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Effects of Gas6 and hydrogen peroxide in Axl ubiquitination and downregulation

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Abstract

The receptor tyrosine kinase Axl has been shown to be activated by its ligand Gas6 and by oxidative stress in the form of hydrogen peroxide. However, the regulatory mechanisms controlling the levels of Axl upon Gas6 binding or oxidative stress have not been elucidated. This report demonstrates that Gas6-induced downregulation of Axl is blocked by inhibitors of endocytosis and lysosomal degradation, but not by inhibitors of proteosomal activity. Furthermore, it is shown that binding of Axl to Gas6 induces the phosphorylation and ubiquitination of Axl and the interaction of Axl with the ubiquitin ligase c-Cbl. Importantly, hydrogen peroxide induces Axl tyrosine phosphorylation but not its ubiquitination, determining the inhibition of Axl downregulation. These results suggest that as shown for other receptor tyrosine kinases, ubiquitination of Axl is needed to ensure its proper degradation in the lysosome, and that oxidative stress may inhibit Axl ubiquitination and downregulation.

Keywords: Axl; Gas6; Hydrogen peroxide; Ubiquitin; c-Cbl; Lysosome; Proteosome

Upon growth factor binding, cytoplasmic tyrosine residues of tyrosine kinases (RTKs) become autophosphorylated and provide docking sites for a variety of phosphotyrosine-binding proteins. The recruitment of these proteins, which harbor various catalytic and/or scaffolding domains, regulates cell proliferation, migration, differentiation or apoptosis [1,2]. Normal cells control the length and intensity of RTK signaling by a major deactivation pathway termed receptor downregulation. This pathway involves RTK ligand-induced internalization by endocytosis, followed by degradation in lysosomes [3–5]. Several RTKs, including epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor (PDGFR), are ubiquitinated and downregulated upon interactions with the c-Cbl family of ubiquitin ligases [3–7]. Multiple monoubiquitination events of EGFR and PDGFR rather than attachment of polyubiquitination chains have been shown to ensure their proper receptor sorting and lysosomal degradation [8–10]. RTKs that are not ubiquitinated can be recycled back to the cell surface and escape lysosomal degradation [3–5].

Deregulation of RTK signaling by chromosomal translocations, increased transcription/translation, point mutations or failure of RTK to be appropriately deactivated can lead to the development and progression of many human malignancies [3–5]. Oxidative stress in the form of hydrogen peroxide (H_2O_2) has also been shown to induce the autophosphorylation of EGFR and inhibit c-Cbl-mediated downregulation of activated EGFR [10]. As a consequence, activated EGFR accumulates in the membrane [10], leading to enhanced cell proliferation [11] and facilitation of tumor promotion processes [12].

AXL (named also *ARK*, *UFO*, or *TYRO7*) [13] was the first discovered member of a subfamily of RTK that shared extracellular regions composed of two immunoglobulin-related domains linked to two fibronectin

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type-III repeats, and cytoplasmic regions that contained an intrinsic tyrosine kinase domain [13]. The Axl ligand, Gas6, can act as a vitamin K-dependent growth-potentiating factor and/or an anti-apoptotic factor depending on the cell type [14–17]. Gas6 contains multiple N-terminal γ-carboxyglutamic acid (Gla) residues, four epidermal growth factor-like repeats, and a C-terminal sex hormone-binding globular like region which is sufficient for Axl activation [18]. Importantly, Axl has been shown to be tyrosine phosphorylated not only by its ligand Gas6 [14–18] but also by oxidative stress in the form of H_2O_2 [19]. The aims of the present work were to gain insight into the molecular machinery controlling the levels of Axl upon Gas6 binding and to determine whether oxidative stress in the form of H_2O_2 could inhibit Axl downregulation, providing a trigger for pathogenic processes in which increased Axl expression and H_2O_2 production are evident [19–26].

Materials and methods

Cell culture conditions. Human lens epithelial cells (HLEC) SRA 01/04 [27] were routinely cultured in a 5% CO_2 atmosphere at 37 °C in DMEM (Life Technologies, Rockville, MD) supplemented with 10% (v/v) fetal bovine serum (FBS; Life Technologies). Cells were serum-starved overnight prior to the addition of Gas6 as previously described [17]. Full-length human recombinant Gas6 (kindly provided by Amgen) or mouse recombinant Gas6 lacking the Gla domain (R&D systems) was used in this study with identical results. Experiments shown in Figs. 1–3 were performed with full-length human recombinant Gas6 and those shown in Fig. 4 with mouse recombinant Gas6 lacking the Gla domain. To confirm specificity for the Gas6/Axl-mediated effects, some experiments were performed in the presence of an antibody against the N-terminus of human Gas6 (1 μg/ml; R&D systems) or in the presence of murine Axl-Fc (1 μg/ml; R&D systems) to block human and murine Gas6/Axl interaction, respectively (data not shown).

One hour prior Gas6 stimulation, cells were pretreated in the presence and absence of inhibitors of proteosomal degradation (10 μM lactacystin) or lysosomal degradation (100 μM cloroquine, 10 mM NH_4Cl or 0.25 μM bafilomycin A1) purchased from Calbiochem. To inhibit endocytosis, cells were pretreated one hour prior Gas6 treatment with the inhibitor of endocytosis monensin (50 μM), that was purchased

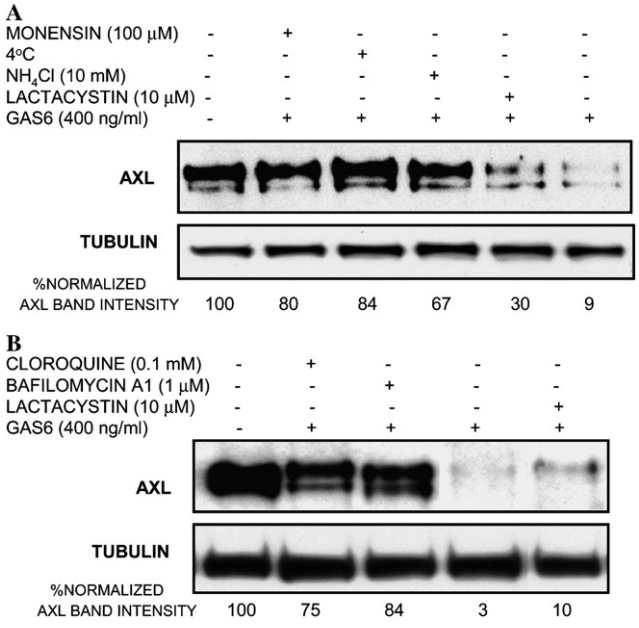

Fig. 1. Inhibitors of endocytosis and lysosomal degradation inhibit Gas6-induced Axl downregulation. HLEC were serum-starved overnight and then preincubated with several inhibitors of lysosomal function (cloroquine, NH_4Cl or bafilomycin A1), inhibitors of endocytosis (monensin) or proteosome inhibitors (lactacystin) for 1 h before treating with Gas6 for 6 h. Cells were also treated with Gas6 in ice (instead of at 37 °C) for 6 h to inhibit internalization. SDS–PAGE and Western blots were performed to evaluate the levels of Axl, which were normalized by assessing the levels of tubulin with an anti-tubulin antibody.

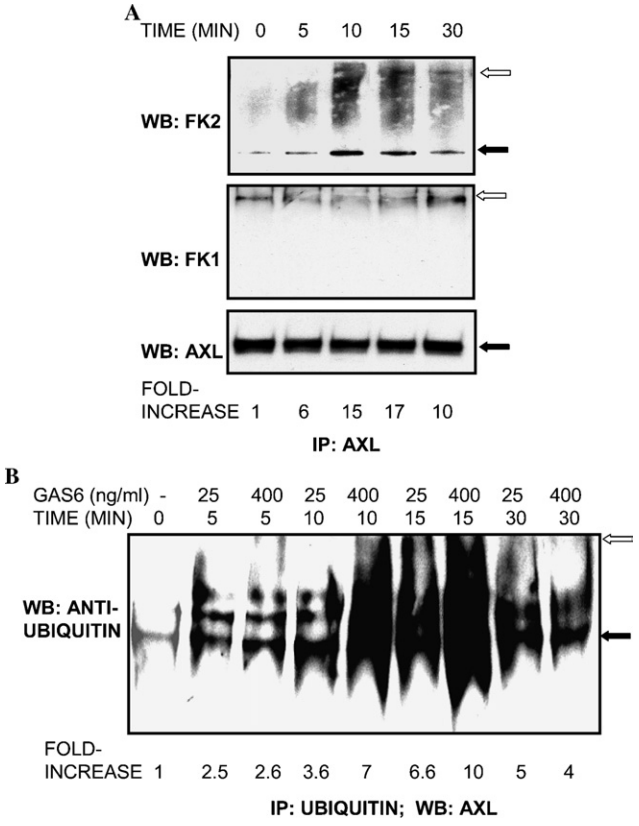

Fig. 2. Gas6 induces Axl ubiquitination. HLEC were serum-starved overnight and treated with Gas6 (400 ng/ml in (A); 25–400 ng/ml in (B)) for different times (0–30 min) to monitor Axl ubiquitination upon Gas6 binding (fold-increase with respect to untreated cells). SDS–PAGE were performed in 4–12% Bis-Tris gradient gels (A) or 3–8% Tris–acetate gels (B). (A) Protein lysates were subjected to immunoprecipitation with anti-Axl antibody followed by Western blotting with anti-Axl antibody and two anti-ubiquitin antibodies; FK1 that only recognizes poly-ubiquitin chains and FK2 that recognizes both mono- and poly-ubiquitin chains. (B) Protein lysates were subjected to immunoprecipitation with anti-ubiquitin antibody (from Santa Cruz Biotechnology that recognizes mono- and poly-ubiquitin chains) followed by Western blotting with anti-Axl antibody. The location of the stacking gel and of the mature form of Axl (140 kDa) is shown by a white arrow and a black arrow, respectively.

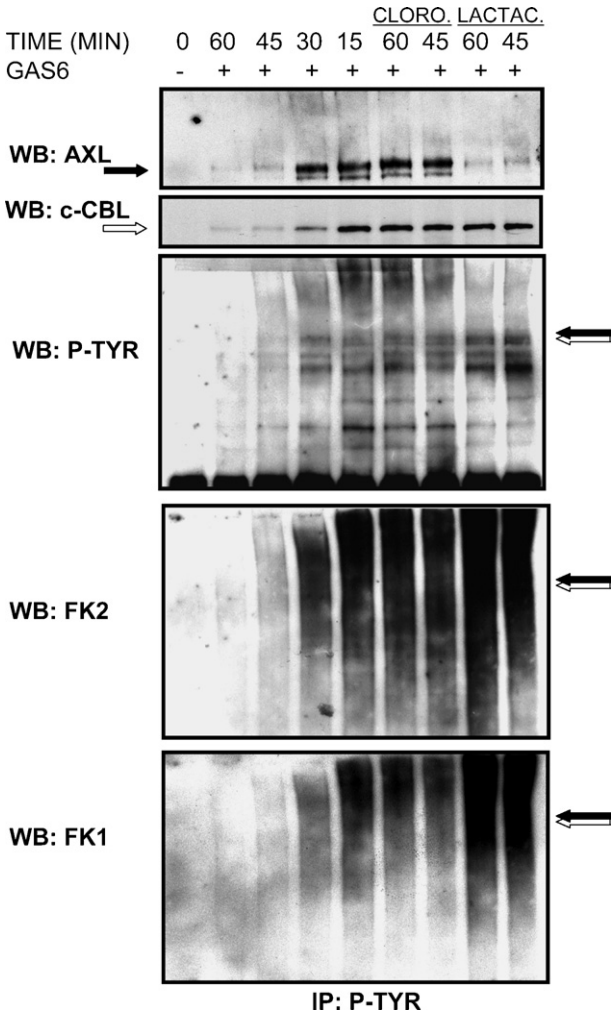

Fig. 3. Gas6 promotes Axl and c-Cbl phosphorylation. HLEC were serum-starved overnight and treated with Gas6 for different times. Some cells were also treated with the proteosome inhibitor lactacystin (10 μM) or the inhibitor of lysosomal degradation cloroquine (100 μM). SDS–PAGE were performed in 4–12% gradient gels. Protein lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibody (from Santa Cruz Biotechnology) followed by Western blotting with anti-Axl, c-Cbl, anti-phosphotyrosine antibodies, FK1 and FK2. The location of the the mature form of Axl (140 kDa) and of c-Cbl (120 kDa) is shown by a black arrow and a white arrow respectively.

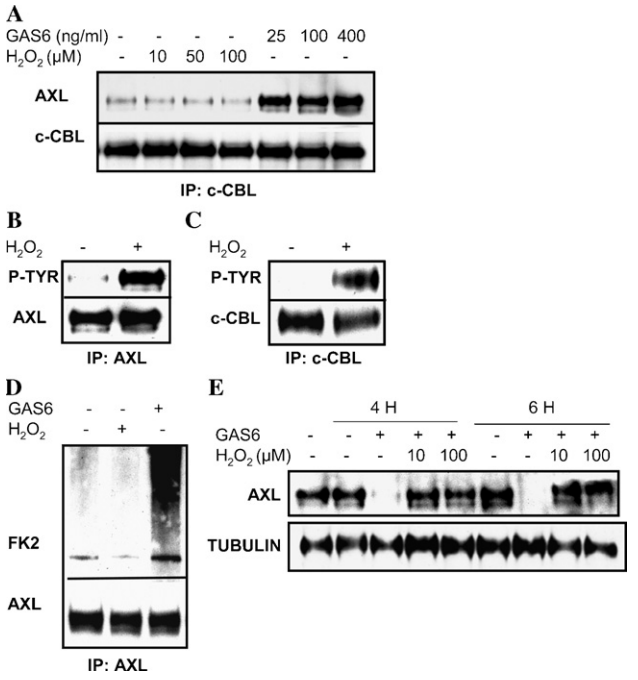

Fig. 4. H_2O_2 induces the phosphorylation of Axl and Cbl but not the interaction of Axl and Cbl, Axl ubiquitination or Axl downregulation. HLEC were serum-starved overnight and treated with either Gas6 (25–400 ng/ml) or H_2O_2 (10–100 μM) for 15 min (A–D) or 15 min followed by washing with PBS and change of media with further incubation for 4 or 6 h in the presence of Gas6 (E). Untreated cells were also isolated at time 0 for comparison purposes (E, lane 1). (A) Gas6 but not H_2O_2 induces the interaction between Axl and Cbl: lysates were subjected to immunoprecipitation with anti-Cbl and WB with anti-Cbl and anti-Axl antibodies. (B) H_2O_2 induces the phosphorylation of Axl: lysates were subjected to immunoprecipitation with anti-Axl and immunoblotting with anti-Axl and anti-phosphotyrosine antibodies. (C) H_2O_2 induces the phosphorylation of c-Cbl: lysates were subjected to immunoprecipitation with anti-Cbl and immunoblotting with anti-Cbl and anti-phosphotyrosine antibodies. (D) Gas6 but not H_2O_2 induces Axl ubiquitination: lysates were subjected to immunoprecipitation with anti-Axl and WB with anti-Axl and FK2. (E) Inhibition of Gas6-mediated Axl downregulation by H_2O_2: lysates were subjected to immunoblotting with anti-Axl or anti-tubulin.

from Sigma. Axl internalization and degradation were also blocked by performing Gas6 treatments in wet ice or 4 °C instead of at 37 °C.

H_2O_2 was purchased from Sigma. Treatments involving H_2O_2 (with or without Gas6) were performed in serum-, pyruvate-, and phenol red-free DMEM (Life Technologies).

Antibodies. Primary antibodies for Axl, c-Cbl, tubulin, and phosphotyrosine were obtained from Santa Cruz Biotechnologies, Inc. Monoclonal antibodies that recognize poly-ubiquitinated proteins (clone FK1) or mono- and poly-ubiquitinated proteins (clone FK2) were obtained from Biomol. An additional antibody to immunoprecipitate mono- and poly-ubiquitinated proteins was obtained from Santa Cruz Biotechnologies. All horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Santa Cruz Biotechnologies, except the goat anti-rabbit IgG that was obtained from Jackson Laboratories.

Lysate preparation, immunoprecipitation (IP), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein lysates were prepared in commercially available lysis buffer (Mammalian Cell-PE LB from GenoTechnology, Inc) containing a cocktail of protease and phosphatase inhibitors (Sigma). After incubation for 30 min on ice, and vortexing for intervals of 10 min, the homogenized samples were centrifuged at 13,000*g* for 15 min at 4 °C to remove insoluble material. Equal amounts of total protein, determined with the Bio-Rad protein assay kit, were used for immunoprecipitation experiments essentially as described previously [17]. Briefly, 100 μg of protein lysates was precleared by incubating with 0.25 μg of the appropriate control IgG, together with 20 μl of 25% v/v protein A/G plus agarose beads (Santa Cruz Biotechnology, Inc) at 4 °C for 30 min. Precleared protein lysates were incubated with 2 μg anti-Axl antibodies for 1 h at 4 °C and then mixed with 20 μl protein A/G plus agarose beads overnight at 4 °C. Alternatively, precleared protein lysates were incubated with 2 μg anti-Cbl, anti-phosphotyrosine (anti-P-Tyr) or anti-ubiquitin antibodies (Santa Cruz Biotechnologies). Beads were washed 5 times in PBS, solubilized in 40 μl of 4× Laemmli sample buffer with 2-mercaptoethanol as previously

described [17], resolved by SDS–PAGE on NuPAGE 4–12% Bis-Tris gradient gels or 3–8% Tris–acetate gels (Invitrogen), and then subjected to Western blot analyses. Since the bands corresponding to Axl (140 and 120 kDa for the mature and partially glycosylated forms, respectively) and c-Cbl (120 kDa) tend to comigrate in these gradient gels, electrophoresis were conducted for an additional 30 min to increase the separation between each other (except in experiment shown in Fig. 2A).

Western blot and image analyses. Western blot (WB) analyses were performed as previously described [17]. In some experiments, membranes were reused for the serial detection of several proteins by stripping in Re-Blot plus strong solution (Chemicon International) as recommended by the manufacturer. In other experiments, several SDS–PAGE with the same lysates were conducted in parallel and used to detect different proteins. Image analysis was performed using Scion Image Beta 4.02 (Scion Image, Frederick, Maryland) by determining the mean pixel value of the bands of interest and then substracting the background mean pixel value of areas not containing any specific signal. In some experiments, the experimental mean pixel values of Axl bands were normalized with those corresponding to tubulin. In other experiments, the experimental mean pixel values were expressed as fold-increase with respect to those at time 0 or fold-decrease with respect to values of untreated control cells.

Results and discussion

The Gas6-dependent downregulation of Axl is stabilized by inhibitors of endocytosis and lysosomal degradation

HLEC were previously shown to express Axl and respond to Gas6 with an increase of their proliferation and/or with a resistance to undergo apoptosis under a variety of proapoptotic insults [17]. To study the mechanisms of Gas6-induced Axl downregulation, HLEC were treated with inhibitors of endocytosis, or inhibitors of lysosomal or proteosomal activity, prior to stimulation with Gas6. Levels of Axl expression were then analyzed by Western blotting and normalized by the levels of tubulin. As shown in Figs. 1A and B, Gas6 stimulation in the absence of any inhibitor resulted in significant degradation of Axl. Importantly, Gas6-induced downregulation of Axl was inhibited by treatment with the endocytosis inhibitor monensin and when Gas6 was incubated with HLEC at 4 °C instead of 37 °C, that inhibits internalization of RTK (Fig. 1A). When lactacystin was added to the cells, a small stabilizing effect on Axl was observed relative to unstimulated control cells (Figs. 1A and B). Importantly, inhibitors of lysosomal degradation including cloroquine, ammonium chloride (NH_4Cl) or bafilomycin prevented the degradation of Axl. (Figs. 1A and B). These results pointed out to the requirement of lysosomal activity for Gas6-induced Axl degradation. The small effect of lactacystin suggested that proteosomal activity could play a less important role than lysosomal activity in controlling the levels of Axl. However, our result cannot discount the possibility that proteosomal activity might have some involvement in the lysosomal sorting of kinase active Axl as previously described for EGFR [28]. In fact, activated EGFR has been reported to be efficiently transported to internal vesicles of multivesicular bodies in a proteosome-dependent manner, even though EGFR itself is not a substrate for proteosomal degradation [28].

The receptor tyrosine kinase Axl is ubiquitinated upon Gas6 binding

To test whether Gas6 could induce Axl ubiquitination, HLEC were treated with Gas6 for different times and lysates were subjected to two different IP strategies. In one set of experiments, IP was performed with anti-Axl antibody and WB analyses were then used with clones FK2 and FK1 that recognize mono- and poly-ubiquitinilated proteins or only poly-ubiquitinilated proteins, respectively (Fig. 2A). In addition, IP was carried out with an antibody for anti-ubiquitin from a different commercial source (Santa Cruz Biotechnologies) that recognizes mono- and poly-ubiquitinated proteins and then used anti-Axl antibody to monitor the levels of ubiquitinated Axl by WB analysis (Fig. 2B). Results obtained through both approaches indicated that Gas6 increased Axl ubiquitination in a time-dependent and dose-dependent fashion up to 15 min of stimulation, and that levels of total Axl ubiquitination tended to decrease after 30 min (Figs. 2A and B). Axl immunoprecipitates exhibited a very small immunoreactivity with clone FK1 between the stacking and separating gels in some experiments, with cells treated with Gas6 for 30 min but not at other time points (Fig. 2A and data not shown). These results suggested that the majority of Axl ubiquitination was due to mono-ubiquitin chains although a very small proportion of the receptor could be subjected to poly-ubiquitination in a very transient fashion. The smeary pattern of ubiquitination found for Axl has been previously reported for other RTK to be a consequence of receptor glycosylation and phosphorylation together with multiple monoubiquitination of the activated receptors [9].

Gas6 promotes c-Cbl phosphorylation

To address the possible role of c-Cbl in the regulation of the Gas6/Axl signaling, the tyrosine phosphorylation of c-Cbl and Axl upon Gas6 stimulation was assessed by anti-P-Tyr IP with lysates from unstimulated or Gas6-stimulated cells, followed by WB analyses with anti-P-Tyr, anti-Cbl or anti-Axl antibodies. Results indicated that Gas6 treatments led to Axl and c-Cbl phosphorylation, reaching the highest levels around 15 min and showing background levels of phosphorylation at 1 h after Gas6 addition (Fig. 3). Phosphorylation of c-Cbl decreased to a higher extent than that of Axl at 30 min. Importantly, Gas6-induced phosphorylation of Axl was stabilized by inhibition of lysosomal

degradation with cloroquine whereas the Gas6-induced phosphorylation of Cbl was stabilized by inhibition of either lysosomal or proteosomal degradation (Fig. 3). In these experiments, the levels of tyrosine phosphorylation paralleled those of ubiquitination in cells treated with Gas6 in the absence of any inhibitor or in the presence of cloroquine. In cells treated with lactacystin levels of ubiquitination were higher than those of tyrosine phosphorylation (Fig. 3). These results suggested that lysosomal and proteosomal inhibitors could stabilize phosphorylated c-Cbl but only the former could stabilize active Axl. Similar results were previously reported in a manuscript about c-Cbl-mediated EGFR degradation [29]. The authors of that manuscript suggested that proteosomal activity could be required for de-ubiquitination of the EGFR prior to its lysosomal degradation [29]. However, it has also been shown that c-Cbl can be targeted for proteosomal degradation by Nedd4 and Itch ubiquitin ligases [30]. Therefore, it is possible that ubiquitin ligases other than c-Cbl could influence the lysosomal degradation of Axl, by indirectly modulating the levels of c-Cbl or by more direct mechanisms that require further investigation.

Gas6 but not H_2O_2 promote Axl and c-Cbl interaction

In order to test whether Gas6 induced not only the phosphorylation of Axl and c-Cbl, but also their interaction with each other, anti-Cbl antibody was used in IP, followed by WB analyses with anti-Axl antibody. As shown in Fig. 4A, different concentrations of Gas6 induced the interaction of Axl and c-Cbl. By contrast, treatments with H_2O_2, that were able to increase the tyrosine phosphorylation of Axl (Fig. 4B and data not shown) and c-Cbl (Fig. 4C and data not shown), did not induce the interaction between Axl and Cbl (Fig. 4A) nor Axl ubiquitination (Fig. 4D). Consistent with these results, cells treated with Gas6 for 4 or 6 h exhibited a significant downregulation of Axl, whereas those pretreated with 10 or 100 μM H_2O_2 for 15 min prior to the addition of Gas6 for 4 or 6 h exhibited a significant Axl accumulation (Fig. 4E and data not shown). These results were in agreement with the paradigm that the pattern of tyrosine phosphorylation induced by H_2O_2 or Gas6 might be different, affecting Axl interactions with other proteins (i.e., Axl/c-Cbl direct or indirect interaction) involved in Axl internalization, degradation, and/or ubiquitination. A similar model was previously hypothesized for the EGFR [10]. Briefly, tyrosine 1045 of EGFR, which is phosphorylated by its ligand EGF and is the major docking site for c-Cbl [4], is not phosphorylated by treatment with H_2O_2 [10]. Lack of tyrosine phosphorylation at residue 1045 of EGFR was associated with the blockade of c-Cbl/EGFR interaction, inhibition of c-Cbl-mediated EGFR internalization, and degradation or EGFR ubiquitination [10]. Additional research will be needed to identify the tyrosine residues whose phosphorylation is induced by Gas6 but not by H_2O_2, that ensure proper Axl ubiquitination and downregulation by c-Cbl-dependent and perhaps by c-Cbl-independent mechanisms. Lack of phosphorylation at one or several of those tyrosine residues may contribute to Axl accumulation, which is evident in a variety of cancers and other pathological processes often characterized by the abundant production of H_2O_2.

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