Heregulin β1 induces the down regulation and the ubiquitin-proteasome degradation pathway of p185^{HER2} oncoprotein

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Abstract Analysis of the fate of the p185^{HER2} oncoprotein following activation by heregulin β1 revealed the induction of the tyrosine-phosphorylation, down-modulation, and polyubiquitination of p185^{HER2}. Receptor ubiquitination was suppressed in cells treated with heregulin β1 in the presence of sodium azide, an inhibitor of ATP-dependent reactions, or genistein, a tyrosine kinase protein inhibitor, indicating the requirement for kinase activity and ATP in p185^{HER2} polyubiquitination. Ubiquitinated p185^{HER2} was degradated by the 26S proteasome proteolytic pathway. Kinetics and inhibition experiments indicated that endocytosis of the receptor occurs downstream of the initiation of the degradation process.

Key words: p185^{HER2} oncoprotein; Down-modulation; Ubiquitination; Proteasome enzyme

1. Introduction

The c-erbB-2 protooncogene encodes a 185-kDa transmembrane glycoprotein (p185^{HER2}) with intrinsic tyrosine kinase activity belonging to the epidermal growth factor receptor (EGFR) family [1]. This receptor, whose specific ligand has not yet been identified, can be activated by epidermal growth factor (EGF) through the formation of heterodimers between HER1 and HER2 [2], or by heregulin through heterodimerization with HER3 or HER4 [3,4]. The first event which occurs rapidly following heregulin treatment is the tyrosine phosphorylation of p185^{HER2} [5], but nothing is known about the processes that are responsible for degradation of this oncoprotein following its activation.

The ubiquitination pathway has recently been established as an important mechanism of degradation of cytoplasmic, nuclear, and even cell membrane proteins [6]. The presence of polyubiquitin chains accelerates the rate of protein degradation by a complex multicatalytic proteinase called 26S proteasome [7]. This proteasome breaks down the ubiquitinated protein to short peptides but recycles the ubiquitin molecules. Monomeric receptors belonging to different receptor tyrosine kinase (RTK) families such as the platelet-derived growth factor receptor [8,9], EGFR, colony-stimulating factor receptor, fibroblast growth factor receptor are polyubiquitinated after ligand stimulation [10], supporting the hypothesis that ubiquitination of the receptor is a general phenomenon for RTK degradation. For all these molecules, the binding of the specific ligand rapidly induces the clustering of ligand-receptor complexes, internalization and ultimately degradation of both ligand and receptor [11]. Using a chimeric receptor composed of the extracellular EGFR binding domain and the cytoplasmic domain of the HER2 molecule, it has been shown that EGFR/HER2 receptors internalize EGF much more slowly than EGFR [12,13]. A more recent study [14] showed that the wild-type HER2 receptor failed to associate with AP2, a clathrin-associated protein complex which interacting with the carboxyl terminus of membrane receptors, facilitates receptor internalization through coated pits.

In the present study, we showed that heregulin β1 treatment induced tyrosine phosphorylation, immediately followed by polyubiquitination and degradation of p185^{HER2} through 26S proteasome proteolytic pathway and that down-modulation of the membrane receptor after heregulin β1 treatment required initiation of p185^{HER2} proteolytic degradation.

2. Materials and methods

2.1. Cell culture

MDAMB453 human breast carcinoma cells were obtained from American Type Culture Collection (Rockville, MD) and maintained in RPMI medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 0.4 mg/ml gentamycin.

2.2. Reagents

Human purified recombinant heregulin β1 protein (NeoMarkers, Freemont, CA) was used at 10 ng/ml. Herbimycin A (HA) (Calbiochem, La Jolla, CA), the peptidyl aldehyde inhibitor of the 26S proteasome complex *N*-acetyl-Leu-Leu-norleucinal (LLnL) (kindly provided by Dr. G. Draetta) and Genistein (Sigma, St. Louis, MO), all-dissolved in methylsulfoxide (Me_2SO) were used at 3 μM, 50 μM and 40 μg/ml, respectively.

Mouse monoclonal antibody (MAb) Ab3, directed against the peptide sequence of the carboxyl domain of the human HER2 gene product (Oncogene Science Inc., Manhasset, NY), was used for immunoblotting. Mouse MAb MGR2, directed against the extracellular domain of the p185^{HER2} [15], was used for immunoprecipitation and fluorimetric analysis. Mouse anti-phosphotyrosine MAb, directed against phosphotyramine-KLH, was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-ubiquitin serum, derived from rabbits immunized with bovine red blood cell ubiquitin conjugated to KLH, was purchased from Sigma (St. Louis, MO).

2.3. Immunoprecipitation and immunoblotting

MDAMB453 cells in log phase in 6-well plates were starved for 24 h and treated or not for 6 h with HA, overnight with LLnL, 1 h with genistein or 15 min with 0.2% sodium azide. Cells were then stimulated with heregulin β1 (10 ng/ml) at different times and lysed by scraping into lysis buffer consisting of 1% NP40, 0.1% SDS, 50 mM Tris-HCl, pH 7.5, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM pepstatin, 1 mM $NaVO_4$, 5 mM. Lysates were immunoprecipitated as described [16] with MAb MGR2. Immunoprecipitates

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Abbreviations: EGF, epidermal growth factor; NDF, neu differentiation factor; KLH, keyhole-limpet hemocyanin; PDGF, platelet-derived growth factor; TCR, T cell receptor; MHC, major histocompatibility complex

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were subjected to immunoblot analysis with MAb Ab3 or anti-phosphotyrosine (1.5 μg/ml) or with anti-ubiquitin antiserum (1 μg/ml) and the proteins were visualized using the ECL Western blotting detection system (Amersham, Little Chalfont, UK).

2.4. Flow cytometric analysis

Indirect immunofluorescence assays on cells treated with heregulin β1 (10 ng/ml) for various periods of time at 37°C with or without biochemical inhibitors, were performed using MAb MGR2 (10 μg/ml) and fluorescein isothiocyanate (FITC)-conjugated sheep-anti-mouse IgG secondary antibody (Keerkegaard & Perry Laboratories Inc., Gaithersburg, MD). Fluorescence was evaluated by FACScan (Becton Dickinson, Mountain View, CA) using Lysis II software.

3. Results and discussion

3.1. Heregulin β1 causes phosphorylation, down-modulation and ubiquitination of p185^{HER2} receptor

When MDAMB453 cells were stimulated with heregulin β1, the tyrosine phosphorylation of the immunoprecipitated p185^{HER2} oncoprotein reached a peak within 1 min of stimulation and returned to basal levels after 15 min of treatment (Fig. 1A, compare lanes 2 and 4). The receptor was also down-modulated: after 60 min of treatment, the quantity of oncoprotein was reduced by half (Fig. 1B, lane 6). The p185^{HER2} immunoprecipitates were also stained with anti-ubiquitin antibodies. Polyubiquitinated p185^{HER2}, resolving as smeared high molecular weight bands, was detectable within 5 min of heregulin β1 treatment and decreased by 10 min after stimulation (Fig. 1C). The kinetics of tyrosine phosphorylation, down-modulation and ubiquitination suggests that the first event is phosphorylation, followed by ubiquitination, and finally down-modulation.

3.2. Heregulin β1-induced membrane down-modulation and ubiquitination of p185^{HER2} requires intact kinase activity and the presence of ATP

Flow cytometric analysis showed that p185^{HER2} membrane expression on MDAMB453 cells was down-modulated after 10 min of treatment of the cells with heregulin β1 (Fig. 2A). By contrast, cells stimulated with heregulin β1 in the presence of the kinase inhibitor genistein or in the presence of sodium azide, an inhibitor of ATP-dependent reactions, showed no change in membrane expression (Fig. 2B). In the presence of these two inhibitors, heregulin β1-induced polyubiquitination was almost completely inhibited (Fig. 3). These data indicate that for the p185^{HER2} receptor, tyrosine phosphorylation is a key event in the activation of the ubiquitination process, as shown previously for other receptors such as EGFR, PDGF receptor, and TCR [17–19]. Consistent with Miwazawa's report on c-kit [20], sodium azide suppressed the polyubiquitination of p185^{HER2}, indicating the requirement for ATP in this phenomenon. Membrane down-modulation of p185^{HER2} was also blocked by sodium azide, suggesting that the first event after kinase activation is ubiquitination, which then leads to endocytosis.

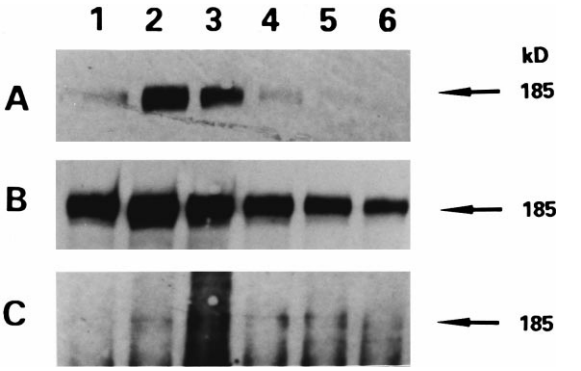

Fig. 1. Western blot analysis of p185^{HER2} phosphorylation (A), expression (B), and polyubiquitination (C) in MDAMB453 cells stimulated with heregulin β1 for different times (min): 0 (lane 1), 1 (lane 2), 5 (lane 3), 15 (lane 4), 30 (lane 5), 60 (lane 6).

3.3. Heregulin β1-induced p185^{HER2} degradation involves the 26S proteasome and is necessary for endocytosis

To identify the proteolytic pathway involved in p185^{HER2} degradation, MDAMB453 cells were treated with LLnL, an inhibitor of the 26S proteasome. Herbimycin A, a tyrosine kinase inhibitor, known to induce EGFR and p185^{HER2} degradation [21], was used as a positive control. In fact, herbimycin A treatment caused the complete disappearance of p185^{HER2} and the presence of LLnL prevented herbymicin A-induced degradation (Fig. 4). When the cells were stimulated with heregulin β1 in the presence of the 26S proteasome inhibitor, the ligand-induced down-modulation was completely inhibited (Fig. 5A), and the quantity of polyubiquiti-

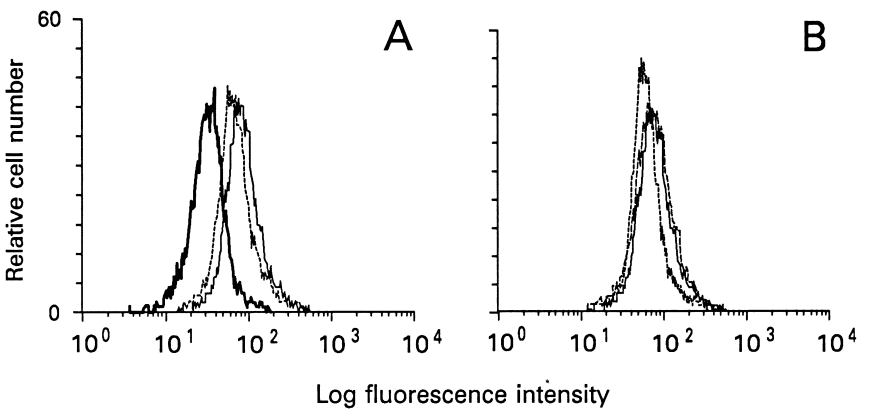

Fig. 2. Flow cytometric analysis of p185^{HER2} membrane expression in: (A) MDAMB453 cells untreated (light line) or treated with heregulin β1 for 10 min (bold line) or pretreated with LLnL overnight followed by heregulin β1 for 10 min (dotted line); (B) MDAMB453 cells untreated (light line) or treated with genistein (dashed line) or with sodium azide (dotted line) and then stimulated with heregulin β1 for 10 min.

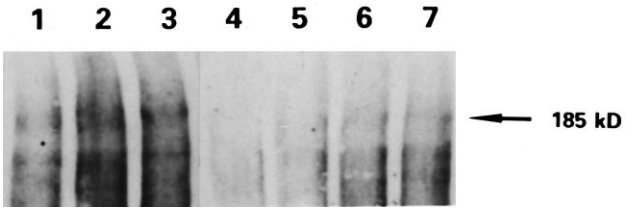

Fig. 3. Western blot analysis of p185^{HER2} ubiquitination in MDAMB453 cells untreated (lanes 1, 2) or treated with sodium azide (lanes 4, 5) or with genistein (lanes 6, 7) and then stimulated with heregulin β1 for 0 (lane 1), 10 (lanes 2, 4, 6) or 60 min (lanes 3, 5, 7).

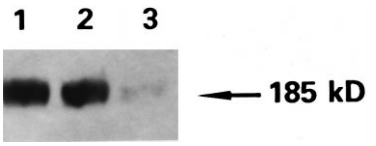

Fig. 4. Western blot analysis of p185^{HER2} expression in MDAMB453 cells untreated (lane 1) or treated with herbimycin A alone (lane 3) or in the presence of LLnL (lane 2).

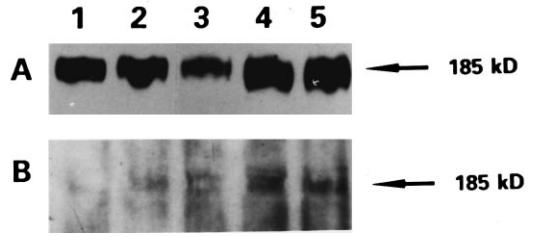

Fig. 5. Western blot analysis of p185^{HER2} expression (A) and ubiquitination (B) in MDAMB453 cells untreated (lanes 1–3), or pretreated with LLnL overnight (lanes 4, 5) and stimulated with heregulin β1 for 10 (lanes 2, 4) or 60 min (lanes 3, 5).

nated receptor increased (Fig. 5B). These data show that p185^{HER2} is degraded by the 26S proteasome pathway, consistent with findings for other receptors [22].

To determine whether the degradation of p185^{HER2} occurs upstream or downstream of its endocytosis, we evaluated the membrane receptor expression of cells treated with heregulin β1 in the presence or absence of the 26S proteasome inhibitor LLnL, by cytofluorimetry. As shown in Fig. 2A, membrane down-modulation induced by heregulin β1 was completely abrogated by LLnL. Thus, endocytosis requires the initiation of degradation. To date, ubiquitination has been considered the key event in the induction of endocytosis. However, we observed an accumulation of polyubiquitinated p185^{HER2} but a block of endocytosis following treatment with LLnL, clearly showing that ubiquitination is not sufficient per se to activate endocytosis and that degradative pathways must also be activated.

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