RNF41 (Nrdp1) controls type 1 cytokine receptor degradation and ectodomain shedding

Joris Wauman1,2,*, Leentje De Ceuninck1,2,*, Nele Vanderroost1,2, Sam Lievens1,2 and Jan Tavernier1,2,‡

1Department of Medical Protein Research, Flanders Interuniversity Institute for Biotechnology (VIB), Ghent University, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium
2Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium
*These authors contributed equally to this work
‡Author for correspondence (jan.tavernier@vib-ugent.be)

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Summary

Cytokines, such as interferons, erythropoietin, leptin and most interleukins, signal through type 1 cytokine receptors and activate the canonical JAK–STAT pathway. Aberrant cytokine signalling underlies numerous pathologies and adequate, temporary receptor activation is therefore under tight control. Negative-feedback mechanisms are very well studied, but cellular sensitivity also depends on the number of receptors exposed at the cell surface. This is determined by the equilibrium between receptor synthesis and transport to the plasma membrane, internalisation and recycling, degradation and ectodomain shedding, but the molecular basis of how cells establish steady state receptor levels is poorly understood. Here, we report that ring finger protein 41 (RNF41, also known as E3 ubiquitin-protein ligase Nrdp1) interacts with JAK2-associated cytokine receptor complexes and modulates their cell surface exposure upon phosphorylation of its cytosolic domain (Diaz-van Kerkhof, 2002). RNF41, determining the balance.

Key words: Cathespin L cleavage, Ectodomain shedding, MAPPIT, RNF41, Nrdp1, Type 1 cytokine receptor, ADAM

Introduction

Cytokines such as interferons, erythropoietin, leptin and most interleukins signal through type 1 cytokine receptors. Because aberrant cytokine signalling underlies many pathologies, including immunodeficiency, metabolic disorders and cancer, temporary cytokine receptor activation is tightly regulated. Negative-feedback mechanisms that operate to dampen signalling after receptor activation are very well studied. These include phosphatases (Xu and Qu, 2008), protein inhibitors of activated STAT (PIAS) proteins (Shuai and Liu, 2005) and the suppressor of cytokine signalling (SOCS) family (Lavens et al., 2006). Cellular sensitivity to cytokine signals, however, also depends on the number of receptors present at the time of stimulation. The molecular basis of how cells establish an appropriate number of receptors at their cell surface is far less well understood. Cell surface exposure of type 1 cytokine receptors is a highly dynamic process that, besides de novo synthesis, involves endocytosis followed by recycling or degradation. Internalised receptors, irrespective of ligand triggering, are targeted to early endosomal compartments, followed by sorting to recycling vesicles, signalling endosomes or multivesicular bodies (MVBs) and lysosomes for degradation (Jovic et al., 2010). Although ligand-induced endocytosis was originally thought to be solely a mechanism of receptor breakdown, receptors can remain active within endosomes and can couple to endosome-specific signalling pathways (Sorkin and von Zastrow, 2009). Protein ubiquitylation is a major mechanism that controls receptor internalisation, intracellular trafficking and proteasomal and lysosomal degradation (d’Azzo et al., 2005; Hicke and Dunn, 2003). Mono-ubiquitylation of cytokine receptors and the recruitment of ubiquitin-binding proteins serve as a regulated sorting mechanism for internalisation and sorting into the endocytic pathway. However, the specific ubiquitin ligases, ubiquitin-sorting signals and ubiquitin-binding proteins that control cytokine receptor trafficking and turnover remain poorly characterised and vary considerably between different cytokine receptor systems (Belouzard and Rouille, 2006; Irandoust et al., 2007; Strous and van Kerkhof, 2002).

The number of signalling-competent receptors at the cell surface is also determined by ectodomain shedding. Proteolytic cleavage of cell surface receptors by transmembrane metalloproteases of the ADAM (a disintegrin and metalloproteinase) family releases the receptor ectodomains and renders the cells desensitised to stimulation. ADAM10 and ADAM17 (or TNFα converting enzyme, TACE), the prototypical receptor sheddases, have been implicated in ectodomain cleavage of cytokine receptors of different families, including the tumor necrosis factor (TNF) and interleukin-1 (IL-1) receptor families and the type 1 cytokine receptors (Levine, 2008). The activity of ADAM17 is regulated by several mechanisms (for a review, see Huovila et al., 2005) including catalytic activation and cell surface exposure upon phosphorylation of its cytosolic domain (Diaz-Rodriguez et al., 2002; Xu and Derynick, 2010). In addition, because ADAM10 and ADAM17 activity is confined to cholesterol-rich membrane microdomains (lipid rafts) (Matthews et al., 2003; Tellier
et al., 2006), substrates must be targeted to these rafts, but the molecular mechanisms regulating this routing remain unknown.

Ring finger protein 41 (RNF41), also referred to as E3 ubiquitin-protein ligase neuregulin receptor degradation protein-1 (Nrdp1) or fetal liver ring finger (FLRF) belongs to the family of single RING (really interesting new gene) finger-containing proteins. Members of this protein family function as E3 ubiquitin ligases. RNF41 serves as a scaffold by coordinating ubiquitin transfer from a ubiquitin-conjugating enzyme (E2) recruited by its N-terminal RING domain to a specific substrate that interacts with its C-terminal substrate binding domain. RNF41 has been implicated in the ubiquitylation and degradation of two other E3 ubiquitin ligases: BRUCE (Qiu et al., 2004), an inhibitor of apoptosis protein and parkin (Zhong et al., 2005), a protein involved in the onset of Parkinson’s disease. More recently, RNF41 was found to control Toll-like receptor (TLR)-mediated responses through ubiquitylation of the central adaptor MyD88 and the kinase TBK1 (Wang et al., 2009). RNF41 also functions as a key regulator of steady-state cell surface levels of ErbB3 and ErbB4, two receptors that are closely related to the epidermal growth factor receptor (EGFR). RNF41 associates with these receptors independently of receptor stimulation (Diamonti et al., 2002; Qiu and Goldberg, 2002) and elicits ligand-independent ErbB3 ubiquitylation and degradation (Qiu and Goldberg, 2002). The biological significance of RNF41 in ErbB3 receptor signalling is underscored by the observation that perturbation of RNF41 activity results in enhanced ErbB3-activated cell growth and motility of human breast carcinoma cells. Moreover, loss of RNF41 expression correlates with in vivo overexpression and hyper-signalling of ErbB3, as commonly found in primary human breast cancer tissue, and is related to tumor malignancy (Yen et al., 2006). Similarly to its impact on ErbB3 and ErbB4 receptor expression and function, RNF41 can modulate ligand-independent expression of the interleukin-3 (IL-3) and erythropoietin (Epo) cytokine receptors (Jing et al., 2008), but the underlying mechanism is unknown. Here, we demonstrate that RNF41 has a generic role in type 1 cytokine receptor signalling by controlling receptor degradation and shedding.

**Results**

**RNF41 is an interaction partner of the leptin receptor complex in MAPPIT screening experiments**

MAPPIT (mammalian protein-protein interaction trap) is a cytokine-receptor-based two-hybrid method that allows detection of protein interactions in mammalian cells (Eyckerman et al., 2001). In brief, a cytokine receptor, e.g. the leptin receptor (LR), is rendered inactive by mutating all cytosolic tyrosines (LR-F3): activation of the JAK–STAT signalling pathway thus depends on a bait–prey interaction, whereby the prey is fused to functional STAT recruitment sites (Fig. 1A). Using a FACS-based protocol, complex prey cDNA libraries can be screened for novel interaction partners of a selected bait (Lievens et al., 2004). It is intrinsic to this method that preys that bind to the receptor complex independently of the bait (e.g. to the LR cytoplasmic tail or JAK2) are identified as technical ‘false positives’. One such frequently identified protein

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**Fig. 1. Identification of RNF41 as a LR-complex-interacting protein.**

(A) Schematic outline of the MAPPIT technique using a chimeric EpoR-LR bait receptor (ELR-F3).

(B) HEK293T cells transfected with an RNF41 prey arising from a MAPPIT screen and the indicated baits, were stimulated with Epo or left untreated. Luciferase data of triplicate measurements from a representative experiment are expressed as fold induction (stimulated/non stimulated) ± s.d.

(C) Human RNF41 structure and sequence of its RING domain. Arrows indicate in-frame fusion positions with gp130 in the RNF41 prey constructs identified in MAPPIT screens. The eight residues constituting a functional RING domain are underlined.
in screening experiments corresponded to RNF41. Fig. 1B shows a standard MAPPIT experiment demonstrating the interaction of an RNF41 prey with the chimeric receptor complex, irrespective of the bait (p53 or FKB12). Significantly, all 13 RNF41-encoding preys identified in MAPPIT screens featured a truncated N-terminal RING domain (Fig. 1C). An RNF41 protein lacking a functional RING domain acts as a dominant-negative inhibitor, potentiating ErbB3 signalling by interfering with receptor degradation (Diamonti et al., 2002; Qiu and Goldberg, 2002). Likewise, truncated RNF41 preys might stabilise the LR–bait complexes, readily explaining their efficient identification in MAPPIT screens.

RNF41 modulates type 1 cytokine receptor cell surface expression and signalling

We next investigated whether modulation of RNF41 levels affected the expression of signalling competent LRs on the cell surface. Ectopic expression of full-length RNF41 or the dominant-negative RNF41 ΔRING clearly suppressed or enhanced LR cell surface expression (Fig. 2A) and signalling (Fig. 2B), respectively. Modulation of receptor expression was ligand independent (Fig. 2A). In addition, vector-based delivery of a short-hairpin oligonucleotide targeting endogenous RNF41 (supplementary material Fig. S1) enhanced LR surface expression and leptin signalling (Fig. 2C,D). These effects were specific to RNF41 because co-expression of a knockdown-resistant RNF41 construct (rscRNF41) (supplementary material Fig. S1) completely normalised LR surface expression and signalling (Fig. 2C,D). Modulation of RNF41 levels also affected STAT3 phosphorylation, as expected (Fig. 2E). Collectively, these results show that RNF41 regulates basal cell surface expression and signalling of the LR.

Similar effects were observed for other type 1 cytokine receptors: RNF41 expression attenuated signalling in HEK293T cells via the endogenous leukaemia inhibitory factor receptor (LIFR) and ectopically expressed EpoR (Fig. 3A) or via the endogenous IL-3R complex in hematopoietic Ba/F3 cells (Fig. 3B), demonstrating that this effect of RNF41 is not restricted to HEK293T cells. Conversely, RNF41 ΔRING acted as a dominant-negative inhibitor that potentiated signalling. Similarly to the LR, knockdown of endogenous RNF41 potentiated LIFR signalling, and expression of the rscRNF41 construct completely reversed this effect (Fig. 3C). The common denominator of all these receptor complexes is the receptor-associated JAK2 kinase, suggesting a direct impact of RNF41 on JAK2. Overexpression of RNF41 can suppress JAK2 levels (Fig. 3D), but we could not demonstrate efficient co-immunoprecipitation between RNF41 and JAK2, or RNF41-induced JAK2 ubiquitylation (data not shown).

Cathepsin L cleaves the leptin receptor

We next investigated the effect of RNF41 on ligand-independent LR turnover. Both the long (LRlo) and short (LRsh) isoforms of the LR are constitutively endocytosed and degraded in lysosomes (Belouzard et al., 2004). To monitor LR degradation, we incubated HEK293T cells expressing a human LRlo carrying a C-terminal HA-tag (hLRlo–HA) with chloroquine, an inhibitor of intralysosomal degradation. This led to the stabilisation of two C-terminal fragments (Fig. 4A). These degradation products of about 42 and 46 kDa, further referred to as the C-terminal LR stubs (LR-CTS), were generated independent of leptin stimulation. This cleavage is conserved between species and between different LR isoforms as a comparable 12 kDa LR-CTS was observed for the mouse LRsh fused to a C-terminal FLAG tag (mLRsh–FLAG) (Fig. 4B). We identified the protease involved in this cleavage using a series of pharmacological inhibitors. HEK293T cells expressing hLRlo–HA were first incubated with cell-permeable broad-spectrum protease blockers, each of which inhibited one of the four large protease classes (calpeptin and E-64d for cysteine proteases; leupeptin and PMSF for serine proteases; pepstatin A for aspartic proteases; GM6001 for metalloproteases). Only calpeptin and E-64d inhibited LR-CTS formation (Fig. 4C). Cathepsins are well-characterised cysteine proteases that are involved in protein degradation. The cathepsin B and L inhibitor z-Fa-fmk and three different specific cathepsin L inhibitors completely blocked LR cleavage, whereas specific inhibitors of cathepsin B, cathepsin K or cathepsin S had no clear effect, demonstrating that cathepsin L causes cleavage of the LR (Fig. 4D). Similar results were obtained with mLRsh–FLAG (Fig. 4E). The size of the LR-CTS predicted cleavage close to the transmembrane region. Subcellular fragmentation revealed that the LR-CTS was associated with the membrane fraction (Fig. 4F), in line with juxtanembrane cleavage. Previous studies have shown that the hLR can undergo ectodomain shedding resulting in the generation of soluble LR. This shedding by a membrane-associated metalloprotease was enhanced by the phorbol ester PMA and N-ethylmaleimide (NEM) and could be blocked by the metalloprotease inhibitor TAPI-1 (Maamra et al., 2001). However, incubation of hLRlo–HA expressing HEK293T cells with PMA or NEM, or co-incubating these shedding enhancers with TAPI-1 had no effect on the generation of LR-CTS, whereas calpeptin or a specific cathepsin L inhibitor completely blocked its formation (Fig. 4G). This indicates that, although processing must occur at very closely positions in the LR ectodomain, shedding and LR-CTS cleavage are two independent mechanisms. Receptor trafficking is necessary to reach the correct subcellular location for cleavage, because incubation with Brefeldin A (BFA), an inhibitor of Arf1 activation that blocks transport from the ER to the Golgi, completely inhibited formation of the LR-CTS, supporting the notion that cleavage occurs in a post-ER subcellular compartment such as the MVBs or lysosomes (Fig. 4H).

RNF41 blocks LR-CTS cleavage and enhances leptin receptor ectodomain shedding

Importantly, RNF41 expression completely blocked the formation of the LR-CTS in hLRlo–HA-expressing HEK293T cells, without affecting the level of full-length LRs. No similar effect was seen for RNF41 ΔRING, implying a crucial role for the RING domain (Fig. 5A). Similar findings were obtained for mLRsh–FLAG (Fig. 5B). Direct inhibition of cathepsin L was ruled out because RNF41 or RNF41 ΔRING expression did not alter the levels of procathepsin L (42 kDa) or of its processed intermediate and active forms (34 kDa and 26 kDa, respectively) (Fig. 5C). We conclude that RNF41 inhibits lysosomal targeting of LRs.

Cell culture supernatants were collected from the same transfected cells to analyse LR shedding. RNF41 expression significantly increased soluble LR levels from hLRlo–HA-expressing HEK293T cells, whereas expression of RNF41 ΔRING had no effect (Fig. 5D). As mentioned above, LR ectodomain shedding can be blocked by the metalloprotease inhibitor TAPI-1 (Maamra et al., 2001). Incubation of hLRlo–HA-expressing HEK293T cells with TAPI-1 reversed the enhancing effect of RNF41 on LR shedding (Fig. 5E). In contrast to TAPI-1, incubation with a specific cathepsin L inhibitor had no significant effect on basal or RNF41-induced soluble LR levels, confirming that LR ectodomain shedding by a metalloprotease and LR cleavage by cathepsin L are two
distinct proteolytic events. TAPI-1 is known to inhibit several members of the ADAM family. Therefore, we tested the effect of silencing ADAM10 and ADAM17, two typical cytokine receptor sheddases, on RNF41-induced ectodomain shedding. Silencing of ADAM10 and ADAM17 gene expression reversed the enhancing effect of RNF41 on LR shedding (Fig. 5F; supplementary material
Fig. S2. Importantly, because shedding and cleavage of the LR were studied simultaneously in the same cells, these observations provide evidence that RNF41 re-routes the LR from lysosomal degradation to subcellular microdomains where it is targeted by metalloproteases of the ADAM family, resulting in ectodomain release.

To visualise the intracellular LR redistribution predicted by such a scenario, we performed confocal microscopy on HeLa cells that expressed LR–FLAG. Following overnight incubation with chloroquine, RNF41 expression impaired colocalisation between the C-terminally FLAG-tagged LR and LAMP-1, which is a typical lysosomal marker (Fig. 5G). Together, these findings suggest an important impact of RNF41 on the intracellular routing of the LR.

RNF41 controls ectodomain shedding of the LIFRα and IL-6Rα

We next questioned whether RNF41 controlled degradation and shedding in a generic way, as we previously observed for JAK2-associated cytokine receptor exposure and signalling. As seen for the LR, chloroquine stabilised C-terminal stub fragments in HEK293T cells expressing LIFRα–HA or IL-6Rα–HA (Fig. 6A,B). Incubation with chloroquine also stabilised another IL-6Rα C-terminal fragment (25 kDa) that was insensitive to cathepsin L inhibition. However, the formation of this fragment was also blocked by RNF41 expression, suggesting that the responsible protease resides at the same subcellular location. Soluble LIFRα and IL-6Rα can be generated by alternative splicing (Horiuchi et al., 1994; Tomida et al., 1994) or, in case of the IL-6Rα, also by ectodomain shedding (Mullberg et al., 1995). Again, as seen for the LR, RNF41 increased soluble LIFRα and IL-6Rα levels, whereas RNF41 ΔRING had no similar effect (Fig. 6C,D). This ectodomain shedding was caused by a sheddase activity because incubation with GM6001, a general metalloprotease inhibitor, reversed RNF41-induced ectodomain shedding of LIFRα and IL-6Rα (Fig. 6E,F). Induced ectodomain shedding of the IL-6Rα is mainly mediated by ADAM17, whereas constitutive shedding is mediated by ADAM10 (Matthews et al., 2003). In contrast to silencing of ADAM17, ADAM10 silencing reversed RNF41-induced IL-6Rα ectodomain shedding, which is in line with the constitutive nature of the effects of RNF41 on receptor shedding (Fig. 6G). TAPI-1, which inhibits shedding of LR (Maamra et al., 2001) and IL-6Rα (Mullberg et al., 1995), had
Fig. 4. The LR is cleaved by cathepsin L. Cell lysates of HEK293T cells transiently expressing the long isoform of the human LR with a C-terminal HA-tag (hLR–HA) or the short isoform of the mouse LR with a C-terminal FLAG-tag (mLRsh–FLAG) were analysed using western blotting (WB). (A) Cells expressing hLR–HA were left untreated (DMSO) or were incubated overnight with chloroquine, in the absence of stimulus or in combination with leptin for 0.5 or 24 hours. (B) Cells expressing mLRsh–FLAG were left untreated (DMSO) or were incubated overnight with chloroquine. (C, D) Cells expressing hLR–HA were left untreated (DMSO) or were incubated overnight with chloroquine alone or together with the indicated protease inhibitors. (E) Cells expressing mLrsh–FLAG were left untreated (DMSO) or were incubated overnight with chloroquine alone or together with cathepsin L inhibitor III. (F) The remaining C-terminal fragment is membrane anchored. Untransfected HEK293T cells or HEK293T cells transiently expressing hLR–HA were incubated overnight with chloroquine. Cell homogenates were separated in a nuclear (NUC), cytoplasmic (CYT), membrane (MEM) and rest fraction by differential centrifugation and analysed for the presence of the FL hLR (top square) or the CTS (bottom square) by western blotting (WB). M, molecular weight marker. (G) Activation or inhibition of ectodomain shedding has no effect on LR cleavage by cathepsin L. Cells expressing hLR–HA were left untreated (DMSO) or were incubated overnight with chloroquine alone or in combination with calpeptin, cathepsin L inhibitor III, NEM or PMA with or without TAPI-1. (H) Brefeldin A (BFA) incubation blocks LR-CTS formation. Cells expressing hLR–HA were left untreated (DMSO) or were incubated overnight with chloroquine alone or in combination with BFA. FL, full length; CTS, C-terminal stub.
Fig. 5. RNF41 blocks formation of the LR CTS and concomitantly enhances LR shedding. (A) HEK293T cells were cotransfected with hLR–HA and increasing amounts of Etag–RNF41 or Etag–RNF41 ΔRING. After overnight chloroquine incubation, cell lysates were analysed using western blotting (WB). (B) HEK293T cells were cotransfected with mLrsh–FLAG and Etag–RNF41 or Etag–RNF41 ΔRING. After overnight chloroquine incubation, cell lysates were analysed using western blotting (WB). FL: full length; CTS: C-terminal stub. (C) Analysis of procathepsin L (42 kDa) and cathepsin L (34 and 26 kDa) expression after RNF41 (ΔRING) expression. (D) Cell media supernatants from the transfectants in Fig. 5A were analysed for soluble LR levels. (E) RNF41-enhanced LR shedding is reversed by TAPI-1. HEK293T cells transfected with hLR–HA with or without full length Etag–RNF41 were incubated overnight in starvation medium with TAPI-1 or cathepsin L inhibitor III and soluble LR levels in the cell media supernatants were quantified. (F) RNF41-enhanced LR shedding is reversed by silencing of ADAM10 and ADAM17. HEK293T cells, reverse transfected with siRNA targeting ADAM10 or ADAM17, were cotransfected the next day with hLR–HA with or without full-length Etag–RNF41 and soluble LR levels in the cell media supernatants were quantified. Values are means ± s.d. (n=3). *P<0.01 or **P<0.05 (Student’s t-test) compared with –RNF41. §P<0.01 or §§P<0.05 (Student’s t-test) compared with +RNF41. (G) RNF41 reduces colocalisation of LR–FLAG with LAMP-1. HeLa cells cotransfected with hLR–FLAG together with (bottom) or without (top) RNF41 were immunostained with anti-FLAG (green) and anti-LAMP-1 (red) antibodies following overnight chloroquine incubation. DAPI (blue) was used to visualise the nuclei. Images were acquired by confocal microscopy.
Fig. 6. RNF41 blocks LIFRα and IL-6Rα cleavage by cathepsin L and enhances their shedding. (A,B) Chloroquine stabilises C-terminal LIFR and IL-6Rα fragments, which are blocked by cathepsin L inhibition (left panels) or RNF41 expression (right panels). HEK293T cells transiently transfected with C-terminal HA-tagged human LIFRα (LIFRα–HA) (A) or IL-6Rα (IL-6Rα–HA) (B) were left untreated (DMSO) or were incubated overnight solely with chloroquine or together with the indicated protease inhibitors (left panels); or were cotransfected with a full-length Etag–RNF41 or Etag–RNF41 ΔRING construct and left untreated or incubated with chloroquine (right). Cell lysates were analysed by western blotting (WB). FL, full length; *, intermediate IL-6Rα cleavage product; CTS, C-terminal stub. (C,D) RNF41 enhances LIFRα (C) and IL-6Rα (D) shedding. Cell media supernatants from the transfectants in the right panels of Fig. 6A,B were analysed for soluble LIFRα and IL-6Rα levels. E-tagged RNF41 or RNF41 ΔRING expression was verified by western blotting. (E,F) RNF41-enhanced LIFRα (E) and IL-6Rα (F) shedding is reversed by metalloprotease inhibitors. HEK293T cells transfected with hLIFRα–HA or hIL-6Rα–HA with or without full-length Etag–RNF41 were incubated overnight in starvation medium with TAPI-1 or GM6001 and soluble receptor levels in the cell medium supernatants were quantified. E-tagged RNF41 expression was verified by western blotting. (G) RNF41-enhanced IL-6Rα shedding is reversed by ADAM10 silencing. HEK293T cells, reverse transfected with siRNA targeting ADAM10 or ADAM17, were cotransfected the next day with hIL-6Rα–HA with or without full-length Etag–RNF41 and soluble IL-6Rα levels in the cell medium supernatants were quantified. Values are means ± s.d. (n=3). *P<0.01 or **P<0.05 (Student’s t-test) compared with –RNF41; §P<0.01 or §§P<0.05 (Student’s t-test) compared with +RNF41.
only a partial effect on levels of soluble LIFR\(\alpha\), indicating that LIFR\(\alpha\) shedding involves a different metalloprotease(s). LIFR\(\alpha\) shedding has not been shown before as a mechanism for the generation of soluble LIFR\(\alpha\). Together, these results support the conclusion that RNF41 controls the routing and processing of many type 1 cytokine receptors in a similar way.

Discussion

The E3 ubiquitin ligase RNF41 is implicated in regulating the expression of a number of proteins with varying functions, including membrane receptors such as the EGFR family members ErbB3 and ErbB4 (Diamonti et al., 2002; Qiu and Goldberg, 2002) and the Epo and IL-3 cytokine receptors (Jing et al., 2008). As an alternative to the RNF41-mediated degradation of these receptors, we assign a role for RNF41 in restricting basal surface expression and signalling of three different type 1 cytokine receptors and provide evidence that RNF41 has a crucial role in ectodomain shedding. We initially identified RNF41 as a novel interaction partner of the leptin receptor in MAPPIT screens, whereby its N-terminal RING domain was invariably interrupted. Expression of such truncated RNF41 or silencing of its transcript enhanced LR cell surface expression and potentiated STAT signalling, whereas expression of full-length RNF41 had the opposite effect. In line with this, the recurrent identification of the truncated RNF41 preys isolated in the screens might be explained by a stabilised surface expression of the LR-derived MAPPIT bait receptor, generating pronounced MAPPIT signals. We confirmed this initial observation for different wild-type cytokine receptors, either transfected or endogenously expressed in different cell types. How RNF41 physically interacts with the type 1 cytokine receptor complexes is unclear at present. The common denominator of all receptor complexes analysed in this study is the receptor-associated JAK2 kinase, suggesting a direct impact of RNF41 on JAK2. JAK kinases have been shown to facilitate receptor folding, maturation and cell surface delivery by operating as a chaperone, constituting a quality control checkpoint in the ER or Golgi (Huang et al., 2001; Radtke et al., 2002). In addition, JAK kinases can promote receptor expression by decreasing basal internalisation rates (Kumar et al., 2008; Ragimbeau et al., 2003) or enhancing recycling (Royer et al., 2005). Furthermore, the human kinome tree shows that JAK kinases are most closely related to the EGFR family members, which are known targets of RNF41 (Manning et al., 2002). Although RNF41 can suppress levels of JAK2, we could not demonstrate efficient co-immunoprecipitation between RNF41 and JAK2, or RNF41-induced JAK2 ubiquitylation. This suggests that the JAK2–RNF41 interaction might be indirect and that other, so far unknown proteins bridge RNF41- and JAK2-associated cytokine receptor complexes. Additional studies involving protein–protein interaction analysis, subcellular localisation of RNF41 and JAK2 and the possible role of JAK2 in receptor routing are required to further clarify this issue.

The key finding of our study is that RNF41 controls the balance between type 1 cytokine receptor degradation and ectodomain shedding. For three different receptor systems (LR, LIFR\(\alpha\) and IL-6R\(\alpha\)) we observed simultaneous inhibition of cathepsin-L-dependent cleavage and enhanced ectodomain shedding. In line with this, confocal imaging revealed a decrease in colocalisation of a C-terminally tagged LR with the lysosomal marker LAMP-1 upon RNF41 expression. Together, this implies an important role for RNF41 in cytokine receptor trafficking. Such a role for RNF41 in cargo trafficking is underscored by recent studies. RNF41 was shown to interact with Tsg101 (Vps23), a component of the endosomal sorting complex required for transport (ESCRT-I) that is involved in protein sorting to late endosomal membranes and MVBs (Markson et al., 2009; Williams and Urbé, 2007). Also, RNF41 can interact with the deubiquitylating enzyme USP8 (also called UBP\(y\)), which is required for maintenance of ESCRT-0 stability and endosomal sorting of ErbB3 and EGFR (Niendorf et al., 2007; Wu et al., 2004). Its role in preventing receptor degradation might also help to explain recent findings by Cao and co-workers, who reported that RNF41 could differentially modulate Toll-like receptor (TLR)-mediated responses. TLRs that reside at the plasma membrane engage the Mal/MyD88 adaptors leading to NF-\(\kappa\)B activation, whereas activation of IRF3 is triggered by TRAM and TRIF adaptor-coupled endosomal TLRs. Next to ubiquitylation of crucial components in either pathway (Wang et al., 2009), RNF41 might promote TRAM- and TRIF-driven signalling by preventing targeting of the endosomal TLRs to the lysosomal degradation pathway.

In addition to alternative splicing, ectodomain shedding is a common mechanism for the generation of soluble receptors. We show here for the first time that LIFR\(\alpha\) is susceptible to metalloprotease-dependent ectodomain shedding, as shown before for LR and IL-6R\(\alpha\) (Maamra et al., 2001; Mullberg et al., 1995). Silencing of both ADAM10 and ADAM17 (also called TACE) in the case of the LR, or ADAM10 in case of the IL-6R\(\alpha\) reverses the effects of RNF41 on receptor shedding. ADAM10 is responsible for the constitutive shedding of the IL-6R\(\alpha\) (Matthews et al., 2003), which is in line with the ligand independency of the RNF41 effects. The sheddase activity of ADAM10 and ADAM17, was shown to be sequestered in well-ordered membrane microdomains that are rich in cholesterol and sphingolipids, called lipid rafts (Matthews et al., 2003; Tellier et al., 2006). Furthermore, ADAM substrates are present in different proportions in lipid rafts, suggesting that the entry of these substrates in these particular membrane microdomains is specifically regulated (Tellier et al., 2006). However, the mechanisms or post-translational modifications modulating the entry of ADAM substrates to these lipid rafts are still unknown. Our findings imply that RNF41 sends receptors to such cellular domains for ectodomain shedding. Because rerouting by RNF41 depends on its membrane and ubiquitin was shown to act as a sorting signal (Acconcia et al., 2009), (mono-)ubiquitylation of cargo proteins and/or proteins involved in the sorting machinery is probably involved. Future studies identifying the specific ubiquitylation targets of RNF41 (e.g. members of the ESCRT complex) will be required to unravel the precise molecular sorting mechanism. Enhanced shedding mediated by RNF41 expression might be directly correlated with decreased detection of surface receptors in our FACS assay and the attenuated signalling. However, additional effects of RNF41 on receptor stability and degradation cannot be ruled out at present and need further investigation.

In addition to its potentiating effect on metalloprotease-dependent ectodomain shedding, RNF41 also affects cleavage of the LR, LIFR and IL-6R\(\alpha\). As shown before for the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) (Yang et al., 2007), we demonstrate using specific cathepsin inhibitors that both LR (long and short isoform), LIFR\(\alpha\) and IL-6R\(\alpha\) are cleaved by the cysteine protease cathepsin L, leading to the formation of a membrane-anchored C-terminal stub (CTS). Brefeldin A, an inhibitor of ER to Golgi transport, completely blocks formation of the LR-CTS, indicating that cleavage occurs in a post-ER subcellular compartment such as the MVBs or lysosomes.
Interestingly, gene deletion or pharmacological inhibition of cathepsin L was shown to reduce body weight gain and glucose intolerance, partly because of increased levels of muscle IR (Yang et al., 2007). Cleavage by cathepsin L of cytokine receptors such as the LR, might contribute to this phenotype. Increased levels of cathepsin L in obese and diabetic patients suggest that this protease is a novel target for these metabolic disorders.

Future studies will have to determine how endogenous RNF41 expression is regulated. RNF41 expression was shown to be a TLR-inducible protein in RAW264.7 macrophages upon LPS stimulation (Wang et al., 2009). Moreover, auto-ubiquitylation and other post-transcriptional mechanisms are involved in suppressing RNF41 stability (Ingalla et al., 2010; Wu et al., 2004).

In conclusion, we have shown that a single protein, RNF41, can control the balance between JAK2-associated cytokine receptor degradation and ectodomain shedding. Our findings imply that RNF41 reroutes receptors from the lysosomal degradation pathway to cellular compartments for ectodomain shedding. As aberrant cytokine receptor expression and shedding has been correlated with the onset of various pathologies (Gooz, 2010; Murphy, 2008), a better understanding of the cellular systems that safeguard correct export and shedding of receptors might contribute to developing therapeutics aimed at harnessing the associated diseases.

Materials and Methods

Constructs

The sequences encoding the human LR (hLR) and human LFIR (hLFIRβx) were amplified and cloned in the pME7 expression vector using 5’-AATCGTCGAC-CATGTAGAATTTTAAATCTCGT-3’, to cellular compartments for ectodomain shedding. As aberrant RNF41 reroutes receptors from the lysosomal degradation pathway to cellular compartments for ectodomain shedding. As aberrant cytokine receptor expression and shedding has been correlated with the onset of various pathologies (Gooz, 2010; Murphy, 2008), a better understanding of the cellular systems that safeguard correct export and shedding of receptors might contribute to developing therapeutics aimed at harnessing the associated diseases.

Cell culture, transfection procedures and protease inhibitors

HEK293T and HeLa cells were cultured in an 8% CO2 humidified atmosphere at 37°C and grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) with 10% fetal calf serum (FCS; Gibco) and WEHI-3B conditioned medium (containing IL-3). Transfections were performed by electroporation (300 V, 1500 μF) (Clontech) with the RNF41-expressing plasmids (22 μg) and the pGL3-β-casein-luc reporter construct (8 μg).

Western blot analysis

HEK293T cells were washed twice with ice-cold PBS and lysed in modified RIPA buffer [200 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, 2 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholy acid, 1 mM Na3VO4, 1 mM NaF, and Complete protease inhibitor cocktail (Roche)]. Lysates were cleared by centrifugation at 14,000 × g for 30 minutes at 4°C. The supernatants were collected and the membrane pellet was resuspended in 40 μl TSE buffer [200 mM Tris-HCl, pH 7.5, 300 mM sucrose, 1 mM EDTA, 1% Nonidet P-40, and Complete protease inhibitor cocktail (Roche)]. Dounce homogenisation was performed 50 times and centrifuged (5 minutes, 4°C) at 7500 r.p.m., the supernatant (cytosol fraction) was collected and the nuclear pellet was resuspended in 40 μl TSE buffer [156 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue sodium salt, 5% 3-mercaptoethanol] and was added to the cell lysates, which were then resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences). Blotting efficiency was checked by preincubation with 5% milk (Sigma). Blots were blocked in blocking buffer (5% milk, 0.1% Tween 20, 1X Tris-buffered saline) and then incubated with primary antibodies overnight at 4°C. The next day, blots were incubated with HRP-conjugated secondary antibodies (Dako) and visualised with enhanced chemiluminescence (ECL) detection kit (Amersham). Membrane proteins were quantified with the Odyssey Imaging System and bands intensities were normalized to β-actin levels. 

Non-specific bands were revealed using a rabbit anti-β-actin antibody (Sigma), rabbit anti-pSTAT3 antibody (Cell Signaling Technology), or mouse anti-β-actin antibodies (Sigma). Blots were incubated with 5% milk (Sigma) and HRP-conjugated secondary antibodies (Dako) and visualised with enhanced chemiluminescence (ECL) detection kit (Amersham). Membrane proteins were quantified with the Odyssey Imaging System and bands intensities were normalized to β-actin levels. 

Cellular subfractionation

IL-6R-α-expressing HEK293T cells were incubated overnight with chloroquine and harvested into 500 μl cell lysis buffer [10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM EDTA and Complete protease inhibitor cocktail (Roche)]. Cells were allowed to swell for 5 minutes and the supernatants were collected and the cell pellets was resuspended in 40 μl TSE buffer [156 mM Tris-HCl, pH 7.5, 300 mM sucrose, 1 mM EDTA, 0.1% Nonidet P-40 and Complete protease inhibitor cocktail (Roche)]. Cell lysates were then collected and cleaved by centrifugation for 30 minutes and centrifuged (5 minutes, 4°C) at 5000 r.p.m. The supernatant was collected and the nuclear pellet was resuspended in 40 μl TSE buffer (LiCor) with 0.1% Tween-20.

Subcellular fractionation

IL-6R-α-expressing HEK293T cells were incubated overnight with chloroquine and harvested into 500 μl cell lysis buffer [10 mM HEPES, 10 mM NaCl, 1 mM KH2PO4, 5 mM NaHCO3, 1 mM CaCl2, 0.5 mM MgCl2, 5 mM EDTA and Complete protease inhibitor cocktail (Roche)]. Cells were allowed to swell for 5 minutes and the supernatants were collected and the cell pellets was resuspended in 40 μl TSE buffer [156 mM Tris-HCl, pH 7.5, 300 mM sucrose, 1 mM EDTA, 0.1% Nonidet P-40 and Complete protease inhibitor cocktail (Roche)]. Cell lysates were then collected and cleaved by centrifugation for 30 minutes and centrifuged (5 minutes, 4°C) at 5000 r.p.m. The supernatant was collected and the nuclear pellet was resuspended in 40 μl TSE buffer (LiCor) with 0.1% Tween-20.

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ELISA for the detection of soluble cytokine receptors

In the final 24 hours before collection of the cell medium samples, cells were cultured in DMEM without fetal calf serum. The human sLIFR and sIL-6R Quantikine ELISA kit (R&D Systems) and human sLIF-R/gp190 ELISA kit (R&D Systems) were used to determine the levels of soluble IL-6R and soluble LIFR in the cell supernatants.
Luciferase reporter assays

For a typical luciferase experiment, HEK293T cells were transfected with the desired receptor (100 ng) and RNF41 (2 μg; 1 μg for RNAi, pSR and RNF41 RNAi) constructs, together with a STAT3-dependent pEXp13-pAPI or STAT3-responsive pGL3-β-casein luciferase reporter plasmid (200 ng). Cells were additionally transfected with a β-gal reporter construct (200 ng) to correct for transfection efficiency. The pMET7-SVT construct was used to normalise for the amount of transfected DNA and load of the transcriptional and translational machinery. 18 hours after transfection, HEK293T cells were left untreated or stimulated for at least 24 hours with mouse leptin (100 ng/ml), human Epo (5 ng/ml), human IL-3 (1 ng/ml) (R&D Systems) or human LIF (10 ng/ml) (Chembion International). Luciferase activity from triplicate samples was measured by chemiluminescence in a TopCount luminometer (PerkinElmer) and expressed as fold induction (stimulated/non-stimulated relative light units) or as relative light units normalised for transfection efficiency. All luciferase data shown are based on at least three independent experiments (n=3).

FACS analysis

HEK293T cells were transiently transfected with a mock vector or an expression vector encoding the mLR either alone (0.5 μg) or together with the indicated RNF41 constructs (1.5 μg). In addition, all cells were transfected with an EGFP construct (0.1 μg) to allow gating for transfected cells during FACS analysis. The pMET7-SVT construct was used to normalise for the amount of transfected DNA and load of the transcriptional and translational machinery. 18 hours after transfection, HEK293T cells were left untreated or stimulated for another 22 hours with 100 ng/ml mouse leptin. Expression of the LR was monitored using a combination of two rat monoclonal antibodies directed against the extracellular domain of the mLR, which were produced in-house, and subsequent incubation with an anti-rat biotin (KPL) and streptavidin-APC (BioLegend Biocytin) conjugated antibody. Fluorescence-activated cell sorting (FACS) was performed on a FACScalibur (Becton Dickinson).

Silencing of ADAM10 and ADAM17

ADAM10 (ON-TARGETplus SMARTpool, Dharmacon), ADAM17 siRNA (5′-AAGAAACAGAGAAGCAGAUAUUUU-3′, Qiagen) and Stealth RNAi siRNA Negative Control Med GC (Invitrogen) were reverse transfected in HEK293T cells using Dharmafect (Dharmacon) 1 day before transfection of the plasmids expressing the appropriate receptor or RNF41. Silencing efficiency was evaluated using real-time PCR.

Confocal microscopy

24 hours after transfection, cells were rinsed with 1 × PBS and fixed for 15 minutes at room temperature in 4% paraformaldehyde. After three washes with 1 × PBS, cells were permeabilised with 0.1% Triton X-100 in 1 × PBS for 10 minutes and blocked in 1% BSA in 1 × PBS for another 10 minutes at room temperature. Samples were then incubated for 1 hour at room temperature with 1:500 rabbit anti-LAMP-1 (H4A3, Abcam) antibodies. After four washes in 1 × PBS, cells were incubated for 1 hour at room temperature with goat anti-rabbit Alexa Fluor 594 secondary antibody (Alexa Fluor 594 secondary antibody). Nuclei were stained with DAPI. Images were acquired using a 60 × 1.35 NA objective on an Olympus IX-81 laser scanning confocal microscope and analysed using FVview 1000 software.

RNA isolation, cDNA synthesis and quantitative real-time PCR analysis

Total RNA was isolated from HEK293T cells using RNAeasy spin columns and treated with RNase-free DNasel (Qiagen). Concentration and purity of the extracted RNA were determined using the A260/A280 value measured on an ND1000 Spectrophotometer (NanoDrop Technologies). cDNA synthesis was carried out from 5 μg total RNA using SuperScript II Reverse transcriptase (Invitrogen) by the oligo(dT) priming method. Relative mRNA transcript levels of endogenous RNF41 were assessed by real-time quantitative PCR using the LightCycler 480 Probes Master (Roche) in combination with the Universal ProbeLibrary probe #25 (Roche) and PCR primers (5′-TGGCCATATTGGAATGAG-3′ and 5′-CCGGTCAAGACATC-3′) specific for RNF41 and designed using the Probe Library Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). For ADAM10, probe #70 and PCR primers 5′- TGGTCCGAGGATTTTTTCTGT-3′ and 5′-TAAAGGCTTCTCCTGCAG-3′ were used. The PCR cycle parameters were an initial denaturation of 95°C for 5 minutes and then 40 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds and extension at 72°C for 10 seconds. Each sample was run as a technical duplicate. GAPDH (probe #86 and primers 5′-AGGCA-CATGGTCCGAGGAC-3′ and 5′-GCCCGCCGATCT-3′) and HnRNA (probe #94 and primers 5′- TGGCCTTCTGCAGTCTTCTCC-3′) served as internal controls and were used to normalise for differences in each sample. Relative RNF41 mRNA levels were quantified using LightCycler 480 Software 1.5.

Statistical analysis

Data were expressed as mean ± s.d. of at least three independent experiments. For statistical analyses of two groups, two-tailed Student’s t-tests were performed. GraphPad Prism version 5.0 software was used for all statistical data analysis.

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