Cell

Cytohesins Are Cytoplasmic ErbB Receptor Activators

Anke Bill,[1,8] Anton Schmitz,[1,8] Barbara Albertoni,[1] Jin-Na Song,[1] Lukas C. Heukamp,[2] David Walrafen,[3] Franziska Thorwirth,[4] Peter J. Verveer,[4] Sebastian Zimmer,[2] Lisa Meffert,[2] Arne Schreiber,[3] Sampu[illegible]terjee,[5] Roman K. Thomas,[5,6,7] Roland T. Ullrich,[5] Thorsten Lang,[3] and Michael Famulok[1,*]

[1]LIMES Institute, Program Unit Chemical Biology & Medicinal Chemistry, Laboratory of Chemical Biology, Rheinische Friedrich-Wilhelms-Universität Bonn, Gerhard-Domagk-Str. 1, 53121 Bonn, Germany

[2]Institute of Pathology, Universitätsklinikum, Rheinische Friedrich-Wilhelms-Universität Bonn, Sigmund[illegible]ud Stras[illegible]5, 53123 Bonn, Germany

[3]LIMES Institute, Program Unit Membrane Biology & Lipid Biochemistry, Laboratory of Membrane[illegible]ochemi[illegible], Rheinische Friedrich-Wilhelms-Universität Bonn, Carl-Troll-Straße 31, 53115 Bonn, Germany

[4]Department of Systemic Cell Biology, Max-Planck Institute of Molecular Physiology, Otto-H[illegible]-Str.[illegible]4227 Do[illegible]nd, Germany

[5]Max Planck Institute for Neurological Research with Klaus-Joachim-Zülch Laboratories of t[illegible]Max Planc[illegible]ciety[illegible]the Medical Faculty of the University of Köln, Gleueler Str. 50, 50931 Köln, Germany

[6]Chemical Genomics Centre of the Max Planck Society, Otto-Hahn Str. 15, 44227 D[illegible]mun[illegible]rmany

[7]Center of Integrated Oncology and Department I of Internal Medicine, University of Köln, Kerp[illegible]Straße 62, 50937 Köln, Germany

[8]These authors contributed equally to this work

*Correspondence: m.famulok@uni-bonn.de

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SUMMARY

Signaling by ErbB receptors requires the activat[illegible] of their cytoplasmic kinase domains, w[illegible]h is ini[illegible]ated by ligand binding to the recepto[illegible]cte[illegible]mains. Cytoplasmic factors contributing [illegible] the a[illegible]ivation are unknown. Here we identify me[illegible]rs [illegible]hesin protein family as such f[illegible]tors. [illegible]hesin inhibition decreased ErbB rece[illegible]r autopho[illegible]orylation and signaling, whereas cyt[illegible]sin overe[illegible]ression stimulated receptor a[illegible]ivation. M[illegible]itoring epidermal growth factor re[illegible]tor (EGFR) [illegible]formation by anisotropy micr[illegible]opy t[illegible]ether with cell-free reconstitution of cyto[illegible]in-[illegible]pendent receptor autophosphorylation indica[illegible]at cyto[illegible]sins facilitate conformational [illegible]ngeme[illegible]s i[illegible]e intracellular domains of dim[illegible]zed [illegible]ceptors. [illegible]onsistent with cytohesins play[illegible]a pr[illegible]t role in ErbB receptor signaling, we fou[illegible]at cyto[illegible]esin overexpression correlated with EGF[illegible]gnaling pathway activation in human lung adeno[illegible]inomas. Chemical inhibition of cytohesins resulted in reduced proliferation of EGFR-dependent lung cancer cells in vitro and in vivo. Our results establish cytohesins as cytoplasmic conformational activators of ErbB receptors that are of pathophysiological relevance.

INTRODUCTION

ErbB receptors are key regulators of cell differentiation, survival, proliferation, and migration, and aberrant ErbB receptor function is a hallm[illegible]many human cancers (Fischer et al., 2003; Bublil [illegible]arden, 2007). The ErbB receptor family is comprised of four [illegible]the epidermal growth factor receptor (EGFR/ErbB1), [illegible]er2/ErbB2, Her3/ErbB3, and ErbB4. Signaling is initiated by growth factor binding to the extracellular domains of the ErbB [illegible]eceptors. The ligand-induced conformational change in the receptor ectodomains results in the association of the cytoplasmic tyrosine kinase domains of two receptor molecules. This association has been considered to be sufficient for releasing the default autoinhibited state of the kinase domains (Ferguson, 2008; Bose and Zhang, 2009). However, the picture appears to be more complex as only a fraction of the dimerized ErbB receptors are catalytically active (Gadella and Jovin, 1995; Moriki et al., 2001; Cui et al., 2002), and because receptor dimerization seems to occur continuously and reversibly even in the absence of ligand (Chung et al., 2010). Recent crystallographic studies indicate that catalytic activity may be restricted to dimers that show a special arrangement of the kinase domains, the so-called asymmetric dimers (Zhang et al., 2006; Qiu et al., 2008; Jura et al., 2009; Red Brewer et al., 2009). However, determinants defining the fraction of active dimers that form within the entire population of dimerized receptors remain elusive. This fraction may simply depend on the rate of the spontaneous conversion from the symmetric to the asymmetric dimer. Alternatively, the fraction of active dimers may not simply be defined by receptor-inherent properties alone or by an equilibrium between the two receptor dimer populations but be modulated by cytoplasmic activator proteins. Such activators would endow the cell with the possibility to fine-tune the number of actively signaling receptors within a given pool of ligand-occupied receptors according to cellular needs. However, cytoplasmic activators of ErbB receptors have not yet been identified.

Here, we report cytohesins as cytoplasmic ErbB receptor activators. The cytohesin family consists of four highly homologous

members, including ubiquitously expressed cytohesin-1, cytohesin-2 (ARNO), cytohesin-3 (Grp1), and cytohesin-4, which is exclusively found in cells of the immune system (Kolanus, 2007). Cytohesins are guanine nucleotide exchange factors (GEFs) for ADP ribosylation factors (ARFs) that belong to the family of small Ras-like GTPases. As in the case of other small GTPases, ARF function critically depends on activation by GEFs (Bos et al., 2007). Thus, because ARFs are involved in controlling cytoskeletal dynamics, cell migration, vesicular traffic, and signaling (Casanova, 2007; Kolanus, 2007), cytohesins are important regulators of these processes.

We show that cytohesins enhance EGFR activation by directly interacting with the cytoplasmic domains of dimerized receptors and by facilitating conformational rearrangements of these domains. Chemical inhibition and knockdown of cytohesins reduce EGFR activation, whereas cytohesin overexpression has the opposite effect. Our results strongly suggest that EGF and cytohesins concertedly determine the degree of EGFR activation. We propose that whereas EGF exhibits its known function from the extracellular side, namely to relieve the autoinhibition of the unliganded receptor, cytohesins function to adjust EGFR signaling from the cytoplasmic side by increasing the number of EGFR dimers having the active, catalytically competent conformation within the reservoir of ligand-bound EGFR dimers. This model is further supported by the finding th[at] hesin expression levels in human tumors correlate with [EGFR] activation and signaling and that the chemical inhibition of [cyto]-hesins reduces cell proliferation in vitro and tumor grow[th] mice. Thus, cytohesins are introduced as int[racellular] EGFR a[ctiva]vators that are relevant in the patho[physiology] of certa[in] cancers.

RESULTS

Chemical Inhibition and Knock[down] of Cytohesins Reduce ErbB Recept[or] Signaling

To test whether cytohesins are involved in ErbB receptor signaling, we used the specific cytohesin antagonist SecinH3 (Hafner et al., 2006; Bi et al., 2008). For this purpose, EGFR-expressing human lung adenocarcinoma-derived H460 cells were stimulated with EGF in the presence of SecinH3. Using autophosphorylation as a readout, we observed that SecinH3-treated cells showed an about 50% inhibition of EGFR activation (Figure 1A). The effect was also found at the level of the adaptor proteins IRS1 and Shc and of the downstream kinases p44/42 (Erk1/2). A control compound (XH1009) that is structurally related to SecinH3 but does neither bind nor inhibit cytohesins (Bi et al., 2008) had no effect on EGFR activation and signaling (Figure S1A available online). To obtain SecinH3-independent evidence, the cytohesin-specific aptamer M69 (Mayer et al., 2001) or cytohesin-specific siRNAs were used. Inhibition of EGFR activation was observed in both experiments (Figures S1B and S1C). The re-expression of cytohesin-2/ARNO in siRNA-treated cells rescued the effect of ARNO knockdown on EGFR autophosphorylation (Figure S2A, lanes 4 and 6).

We then analyzed whether cytohesins also affected the signaling of Her2 and Her3, two other members of the ErbB receptor family forming a heterodimer. When Her2/Her3-expressing human breast adenocarcinoma-derived SkBr3 cells were treated with heregulin, SecinH3 reduced the phosphorylation of Her3 by about 50% (Figure 1B). This reduction in Her3 activation was mirrored in reduced activation of the adaptor protein IRS1 and the downstream kinases Akt and p44/42.

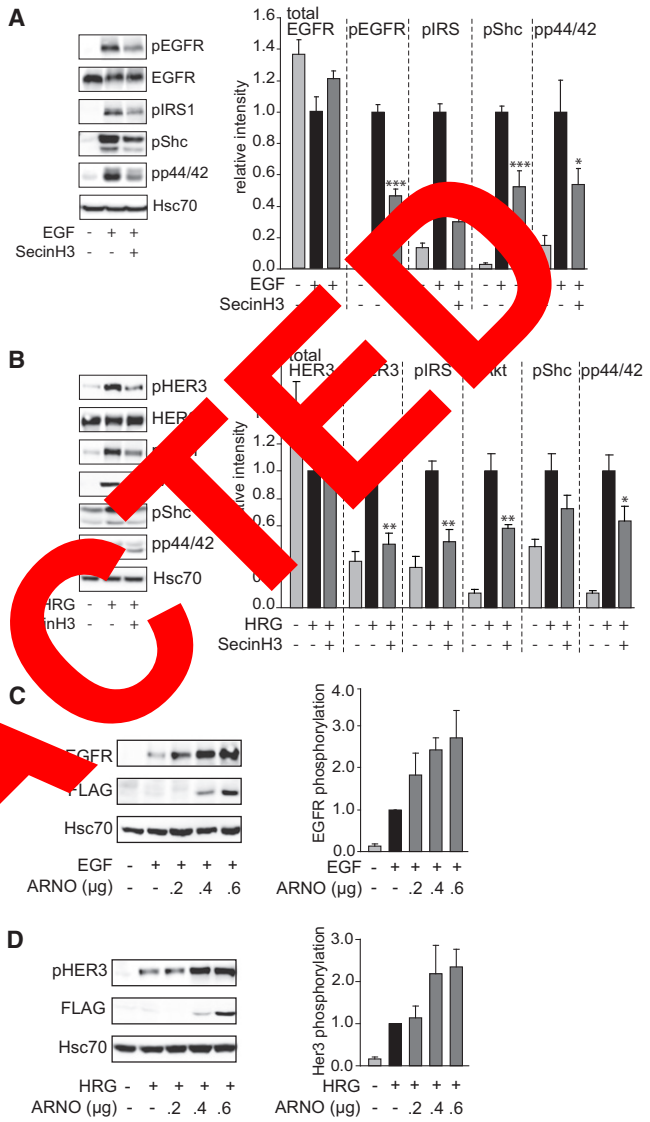

Figure 1. Cytohesins Enhance Activation of ErbB Receptors

(A and B) The cytohesin inhibitor SecinH3 reduces ErbB receptor signaling. Western blot analysis of H460 (A) or SkBr3 (B) cells treated with SecinH3 or solvent and stimulated with EGF or heregulin (HRG), respectively, is shown. Phosphorylation of the indicated proteins was determined by immunodetection using phosphospecific antibodies. Heat shock cognate protein 70 (Hsc70) served as loading control. The diagrams show relative phosphorylation levels after normalization for Hsc70. The untreated ligand-stimulated cells were set as 1 (n = 6).

(C and D) Overexpression of the cytohesin ARNO enhances ErbB receptor autophosphorylation. H460 (C) or SkBr3 (D) cells were transfected with increasing amounts of FLAG-tagged ARNO and stimulated with ligand. Receptor autophosphorylation was analyzed as above (n = 3).

Data are represented as mean ± SEM. See Figure S1 for further information.