Cellular Signalling 26 (2014) 2645–2657

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

Ligand stimulation induces clathrin- and Rab5-dependent downregulation of the kinase-dead EphB6 receptor preceded by the disruption of EphB6-Hsp90 interaction

Odette Allonby [a], Amr M. El Zawily [a], Tanya Freywald [a], Darrell D. Mousseau [b,c], Jennifer Chlan [b,d], Deborah Anderson [e,f], Alexandre Benmerah [g,h], Vishaldeep Sidhu [i], Mohan Babu [i], John DeCoteau [a], Andrew Freywald [a,e,*]

[a] Department of Pathology, College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[b] Department of Psychiatry, College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[c] Department of Physiology, College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[d] Department of Anatomy and Cell Biology, College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[e] Department of Biochemistry, College of Medicine, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[f] Cancer Research Unit, Saskatchewan Cancer Agency, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada
[g] INSERM U1163, Laboratory of Inherited Kidney Diseases, 75015 Paris, France
[h] Université Paris Descartes — Sorbonne Paris Cité, Institut Imagine, 75015 Paris, France
[i] Department of Biochemistry, Research and Innovation Centre, University of Regina, Regina, SK,S4S 0A2, Canada

ARTICLE INFO

Article history:
Received 12 May 2014
Received in revised form 25 July 2014
Accepted 15 August 2014
Available online 23 August 2014

Keywords:
EphB6
EphB4
Hsp90
Internalisation
Receptor tyrosine kinase
Downregulation

ABSTRACT

Ligand-induced internalisation and subsequent downregulation of receptor tyrosine kinases (RTKs) serve to determine biological outputs of their signalling. Intrinsically kinase-deficient RTKs control a variety of biological responses, however, the mechanism of their downregulation is not well understood and its analysis is focused exclusively on the ErbB3 receptor.

The Eph group of RTKs is represented by the EphA and EphB subclasses. Each bears one kinase-inactive member, EphA10 and EphB6, respectively, suggesting an important role for these molecules in the Eph signalling network. While EphB6 effects on cell behaviour have been assessed, the mechanism of its downregulation remains elusive. Our work reveals that EphB6 and its kinase-active relative, and signalling partner, EphB4, are downregulated in a similar manner in response to their common ligand, ephrin-B2. Following stimulation, both receptors are internalised through clathrin-coated pits and are degraded in lysosomes. Their targeting for lysosomal degradation relies on the activity of an early endosome regulator, the Rab5 GTPase, as this process is inhibited in the presence of a Rab5 dominant-negative mutant. EphB6 also interacts with the Hsp90 chaperone and EphB6 downregulation is preceded by their rapid dissociation. Moreover, the inhibition of Hsp90 results in EphB6 degradation, mimicking its ligand-induced downregulation. These processes appear to rely on overlapping mechanisms, since Hsp90 inhibition does not significantly enhance ligand-induced EphB6 elimination.

Taken together, our observations define a novel mechanism for intrinsically kinase-deficient RTK downregulation and support an intriguing model, where Hsp90 dissociation acts as a trigger for ligand-induced receptor removal.

1. Introduction

Molecular mechanisms controlling receptor downregulation that is assured by its internalisation, trafficking and eventually, proteolytic degradation, actively modulate responses cell-surface receptors generate following ligand stimulation, and have a profound influence on cell behaviour [1]. Ligand-induced receptor internalisation is a multipurpose process that gates routes towards both receptor degradation and signal abrogation, as well as to efficient activation of certain signalling pathways, including the Ras-MAPK cascade [2]. In agreement, imbalanced downregulation of a number of receptor tyrosine kinases (RTKs) through stimulation-triggered internalisation and degradation, has been linked to oncogenic transformation [1,3,4]. Thus, disruption of Met receptor degradation, following activation by its ligand, HGF, leads to a sustained activation of the Ras-MAP kinase pathway and oncogenic transformation in non-small-cell lung cancer [5]. Colony-stimulating factor-1 receptor

* Corresponding author at: 4D01.9 107 Wiggins Rd, University of Saskatchewan, Saskatoon, SK S7N 5E5, Canada. Tel.: +1 306 966 5248; fax: +1 306 655 0235.
E-mail addresses: odette.allonby@usask.ca (O. Allonby), amr.elzawily@usask.ca (A.M. El Zawily), tanya.freywald@usask.ca (T. Freywald), darrell.mousseau@usask.ca (D.D. Mousseau), jen.chlan@usask.ca (J. Chlan), deborah.anderson@saskcancer.ca (D. Anderson), alexandre.benmerah@inserm.fr (A. Benmerah), vishaldeep.sidhu@uregina.ca (V. Sidhu), mohan.babu@uregina.ca (M. Babu), john.decoteau@usask.ca (J. DeCoteau), andrew.freywald@usask.ca (A. Freywald).

http://dx.doi.org/10.1016/j.cellsig.2014.08.007
0898-6568/

mutations, which impair its internalisation and degradation, have been linked to myelodysplasia and acute myeloid leukaemia [6], and the mitogenic capacity of c-Kit receptor signalling is greatly enhanced in a mutant lacking a docking site for c-Cbl, a well-described regulator of receptor downregulation [7].

A striking example of the power of naturally inefficient RTK downregulation is presented by a member of the EGF receptor (EGFR) group (conventionally called ErbB group), ErbB2. The high potency of ErbB2 receptor action is in part, assured by its ineffective internalisation and degradation, and by its ability to slow down ligand-induced downregulation of other ErbB receptors in heterodimers [8–11]. This results in highly active and robust signalling, which in pathological situations, drives malignant cell behaviour in a variety of tumours, including those of breast, ovarian, gastric, and lung origins [12,13]. The oncogenic potential that stems from inefficient ErbB2 downregulation, and from its ability to modify the internalisation and degradation of its signalling partners gives a strong indication of the biological importance of modulating the balance in these processes. Although the RTK family contains five intrinsically kinase-inactive members [14–18] and, while some of these molecules are known to have important biological functions [13, 19,20], our current understanding of the mechanism of their ligand-induced downregulation is incomplete and is limited to reports focused exclusively on another member of the EGFR group, the ErbB3 receptor. Furthermore, even the mechanism of ErbB3 downregulation is far from being completely understood, as this receptor appears to behave in an inconsistent fashion, demonstrating both internalisation- and downregulation-deficiency, or efficient downregulation in various models [21–26].

Interestingly, two of five known kinase-deficient RTKs belong to the Eph group of receptors [16,17,27]. The *eph* gene was initially identified in the late 1980's by Hirai et al. during a screen of the human genome for tyrosine kinase domains [28]. Today, sixteen Eph receptor tyrosine kinases have been described, including ten EphA (EphA1-EphA10) and six EphB receptors (EphB1-EphB6), and most of them, with the exception of EphA9 and EphB5, are expressed in mammals. Stimulation of Eph receptors with their ligands, ephrins, triggers receptor dimerization or oligomerization, auto-phosphorylation, and the initiation of cytoplasmic signalling [29]. Through their active signalling, Eph receptors control a wide range of responses, including rearrangements of the actin cytoskeleton, cell attachment, repulsion, migration, proliferation and survival, in a variety of normal and malignant cell types [30,31]. In agreement, Eph receptors are actively involved in multiple biological processes, such as embryo development [32], angiogenesis [33], bone homeostasis [34], regulation of T-cell functionality [35–37], insulin production [38] and synaptic plasticity [31,39]. Eph receptors also play an important role in human malignancies, including breast, colorectal, prostate, brain, skin, lung, hepatocellular, and gastric cancers [40]. As both the EphA and EphB subclasses of Eph receptors contain an intrinsically kinase-dead member, EphA10 and EphB6, respectively [16,17,27], it is likely that these kinase-inactive players may have an important role in Eph receptor signalling network. While, little is known about the mechanism or impact of EphA10 signalling, the EphB6 receptor is actively involved in a number of biological responses in normal physiological conditions [41–43] and in human malignancies [19,44–48]. Being kinase-dead, EphB6 relies on ligand-triggered tyrosine phosphorylation provided by kinase-active family members, including EphB1 and EphB4, for signal transduction [45,49]. This raises interesting questions about how stimulation-dependent EphB6 receptor downregulation is organized and whether EphB6 follows the same route of elimination as its kinase-active partner, EphB4 [45].

In this work, we dissect the mechanisms responsible for the ligand-induced downregulation of the kinase-dead EphB6 receptor, comparing EphB6 removal with the downregulation of the EphB4 receptor, which also has not been previously assessed. According to our observations, both receptors follow the same route in their downregulation, their degradation is preceded by internalisation through clathrin-coated pits and once internalised, both are targeted in a Rab5-dependent manner towards their degradation in the lysosomal compartment. In agreement with these observations, EphB6 co-expression does not alter the rate at which EphB4 is eliminated. Most notably, our work reveals that EphB6 interacts with the Hsp90 chaperone and this interaction appears to ensure EphB6 stability, as treatment with an Hsp90 inhibitor, geldanamycin (GA), reduces EphB6 presence, with the rate of EphB6 removal closely resembling ligand-induced downregulation. Moreover, stimulation of the EphB6 receptor with its ligand, ephrin-B2 results in a rapid disruption of EphB6-Hsp90 interaction at the cell membrane that precedes EphB6 removal. Both ligand-induced and GA-triggered EphB6 degradation responses are likely to be mediated by the same molecular mechanism, as GA treatment does not provide a consistent significant enhancement to stimulation-initiated EphB6 elimination. These observations support an intriguing model, where the process of EphB6 downregulation is initiated by EphB6-Hsp90 dissociation.

Overall, our data provide the first description of the routes of the stimulation-dependent downregulation of the kinase-deficient EphB6 receptor and its kinase-active partner, EphB4, and highlight a novel mechanism for RTK removal, which is associated with the ligand-induced disruption of a receptor-Hsp90 complex. This clarifies one of the pathways used by kinase-dead RTKs in their downregulation and may also help in understanding EphB6 action in normal physiological responses and in malignancy.

2. Materials and methods

2.1. Antibodies

Antibodies for Myc, Erk2, β-Tubulin, EphB4, Clathrin heavy-chain, EphB4, and Eps15 were from Santa Cruz Biotechnology, goat anti-human Fc (anti-hFc) was from Pierce Biotechnology, anti-EphB6 were from Santa Cruz, R&D Systems, and Sigma-Aldrich, anti-Hsp90 was from StressGen, and Goat F(ab')2 Anti-Human IgG (Fc) FITC was from Beckman Coulter. Ephrin-B2-Fc and fluorescein labelled anti-sheep IgG were purchased from R&D Systems. Secondary antibodies linked to IR-dyes for Western blotting (anti-goat, anti-rabbit, anti-rat and anti-mouse) were purchased from Mandel Scientific. Anti-rat Alexafluor 594 was purchased from Cell Signalling.

2.2. Expression constructs and shRNA

The wild-type EphB6 receptor and EGFP-tagged EphB6 constructs were kindly provided by Dr. C.M. Roifman (Sick Children's Hospital, Toronto, ON). EphB4 and EphB4-EGFP constructs were purchased from OriGene Technologies, Inc. Myc-tagged EphB6 and an EphB6 mutant, lacking the cytoplasmic domain (ΔEphB6) and a tyrosine to phenylalanine substitution mutant of EphB6 (EphB6 Y->F) were described previously [43,45,49]. Clathrin heavy chain (CHC) shRNA lentiviral particles and control non-silencing shRNA were purchased from Santa Cruz. RFP-tagged Rab5 was provided by Dr. Anderson from the University of Saskatchewan. An Eps15 mutant, DIII, was provided by Dr. Benmerah from the Institut Imagine, Paris, France.

2.3. Cell culture and generation of stable cell lines

Stable cell lines of HEK-293 cells [American Type Culture Collection (ATCC)], expressing either Myc-tagged EphB4 (HEK-B4-M), EphB6 (HEK-B6), ΔEphB6 (HEK-ΔEphB6), EphB6 (Y->F) (HEK-EphB6(Y->F)), EGFP-tagged EphB4 (HEK-B4-EGFP), or Myc-tagged EphB6 (HEK-B6-M) were generated by electroporation using 20 μg of DNA (70 ms, 140 V, ECM 830 electroporator; Harvard Apparatus Inc.). Cells were allowed to rest overnight and were then subjected to G418 (1 mg/ml) (Calbiochem) selection for 30 days. Cells were lysed and screened for Eph receptor expression by Western blotting.