathrin-dependent or caveolar compartments) and the contribution of these pools of IGF-IR in signaling versus degradation remain elusive. Previous results have indicated that upon ligand activation the IGF-IR induces the formation of a complex that includes IRS-1 and Caveolin-1 (Panetta et al., 2004) suggesting that the lipid raft/caveolar compartment may function as a signaling platform for the IGF-IR pathway, versus the clathrin-dependent pathway that would mediate internalization of the IGF-IR for lysosomal degradation. On the other end, recent work by Pelkmans et al. (2004) (Pelkmans et al., 2004) has demonstrated that caveolar vesicles are targeted to early endosomes and formed stable membrane domains, strongly suggesting that there is intersection between caveolar and endosomal pathways. It is possible therefore that after the initial stages of internalization the different pools of IGF-IR may converge into endosomes and then sorted for degradation or recycling to the cell surface.

Inhibition of IGF-IR ubiquitination by dominant negative approaches toward Grb10 and Nedd4 severely reduced but not completely abolished ligand-dependent internalization of the IGF-IR: these results would suggest that ubiquitin-independent mechanisms may additionally contribute to the regulation of IGF-IR internalization.

Significantly, Grb10 contains a -NPF- motif in the amino-terminal region, which is a consensus sequence for EH (Eps15 homology) domain-containing proteins. The EH family of proteins, including Eps15, EHD1 and others, is involved in the internalization and sorting of RTK (Di Fiore and De Camilli, 2001; Dupre et al., 2004). Grb10 has the potential therefore to modulate IGF-IR down-regulation at multiple levels by promoting receptor ubiquitination through the interaction with the ubiquitin ligase Nedd4 and receptor internalization/sorting by mediating the interaction with proteins of the endocytic machinery.
Studies are currently under way to identify additional proteins that may play a role in conjunction with Grb10 and Nedd4 in the regulation of IGF-IR internalization and sorting.
REFERENCES


He W, Rose DW, Olefsky JM, Gustafson TA. 1998. Grb10 interacts differentially with the insulin receptor, insulin-like growth factor I receptor, and epidermal growth factor receptor via the Grb10 Src homology 2 (SH2) domain and a second novel domain located between the pleckstrin homology and SH2 domains. J Biol Chem 273(12):6860-6867.


ACKNOWLEDGMENTS

This work has been supported by the Benjamin Perkins Bladder Cancer Fund and the Martin Greitzer Fund. We are deeply grateful to Dr. Daniela Rotin for the gift of rat Nedd4 and Nedd4(CS) mutant and to Dr. Ivan Dikic for the gift of the pcDNA3.1/Ub wild type and pcDNA3.1/Ub$^{K29,46,63R}$ mutant. We acknowledge Judy Verdone for her skillful assistance in cell culture.

TITLES AND LEGENDS TO FIGURES

Figure 1. Grb10 promotes ligand-dependent multiubiquitination of the IGF-IR. (A, B) p6/Grb10 or p6 (C) cells were plated in triplicate onto 6-well plates and transiently transfected with an 8xHA-tagged ubiquitin construct (HA-Ub Tx) or (C, D) with the pcDNA3.1 HA-Ub (wild type) or pcDNA3.1 HA-Ub$^{K11,29,48,63R}$ (4KR) mutant. After 24 hr cells were shifted to serum free medium (SFM) for additional 24 hr and then stimulated for 10, 30 and 60 min (A) or 10 min (C, D) (with 20 ng/ml of IGF-I in the presence of 20 µM MG132 and 0.4 mM chloroquine. Lysates were pooled and 1 mg of proteins were immunoprecipitated with anti-IGF-IR antibody and blotted with anti-HA antibodies to detect ubiquitinated species (A, C, D). These blots were then stripped and re-probed first with FK1 and then with P4D1 anti-ubiquitin antibodies. Filter was then incubated with anti-IGF-IR antibodies to test for the amount of receptor immunoprecipitated. β-catenin ubiquitination was detected in p6/Grb10 after 6 hr incubation with 10 µM MG132 as described in Material and Methods. Results in panels A, C and D are representative of three independent experiments. Result in panels B is representative of two independent experiments.

Figure 2. Grb10-mediated multiubiquitination of the IGF-IR is required for receptor internalization. The level of cell surface IGF-IR in p6/Grb10 cells transiently transfected with either an empty vector or with the pcDNA3.1 HA-Ub (wild type) or pcDNA3.1 HA-Ub4KR mutant was determined by ELISA at 10 and 30 min of IGF-I stimulation. IGF-I was used at 200
ng/ml to ensure saturating concentration of the ligand. The level of ubiquitin expression in p6/Grb10-transfected cells was determined by immunoblot with anti-HA and anti-ubiquitin monoclonal antibodies (Covance). The data are the average of three independent experiments with SD. (B) P6/Grb10 cells were transiently co-transfected with either an empty vector (V) or a Grb10 SH2-deleted mutant (ΔSH2) as described. Ligand-dependent internalization of the IGF-IR was assessed by ELISA. Results are average of three independent experiments. Lysates were probed with anti-myc antibodies to detect the expression of myc-tagged Grb10 ΔSH2 protein (B), which lacks the epitope (SH2 domain) recognized by anti-Grb10 antibodies.

Figure 3. Nedd4-mediated multiubiquitination of the IGF-IR is required for receptor internalization. (A) P6/Grb10 cells were transiently co-transfected with either an empty vector (V) or a catalytically inactive Nedd4(CS) and pcDNA3.1 HA-Ub4KR mutant. After IGF-I-stimulation, the IGF-IR was immunoprecipitated and levels of its ubiquitination determined as described in legend for fig. 1. Lysates were probed with anti T7 antibodies to detect the expression of Nedd4(CS). Results are representative of three independent experiments. (B) P6/Grb10 cells were transiently co-transfected with either an empty vector (V) or Nedd4(CS) as described. Ligand-dependent internalization of the IGF-IR was assessed by ELISA. Results are average of three independent experiments. Lysates were probed with anti-T7 and anti-Nedd4 antibodies to detect the expression of Nedd4(CS) protein. (C) Endogenous Nedd4-1 was depleted by siRNA strategies as described in Materials and Methods. Ligand-dependent internalization of the IGF-IR was assessed by ELISA. Results are average of three independent experiments. Nedd4-1 expression levels were measured by immunoblot using anti-Nedd4-1 monoclonal antibodies. The amount of protein loaded was monitored with anti-β-actin polyclonal antibodies.
Figure 4. **Nedd4 regulates IGF-I-induced proliferation of p6/Grb10 cells.** P6/Grb10 cells were either transiently transfected with the pRC/CMV-Nedd4-1 plasmid, which express rat Nedd4-1 (A) or depleted of endogenous Nedd4-1 by siRNA approaches (C). Cells were then shifted (time 0) to SFM supplemented with IGF-I (50 ng/ml) and counted after 48 hr in a hemocytometer. Cell growth is expressed as % increase over time 0. At the same time samples were also lysed and the expression of transfected rat Nedd4-1 (B) or endogenous Nedd4 in vehicle (veh), control oligos (con) and siRNA-treated cells (D) was assessed by immunoblot with anti-Nedd4-1 monoclonal antibodies. Results are the average of three independent experiments.

Figure 5. **Grb10 is associated with the internalized IGF-IR in clathrin-dependent and independent pathways.** P6/Grb10 cells were plated onto cover slips and serum-starved for 24 hr. Cells were then untreated (A and C) or stimulated with 200 ng/ml of IGF-I for 10 min (B and D). Cover slips were analyzed and photographed at the Kimmel Cancer Center Confocal Microscopy Core Facility with LSM 510 Meta Confocal Microscope using the ip-lab v3.0 software. Images were merged using Photoshop. Right panels show the merged images of IGF-IR (blue), Grb10 (red), clathrin or caveolin (green) and their colocalization (white). Insets represent enlarged (2.5-fold) views of boxed region. The single cell images represented the result from at least 20 cells from three independent experiments. The white bars represent 10 microns.

Figure 6. **Nedd4 colocalized with the internalized IGF-IR in clathrin-dependent and independent pathways.** P6/Grb10 cells were plated onto cover slips and serum-starved for 24 hr. Cells were then untreated (A and C) or stimulated with 200 ng/ml of IGF-I for 10 min (B and D). Cover
slips were processed for confocal microscopy as described in Materials and Methods. Images were merged using Photoshop. Right panels show the merged images of IGF-IR (blue), Nedd4 (red), clathrin or caveolin (green) and their colocalization (white). Insets represent enlarged (2.5-fold) views of boxed region. The single cell images represented the result from at least 20 cells from three independent experiments. The white bars represent 10 microns.

Figure 7. Depletion of endogenous clathrin and caveolin-1 by siRNA inhibits ligand-dependent internalization of the IGF-IR. Depletion of endogenous clathrin (A) or caveolin-1 (B) in p6/Grb10 cells was achieved by siRNA strategies. Internalization of the IGF-IR was assessed in serum-starved p6/Grb10 cells by ELISA 72 hr post-transfection. Serum-starved p6/Grb10 cells were stimulated for 10 and 30 min with 200 ng/ml of IGF-I. Results are average of three independent experiments. Level of clathrin (A) and caveolin-1 (B) in vehicle (veh), control oligos (con) and siRNA-treated cells was assessed by immunoblot with anti-clathrin (A) and anti-caveolin-1 (B) specific antibodies.

Figure 8. The IGF-IR colocalized with both Grb10 and Nedd4 in EEA1-positive early endosomes. P6/Grb10 cells were plated onto cover slips and serum-starved for 24 hr. Cells were then untreated (A and C) or stimulated with 200 ng/ml of IGF-I for 30 min (B and D). Cover slips were processed for confocal microscopy as described in Materials and Methods. Right panels show the merged images of IGF-IR (blue), Grb10 or Nedd4 (red), EEA1 (green) and their colocalization (white). Insets represent enlarged (2.5-fold) views of boxed region. The single cell images represented the result from at least 20 cells from three independent experiments. The white bars represent 10 microns.
Figure 9. The IGF-IR but not Grb10 or Nedd4 colocalized with lysosomes. P6/Grb10 cells were plated onto cover slips and serum-starved for 24 hr. Cells were then untreated (A and C) or stimulated with 200 ng/ml of IGF-I for 6 hr (B and D) in the presence of chloroquine (0.2 mM). Cover slips were processed for confocal microscopy as described in Materials and Methods. Right panels show the merged images of IGF-IR (blue), Grb10 or Nedd4 (red), Lamp-1 (green) and their colocalization (turquoise, IGF-IR/Lamp1). The single cell images represented the result from at least 20 cells from three independent experiments. The white bars represent 10 microns.

For immunoblot analysis of IGF-IR, Grb10 and Nedd4 protein levels (E), p6/Grb10 cells were serum-starved for 48 hr and then stimulated with 50 ng/ml of IGF-I for 8, 20 and 24 hr. Cells were lysed and 20 µg of proteins were loaded for SDS-PAGE. The IGF-IR was detected by immunoblot with anti-IGF-IR polyclonal antibodies. After stripping the filter was incubated with anti-Grb10 polyclonal antibodies, which recognizes mouse Grb10 β and δ isoforms in p6/Grb10 cells, and anti-Nedd4 monoclonal antibodies. The amount of protein loaded on the gel was monitored using anti-β-actin polyclonal antibodies. Results are representative of three independent experiments.
Figure 1, Monami et al.

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181x203mm (300 x 300 DPI)
Figure 2, Monami et al.

A

% IGF-IR on cell surface

Time (min)

B

% IGF-IR on cell surface

Time (min)

156x165mm (300 x 300 DPI)
Figure 3, Monami et al.

A  P6/Grb10
Blot

HA
UB4KR
V  N(CS)

IGF-IR

IP: IGF-IR

T7

Lysate

113kDa
80kDa

113kDa
80kDa

B

C

% IGFR on cell surface

% IGFR on cell surface

260x138mm (300 x 300 DPI)
Figure 4, Monami et al.

A

% increase over time 0

48 hr

B

V Nedd4

Nedd4 blot

β-actin blot

C

% increase over time 0

Mock control siRNA Nedd4

D

veh con Nedd4

Nedd4 blot

β-actin blot

167x141mm (300 x 300 DPI)
Figure 5, Monami et al.

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196x179mm (300 x 300 DPI)
Figure 6, Monami et al.

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233x173mm (300 x 300 DPI)
Figure 7, Monami et al.

A

B

162x122mm (300 x 300 DPI)
Figure 8, Monami et al.

A | SFM
---|---
IGF-IR | Grb10 | EEA1 | Merge

B | IGF-1
---|---
IGF-IR | Nedd4 | EEA1 | Merge

C | SFM
---|---
IGF-IR | Nedd4 | EEA1 | Merge

D | IGF-1
---|---
IGF-IR | Nedd4 | EEA1 | Merge

195x172mm (300 x 300 DPI)
Figure 9, Monami et al.

![Image](image_url)

262x158mm (300 x 300 DPI)