Angiopoietin-1-induced ubiquitylation of Tie2 by c-Cbl is required for internalization and degradation

Christina WEHRLE*,†, Paul VAN SLYKE* and Daniel J. DUMONT*†

Division of Molecular and Cellular Biology Research, Sunnybrook Research Institute, Toronto, Ontario, Canada, M4N 3M5, and †Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, M5G 2M9

INTRODUCTION

Tie1 and Tie2 [where ‘Tie’ is an acronym from tyrosine kinase with Ig and EGF (epidermal growth factor) homology domains] together make up the two members of the Tie family of RTKs (receptor tyrosine kinases). Expression of Tie2 has been described in several tissues, but its expression is most pronounced in endothelial and haematopoietic cells [1–4]. Mice lacking Tie2 die between embryonic day 9.5 and 12.5 due to a lack of remodelling of the primary capillary plexus, severe heart defects and poor association of endothelial cells with underlying matrix [5]. The angiopoietin ligands bind to Tie2, mediating a range of effects, which include tightening cell junctions to promote anti-permeability and anti-inflammation [6,7], active angiogenesis including tube formation [8], sprouting [9], endothelial cell migration through the adaptor protein Dok-R (downstream of kinase-related) and FAK (focal adhesion kinase) activation [10–12], and cell survival through PI3K (phosphoinositide 3-kinase) and PKB (Akt/protein kinase B) activities [13,14]. Ang1 (angiopoietin-1), the first and the best-characterized member of the angiopoietin family, is secreted by periendothelial cells in quiescent vasculature and can be incorporated into the extracellular matrix [15,16]. Ang1 is essential for vascular development, as mice lacking this ligand have a similar phenotype to Tie2-knockout mice [15,17].

Although much attention has been paid to signal transduction pathways of the Ang1–Tie2 axis, little is known about the spatial organization of Tie2 following activation. Ang1 was demonstrated to induce Tie2 turnover in a dose-dependent manner, whereas Ang-2 had a less dramatic effect on Tie2 degradation. [18].

Uniquely from other RTKs, the angiopoietins were found not to co-internalize with their receptor, suggesting that Tie2 possesses a novel ligand-release mechanism [19].

Recently, two groups examined the spatial distribution of Tie2 under stimulated and non-stimulated conditions [20,21]. They report similar observations of differential localization of Tie2 and Tie2 signalling in response to Ang1. In sparse cells, Tie2 clustered at the basal plasma membrane with extracellular matrix-bound Ang1, whereas in confluent cells Ang1 caused Tie2 to translocate to cell–cell contacts, where Ang1 bridged Tie2 receptors across neighbouring cells. These observations have been critical in explaining this receptor’s involvement in both vascular quiescence and active angiogenesis [20,21].

RTKs are frequently modified by the addition of ubiquitin prior to receptor-mediated endocytosis [22]. Ubiquitin is a highly conserved, small (≈8.5 kDa) globular protein of 76 amino acids [23]. Ubiquitylation, or the covalent attachment of a ubiquitin monomer to a substrate, is a well-conserved, tightly regulated and highly specific three-step process (reviewed in [24]).

The addition of a single ubiquitin molecule to a RTK is sufficient to target the receptor for internalization and degradation [25]. The addition of several monomers of ubiquitin, termed multiquitylation or multifunctional ubiquitylation, was suggested as the tagging method to target proteins for lysosomal degradation [22]. Alternatively, polyubiquitinated proteins which have been modified by the addition of chains of four or more ubiquitin monomers, specifically linked through Lys48 on ubiquitin, are targeted for proteasomal degradation [26,27].

The E3 ubiquitin ligases involved in ubiquitinating most RTKs studied to date are members of the Cbl family of proteins [28,29].

Abbreviations used: Ang1, angiopoietin 1; BCA, bicinchoninic acid; (b)FGF, (basic) fibroblast growth factor; Cbl, Casitas B-lineage Lymphoma; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; RTK, receptor tyrosine kinase; Tie, tyrosine kinase with Ig and EGF (epidermal growth factor) homology domains; TKB, tyrosine kinase binding domain; UBA/LZ, ubiquitin-associated domain with a leucine zipper; VEGF(R), vascular endothelial growth factor (receptor); YFP, yellow fluorescent protein.

† To whom correspondence should be addressed (email dan.dumont@sri.utoronto.ca).
In the present paper, we demonstrate that c-Cbl interacts with activated Tie2 and that this interaction is required for Tie2 ubiquitylation, internalization and degradation.

EXPERIMENTAL

Cell culture

Pooled HUVECs (human umbilical vein endothelial cells; GlycoTech, Rockville, MD, U.S.A.) were grown on 2% gelatin-coated (Sigma) 10 cm tissue culture plates (Corning) in F12K medium (A.T.C.C.) supplemented with 10% (v/v) FBS (fetal bovine serum), 1% penicillin/streptomycin, 0.1 mg/ml heparin sodium, 10 ng/ml EGF, 10 ng/ml VEGF (vascular endothelial growth factor), 5 ng/ml bFGF (basic fibroblast growth factor) and 2 mM L-glutamine (all from Sigma). Cells were used from passages 5 to 8. EA.hy926 cells (a gift from Dr Cora-Jean Edgell, formerly at the Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, U.S.A.) were grown on 10 cm plates (Corning) in DMEM (Dulbecco’s modified Eagle’s medium; Sigma) supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine (all from Sigma) and 1 × HAT (hypoxanthine/aminopterin/thymidine) supplement ( Gibco). (HEK-)293F cells (Invitrogen) were grown on 10 cm plates (Nunc) in DMEM (Sigma) supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine (all from Sigma). Stable transfected cell lines were maintained in selection medium supplemented further with 600 μg/ml G418 (Life Technologies, Inc.).

Cell stimulations

All cell stimulations were performed at 37 °C with 800 ng/ml human recombinant Ang1 fused with a 6 × histidine tag at the C-terminus (obtained from R&D Systems, Minneapolis) for 15 min, unless otherwise indicated. Recombinant Ang1 was pre-incubated with an anti-polylhistidin monoclonal antibody (10 μg/ml; R&D Systems) in cell-culture medium nutating at room temperature for 30 min prior to stimulation to facilitate cross-linking of Ang1 multimers to mimic Ang1 oligomers found in vivo [30]. Mock stimulations were performed with the same method including the anti-polylhistidin monoclonal antibody, while excluding Ang1. Endothelial and 293F cells were approx. 80–90% confluent for the stimulation experiments.

Precipitation, Western blotting and antibodies

Cells were lysed in RIPA lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS and protease inhibitors), and cellular protein was quantified using the BCA protein assay (Pierce). Total cell lysates were resolved by SDS/PAGE and Western blots were performed using anti-Tie2 antibody 33.1 (0.5 μg/ml).

Transient transfections and stable cell lines

293F cells were transfected with Lipofectamine™ (Invitrogen) according to the manufacturer’s instructions. For each 10 cm plate, 2 μg of DNA, Tie2WT and Tie2KD [31] were pre-incubated with 20 μl of Lipofectamine™ in 2 ml of Opti-MEM Reduced Serum Medium (Gibco) for 30 min at room temperature. Cells were washed with PBS, then incubated with the transfection medium supplemented with another 2 ml of Opti-MEM. After 5 h, the medium was changed to 293F medium (described above), without antibiotics and 20% FBS. Approx. 24 h after transfection, the medium was changed again to 293F medium without antibiotics and 10% FBS. All experiments were performed 48 h after transfection. Stable cell lines were created for Tie2WT [31] in the 293F cell line. Transfections were performed as in transient transfections described above. At 48 h after transfection, one 10 cm plate was split to approx. twenty 10 cm plates and cells were grown in selection medium with 600 ng/ml G418, as described above. The medium was changed every 2 days for 2 weeks and select clones were isolated from distinct colonies. The colonies were split to individual plates and screened for Tie2 expression by immunoblotting. YFP (yellow fluorescent protein)-tagged v-Cbl [a viral form of c-Cbl that contains only the TKB (tyrosine kinase binding domain) region] expression vector was kindly given by Dr James Booth (Sunnybrook Health Sciences Centre, Toronto, ON, Canada).

RESULTS

Ang1 stimulation results in the activation and ubiquitylation of Tie2

Towards understanding how Tie2 signalling is attenuated in endothelial cells, we set out to determine whether it was ubiquitylated. Stimulation of HUVECs and EA.hy926 cells with Ang1 results in the activation of Tie2, as indicated by the increased presence of phosphotyrosine in Tie2 (αpY; Figure 1). The levels of Tie2 in each cell did not vary upon stimulation and were quite comparable between cells (Figure 1; αTie2).

Probing
the membranes prepared from Tie2 immunoprecipitates with an antibody specific for ubiquitin revealed a dramatic increase in the ubiquitin content of Tie2 in cells stimulated with Ang1 (Figure 1; αUb). The levels of ubiquitin incorporation did appear marginally different between the two cell types. Whether this reflects differences in levels of Tie2 expression or the extent of stimulation in each cell type is not known; however, it is very evident that there is a clear increase in ubiquitin incorporation in Tie2 upon Ang1 stimulation.

**Tie2-expressing engineered cell lines recapitulate Tie2 activation in endothelial cells**

In order to bypass the extremely poor uptake of DNA by endothelial cells, we established several 293F–Tie2-expressing cell lines (Figure 2A). To ensure uniformity in Tie2 expression, cell lines were cloned and Tie2 expression levels and their response to Ang1 stimulation were established (Figure 2). All cell lines tested had comparable levels of β-actin, demonstrating equal loading of protein from each cell line in the gel (Figure 2A). In addition to several drug-resistant cell lines not expressing detectable levels of Tie2, three cell lines, i.e. WTA, WTE, and WTF, expressed readily detectable levels of Tie2 when compared to HUVECs (Figure 2A). Stimulation of WTA and WTE cells with Ang1 resulted in the activation of Tie2, as indicated by the increased phosphotyrosine signal by Western blotting (Figure 2B). Stimulation of WTE cells (which had levels of Tie2 expression that were the closest to HUVECs) with Ang1 resulted in the Ang1-dependent activation of Tie2 and its ubiquitylation (Figure 2C).

**Tie2 activation with Ang1 results in its loss from the cell surface and co-immunoprecipitation with c-Cbl**

The human endothelial cell line EA.hy926 and WTE cells express comparable levels of Tie2 receptor (Figure 3A). The amount of Tie2 receptor remaining on the cell surface after different times post-stimulation was determined by cell-surface biotinylation and subsequent pull-down of biotinylated proteins with Streptavidin–agarose and Western blotting for Tie2. The amount of precipitated Tie2 decreased dramatically, and at a similar rate, for both cell lines from 15–240 min (Figure 3B). These results demonstrate that Tie2 is lost from the cell surface upon Ang1 stimulation for both of these cell lines with similar kinetics. In many instances, activated RTK internalization is driven by the ubiquitylation of the receptor, and since we demonstrated that Tie2 ubiquitylation was driven by Ang1 stimulation (Figures 1 and 2C), we set out to establish the identity of the ubiquitin ligase responsible for ubiquitylating Tie2. c-Cbl is an E3 ligase that is known to interact and target several tyrosine kinase receptors for ubiquitylation; thus, we set out to establish whether c-Cbl was responsible for the observed ubiquitylation of Tie2. Immunoprecipitation of c-Cbl from cell lysates prepared from either mock or Ang1-stimulated EA.hy926 or WTE cells were Western-blotted for the presence of Tie2 and c-Cbl (Figure 3C). Stimulation of either EA.hy926 or WTE cells with Ang1, and immunoprecipitation of c-Cbl from these cells and immunoblotting for the co-immunoprecipitation of Tie2, demonstrated that c-Cbl and Tie2 associated in an Ang1-stimulation-dependent manner (Figure 3C). This result suggests that c-Cbl may be the E3 ligase responsible for ubiquitylating Tie2 and initiating its loss from the cell surface.

**v-Cbl inhibits Tie2 ubiquitylation**

In order for c-Cbl to catalyse the ubiquitylation of a receptor, it must bind to it and transfer ubiquitin to the receptor. c-Cbl comprises several domains, including the TKB, a RING-finger domain (RING), a proline-rich region (Pro-Rich) and a ubiquitin-associated domain with a leucine zipper (UBA/LZ) [32]. The TKB region is responsible for receptor binding, whereas the other domains are involved in the ubiquitylation process. In order to determine whether the c-Cbl interaction with Tie2 was required for its ubiquitylation, WTE cells were transfected with a dominant-interfering form of c-Cbl, v-Cbl. v-Cbl is a viral form of c-Cbl that contains only the TKB region (Figure 4A), allowing it to bind to activated receptors and displace the endogenous c-Cbl, thus preventing the interaction with the receptor (Figure 4A). WTE cells either v-Cbl-transfected (Figure 4B, two right-hand lanes) or not were stimulated with Ang1 or vehicle (mock-treated...
Figure 3  Activated Tie2 in WTE and EA.hy926 cells is lost on the cell surface and co-immunoprecipitates c-Cbl in response to Ang1 stimulation

(A) The endothelial cell line EA.hy926 and WTE cells express similar levels of total cellular Tie2. (B) EA.hy926 and WTE cells were stimulated with clustered Ang1 for the times indicated (0, 15, 60 and 240 min), after which cell-surface proteins were biotinylated and precipitated with Streptavidin–agarose. The precipitated proteins were Western-blotted for Tie2. (C) EA.hy926 and WTE cells were stimulated with clustered Ang1 for 15 min and immunoprecipitated for c-Cbl and Western-blotted for either c-Cbl or Tie2 illustrates that Tie2 and c-Cbl co-immunoprecipitate only upon activation of Tie2.

cells), and protein cell lysates were immunoprecipitated with anti-Tie2 antibodies and Western-blotted for the presence of ubiquitin, phosphotyrosine and Tie2 with the appropriate antibodies. As demonstrated in previous experiments (Figure 2C), stimulation of WTE cells with Ang1 results in Tie2 ubiquitylation and tyrosine phosphorylation (Figure 4B). However, Tie2 extracted from WTE cells expressing equal amounts of v-Cbl demonstrated that, whereas the activation of Tie2 was unaffected as judged by tyrosine phosphorylation, ubiquitylation of Tie2 was completely abrogated (Figure 4B). This result demonstrates that overexpression of the TKB region of v-Cbl does not affect activation of Tie2, but is able to dramatically inhibit the ubiquitylation of Tie2.

v-Cbl inhibits Tie2 internalization and degradation

Ubiquitylation of RTKs is known to be a key signal for the initiation of receptor internalization and its subsequent degradation [33]. To determine whether Tie2 ubiquitylation was required for Tie2 internalization and degradation, similar experiments as the one described for Figure 3 were performed on WTE v-Cbl-transfected cells. Streptavidin pull-downs of biotinylated cell-surface protein followed by Tie2 Western blot analysis revealed that, upon Ang1 stimulation of WTE mock-transfected cells, Tie2 is rapidly lost on the cell surface, whereas in WTE v-Cbl transfectants Tie2 remained on the cell surface (Figure 5A). Importantly, cycling of the transferrin receptor, which is endocytosed by a c-Cbl-independent mechanism, was unaffected in these cells. The retention of Tie2 on the cell surface in v-Cbl-transfected cells suggests cleavage of the ectodomain of Tie2 to produce sTie2 [34], which does not contribute significantly to the turnover of Tie2 in these cells. Furthermore, blotting whole cell lysates for total Tie2 illustrated that the inhibition of Tie2 ubiquitylation by v-Cbl prevented the marked turnover of Tie2 over 8 h (Figure 5B). Collectively these results support the conclusion that activated Tie2 is down-regulated by c-Cbl-mediated ubiquitylation, leading to its internalization and degradation.
In the present study we have demonstrated that activation of Tie2 with Ang1 results in its ubiquitylation and co-immunoprecipitation with c-Cbl. Blocking this interaction by the expression of the dominant-interfering molecule v-Cbl illustrated that blocking Tie2 ubiquitylation results in the retention of Tie2 on the cell surface and inhibition in Ang1-induced Tie2 turnover. This is the first report of Tie2 ubiquitylation in response to Ang1 stimulation. Furthermore, we find that this covalent modification is required for Tie2 internalization and degradation. Although Tie2 trafficking is poorly understood, several reports published in this topic have been somewhat contradictory. We observed Tie2 internalization in EA.hy926 cells and WTE cells, as was previously reported in HUVECs [19]. Two other groups report that Ang1 preserves Tie2 on the cell surface for an extended period of time in confluent cells [20,21]. A mutual observation that unites these published studies is that Ang1 is not internalized with the receptor. Bogdanovic et al. [19] reported that Ang1 is predominantly released into the culture medium while Tie2 is internalized. Papers by Saharinen et al. [20] and Fukuhara et al. [21] reported that Ang1 maintains Tie2 at the cell surface without significant Ang1 internalization. Therefore it is possible that Ang1 activates Tie2 and preserves it on the cell surface at cell–cell junctions, and at some point Ang1 is released while Tie2 is internalized in an effort to shut down Tie2-mediated signalling. Tie2 internalization was much less apparent in the studies of Saharinen et al. and Fukuhara et al. [20,21], probably due to differing technical approaches. Both of these groups engineered Tie2 overexpression systems and, in some cases, employed mutant variants of Tie2, including a kinase-deficient Tie2 or receptors with deleted intracellular domains. It would be expected that receptors with these mutations would impede internalization kinetics compared with wild-type Tie2, as was observed for EGF receptor mutants [35].

Although c-Cbl is known to play an important role in the trafficking of several RTKs, this is the first report of it being required for Tie2 trafficking. Similarly, trafficking of VEGFR (VEGF receptor) 1 and 2 are thought to involve a complex that contains c-Cbl [36,37]. In contrast, trafficking of the FGF receptor does not seem to require c-Cbl, but uses c-Cbl as a signalling adaptor [38]. Collectively, these results suggest that the role of c-Cbl in angiogenesis may very well depend on the signalling pathway initiating the angiogenic response, and that c-Cbl-targeted therapies will need to take this into consideration. Notwithstanding this, our results suggest that drugs which could specifically target the Tie2–c-Cbl interaction in endothelial cells may have a therapeutic use in pro-angiogenic or vessel-stabilizing therapies. The rationale for this belief rests on the idea that attenuating the trafficking of Tie2 by abrogating its ubiquitylation may result in the maintenance of a signalling cascade from this receptor on the cell surface, ultimately resulting in an increased vessel stabilization and integrity, two known attributes of Tie2-mediated signalling in endothelial cells [39].

One caveat with the experiments described in this paper is the fact that the inhibitory experiments were performed in cells engineered to express Tie2; therefore whether c-Cbl has a similar role in endothelial cells will require further experiments. However, considering that the machinery used to down-regulate RTKs is quite universal, it would be surprising if c-Cbl did not have a similar role in endothelial cells.

**AUTHOR CONTRIBUTION**

Christina Wehrle performed all the experiments. Paul Van Slyke reviewed the manuscript and Dan Dumont wrote the paper.

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