A promiscuous liaison between IL-15 receptor and Axl receptor tyrosine kinase in cell death control

Vadim Budagian1, Elena Bulanova1, Zane Orinska1, Lutz Thon2, Uwe Mamat1, Paola Bellosta3, Claudio Basilico4, Dieter Adam2, Ralf Paus5 and Silvia Bulfone-Paus1,∗

1Research Center Borstel, Borstel, Germany, 2Institute of Immunology, University of Kiel, Kiel, Germany, 3Department of Medical Sciences, Novara, Italy, 4Department of Microbiology, School of Medicine, New York, NY, USA and 5Department of Dermatology, University of Lübeck, Lübeck, Germany

Discrimination between cytokine receptor and receptor tyrosine kinase (RTK) signaling pathways is a central paradigm in signal transduction research. Here, we report a ‘promiscuous liaison’ between both receptors that enables interleukin (IL)-15 to transactivate the signaling pathway of a tyrosine kinase. IL-15 protects murine L929 fibroblasts from tumor necrosis factor α (TNFα)-induced cell death, but fails to rescue them upon targeted depletion of the RTK, Axl; however, Axl-overexpressing fibroblasts are TNFα-resistant. IL-15Rα and Axl colocalize on the cell membrane and co-immunoprecipitate even in the absence of IL-15, whereby the extracellular part of Axl proved to be essential for Axl/IL-15Rα interaction. Most strikingly, IL-15 treatment mimics stimulation by the Axl ligand, Gas6, resulting in a rapid tyrosine phosphorylation of both Axl and IL-15Rα, and activation of the phosphatidylinositol 3-kinase/Akt pathway. This is also seen in mouse embryonic fibroblasts from wild-type but not Axl−/− or IL-15Rα−/− mice. Thus, IL-15-induced protection from TNFα-mediated cell death involves a hitherto unknown IL-15 receptor complex, consisting of IL-15Rα and Axl RTK, and requires their reciprocal activation initiated by ligand-induced IL-15Rα.

The EMBO Journal (2005) 24, 4260–4270. doi:10.1038/sj.emboj.7600874; Published online 24 November 2005
Subject Categories: signal transduction; differentiation & death
Keywords: fibroblast; Gas6; TNFα; transactivation

Introduction

The cytokine interleukin (IL)-15 is a potent apoptosis inhibitor (Bulfone-Paus et al., 1997b, 1999) with many immunomodulatory activities that overlap with those of IL-2 (Bulfone-Paus et al., 1997a; Waldmann and Tagaya, 1999). IL-15 binds to a heterotrimeric receptor complex that shares the IL-2 receptor β (IL-2Rβ) and IL-2 receptor γ (IL-2Rγ/γc) chains, but has a unique IL-15Rα chain that binds IL-15 with high affinity even in the absence of the other two receptor subunits (Giri et al., 1995; Fehninger and Caligiuri, 2001).

Receptor tyrosine kinases (RTKs) are cell-surface receptors that transduce specific signals from the extracellular environment, possesses an intrinsic tyrosine kinase activity, and control growth, differentiation, and survival of cells (Bennasroune et al., 2004). The mammalian Axl RTK family includes Axl, Tyro3, and Mer (Graham et al., 1994; Mark et al., 1994), and is widely expressed (Lai et al., 1994; Lu et al., 1999; Healy et al., 2001; Lu and Lemke, 2001). Axl, Tyro3, and Mer display an extracellular region (two immunoglobulin-related domains linked to two fibronectin type III repeats) and an intracellular region that contains an intrinsic RTK domain. The only known bona fide ligand for the Axl RTK family members is Gas6, the product of growth-arrest-specific gene 6 (Nagata et al., 1996), a soluble member of the vitamin K-dependent protein family (Manfioletti et al., 1993).

Gas6/Axl signaling modulates cell growth and inhibits apoptosis (Bellosta et al., 1997; Melaragno et al., 2004). Axl promotes survival of pulmonary endothelial cells (Healy et al., 2001) and neuronal cells (Allen et al., 1999), and protects murine fibroblasts and human endothelial cells from apoptosis induced by TNFα or other stimuli (Bellosta et al., 1997; Goruppi et al., 1997). Axl is overexpressed in a variety of tumor cells (Chen et al., 1999; Goruppi et al., 2001), and overexpression of Axl can transform fibroblasts even in the absence of ligand (O’Brian et al., 1991; Burchert et al., 1998). Among signaling molecules activated by Axl in various cells types are phosphatidylinositol 3-kinase (PI3K), Akt, Src, extracellular signal-regulated kinase (ERK), and nuclear factor κB (NF-κB) (Goruppi et al., 1997; Lee et al., 2002). For example, in NIH 3T3 mouse fibroblasts, antiapoptotic Gas6/Axl signaling induces NF-κB activity and increases protein level of Bcl-xL (Demarchi et al., 2001).

IL-15 protects mouse L929 fibroblasts from TNFα-induced apoptosis through recruitment of TRAF2 to the IL-15Rα chain and NF-κB activation (Bulfone-Paus et al., 1999). In human Raji cells, IL-15 stimulation reduces ceramide-induced apoptosis, accompanied by the recruitment of Syk, a non-receptor protein-tyrosine kinase, to the IL-15Rα chain, which phosphorylates a single tyrosine in the cytoplasmic tail of IL-15Rα (Bulanova et al., 2001). L929 cells lack Syk, yet IL-15 induces IL-15Rα phosphorylation in these cells.

Therefore, the goal of this study was to investigate whether the IL-15-mediated antiapoptotic signaling in these model cells involves the activity of other, yet unknown kinase(s). Here, we unravel an unexpected alliance between IL-15Rα and Axl RTK, which is indispensable for the IL-15-mediated survival of L929 cells upon TNFα stimulation. Our results point to a so far unprecedented cooperation in signal transduction between a cytokine receptor and an RTK, and demonstrate how direct interactions between heterologous receptor systems can regulate qualitatively distinct cellular responses to death-inducing and death-opposing stimuli.
Results

**Axl RTK is essential for the IL-15-mediated protection of murine fibroblasts from TNFα cytotoxicity**

IL-15 protects mouse L929 fibroblasts from TNFα-mediated cell death via recruitment of TRAF2 to its high-affinity receptor chain, followed by NF-κB activation (Bulleone-Paus et al., 1999). To further dissect the antiapoptotic effects of IL-15, we have established an IL-15-dependent L929 subline that is resistant to TNFα-induced cell death, referred to hereafter as L929R (see Materials and methods). The L929R cells are indistinguishable from the parental cell line, both morphologically and with respect to the expression levels of TNFα receptors, IL-15Rz and IL-2Rγ (Supplementary Figure 1) and the absence of IL-2Rβ (data not shown). However, cDNA microarray revealed a significant upregulation of an RTK, Axl, in L929R cells compared to parental L929 cells (data not shown). High expression of Axl was confirmed by RT–PCR, Western blotting (WB), and flow cytometry analysis (FACS) (Supplementary Figure 1A and B).

Given the high expression of Axl in L929R cells, we tested how IL-15 treatment affects Axl expression in L929 cells. In fact, IL-15 stimulation for 24 h induced a significant upregulation of Axl RTK mRNA and protein to a level comparable to that of L929R cells (Supplementary Figure 2A and B). In L929R cells, IL-15 failed to further elevate Axl expression.

Gas6 did not modulate the expression of its own receptor (neither at the transcriptional nor at the protein level) within 24 h (Supplementary Figure 2A and B), 48, or 72 h (data not shown). Instead, Gas6 significantly upregulated IL-15Rα expression (Supplementary Figure 2A). As previously reported (Kumaki et al., 1996), IL-15 downregulated the expression of its own high-affinity α chain (Supplementary Figure 2A).

The fact that Gas6/Axl signaling protects murine fibroblasts from apoptosis induced by serum withdrawal or treatment with TNFα (Bellosta et al., 1997) raised the possibility that the observed overexpression of Axl in L929R cells was the sole cause for the acquired TNFα resistance. In this case, targeted deletion of Axl was expected to restore TNFα sensitivity in L929R cells. Thus, L929R cells, as well as the parental cells, were transfected with small interfering RNA oligonucleotides (Axl siRNA) to disrupt Axl transcripts and were analyzed by RT–PCR and WB for the knockdown of Axl 48 h post-transfection.

Transfection with Axl siRNA resulted in a significant decrease of Axl at the mRNA (Figure 1A) and protein (Figure 1B) levels in both cell lines. As expected, the targeted depletion of Axl indeed restored TNFα sensitivity in L929R cells, resulting in a dramatic increase in the number of apoptotic cells in the presence of TNFα, as assessed by...
propidium iodide (PI) and annexin V staining and FACS analysis. About 70–80% of Axl-targeted L929R cells died, whereas cells transfected with scrambled control siRNA oligonucleotides remained viable (Figure 1C). Moreover, IL-15 failed to prevent the TNFα-induced apoptosis in parental L929 cells deficient in Axl expression, but effectively rescued non-transfected fibroblasts (Figure 1C). The protective properties of Axl were corroborated by the fact that L929 cells stably overexpressing a vector coding for Axl were resistant to TNFα (Supplementary Figure 2C). Transfection of L929 cells with siRNA oligonucleotides targeting expression of IL-15 was observed in L929 fibroblasts from TNFα-resistant, and Axl-overexpressing L929 cells were treated with TNFα for 4 h or were left untreated. In parallel, cells were cultured in the presence of TNFα in combination with the broad-spectrum caspase inhibitor zVAD-fmk, because such treatment induced a more pronounced increase in intracellular ceramide than TNFα alone (Thon et al., 2005).

Since TNFα induces rapid generation of ceramide in L929 cells, which induces apoptosis in these fibroblasts (Strelow et al., 2000), we asked whether high Axl expression interferes with ceramide generation. Parental, TNFα-resistant, and Axl-overexpressing L929 cells were treated with TNFα for 15 min with IL-15, TNFα, IL-15, and TNFα/IL-15 failed to prevent the TNFα-induced apoptosis in parental L929 fibroblasts (Figure 1D). Importantly, transfection with Axl construct also reduced ceramide generation. TNFα combined with zVAD-fmk induced a dramatic increase in the intracellular ceramide level, yet only in TNFα-sensitive fibroblasts (Figure 1D). Therefore, Axl RTK is required for the survival of mouse fibroblasts in the presence of TNFα, and Axl affects the TNFα-induced generation of intracellular ceramide. Since IL-15 treatment no longer protected Axl-deficient L929 cells from TNFα (Figure 1C), Axl must be an essential player in the IL-15-mediated protection of these fibroblasts from TNFα-induced cell death.

**Axl RTK and IL-15Rα associate and colocalize with each other**

To study whether and how Axl RTK participates in IL-15 signaling, immunoprecipitation (IP) experiments were performed. Parental L929 cells were stimulated with IL-15 or left untreated, and IL-15Rα or Axl was precipitated from the cell lysates using specific antibodies (Abs). Surprisingly, anti-Axl Abs co-immunoprecipitated IL-15Rα, and vice versa, in L929 fibroblasts even in the absence of IL-15 stimulation (Figure 2A). This unexpected physical association between IL-15Rα and Axl was confirmed by a two-site ELISA, where TNFα induced a rapid accumulation of ceramide in L929 cells, whereas ceramide generation was clearly impaired in L929R fibroblasts (Figure 1D). Importantly, transfection with Axl construct also reduced ceramide generation. TNFα overexpressing L929 cells were treated with TNFα, which induces apoptosis in these fibroblasts (Strelow et al., 2000). This indicates that IL-2Rα is dispensable for the antiapoptotic function of IL-15 in L929 cells.

**Figure 2** IL-15 induces phosphorylation of IL-15Rα and Axl, which constitutively associate. (A) L929 cells were stimulated with IL-15 (100 ng/ml). Cell lysates were precipitated with specific Abs against Axl, IL-15Rα, or IL-2Rγ and analyzed by WB. (B) Axl/IL-15Rα coassociation was detected in a two-site ELISA. Plates were coated with anti-Axl Abs. Axl (white bars) or Axl/IL-15Rα complexes (gray bars) were detected in cell lysates using biotinylated anti-Axl or anti-IL-15Rα Abs, respectively. COS-7 cells served as a negative control. (C) L929 cells were treated for 15 min with IL-15, TNFα, or both. Axl was precipitated from lysates and its phosphorylation was assessed with anti-pTyr Abs (upper panel). Axl detection served as a loading control (lower panel). (D) Axl and IL-15Rα colocalization analysis by confocal microscopy. Cells were stained with anti-IL-15Rα Abs (red), anti-Axl Abs (green), and TOTO-3 for nuclei (blue). Yellow color indicates IL-15Rα and Axl colocalization (right panels).
plates were coated with monoclonal Abs directed against Axl and incubated with cell lysates to bind target protein. Next, polyclonal Abs directed against IL-15Rα were added to detect Axl-associated IL-15Rα (Figure 2B). This also allowed quantitative analyses, by comparing results from two-site ELISA with specific ELISA for Axl or IL-15Rα (not shown). Arbitrarily defining the amount of Axl or IL-15Rα detected by specific ELISA as 100%, 63.1% of Axl and 87.4% of IL-15Rα molecules in L929 cells were in complex, whereas in L929R cells characterized by high Axl expression, 60.5% of Axl and 98% of IL-15Rα molecules were in complex. Thus, only a fraction of Axl molecules is associated with IL-15Rα in living cells, which may also depend on the cell type and may be relevant for the IL-15-mediated biological responses. To test whether the observed association occurs only in viable cells, we performed crosslinking experiments. However, no difference in the amount of IL-15Rα associated with Axl was observed after crosslinking, as detected by IP followed by WB, and two-site ELISA (data not shown).

Moreover, both anti-IL-15Rα and anti-Axl Abs precipitated IL-2Rγ, which constitutes (together with IL-15Rα) the high-affinity IL-15R complex in these L929 cells (Figure 2A). Remarkably, co-IP of IL-2Rγ with either receptor was much more prominent in the presence of IL-15 (Figure 2A). To further validate these results, we tested whether anti-γc Abs precipitate Axl or IL-15Rα upon IL-15 stimulation. The γc chain indeed associates with Axl and IL-15Rα upon stimulation with IL-15, as demonstrated by the ability of anti-γc Abs to co-precipitate these molecules (Figure 2A). Isotype-matched control Abs did not precipitate these proteins (data not shown). IL-15 treatment induced phosphorylation of both Axl and the IL-15Rα chain (Figure 2A). The pattern of IL-15-induced Axl phosphorylation was rather similar in the presence or absence of TNFα (Figure 2C). The constitutive association of Axl and IL-15Rα was further supported by confocal microscopy, which suggested that IL-15Rα and Axl colocalize on the cell membrane in the absence of IL-15 (Figure 2D).

To analyze the physical association of IL-15Rα and Axl on the cell membrane in more detail, we next tested by density gradient centrifugation and confocal microscopy whether IL-15Rα and Axl associate in low-density lipid microdomains of the cell membrane (lipid rafts). These are enriched in many signaling molecules and segregate proteins to regulate signaling and membrane trafficking (Marmor and Julius, 2001). Under our experimental conditions, however, we did not detect IL-15Rα or Axl in membrane microdomains by either method (data not shown).

The extracellular portion of Axl contains features that are reminiscent of cell adhesion molecules, and Axl mediates cell aggregation and adhesion by homotypic/homophilic association of its extracellular domains (Bellosta et al, 1995). Moreover, Axl-Fc chimeric protein, which consists of the extracellular domain of Axl (Faust et al, 1992) fused to the carboxy-terminal Fc region of human IgG1, promotes the migration of L929 cells upon immobilization on plastic (Budagian et al, 2005). Given the ability of IL-15Rα to interact with Axl, we asked whether IL-15 stimulation might provide a costimulatory signal for cell adhesion and migration through heterophilic interactions of IL-15Rα with Axl. To test this hypothesis, plates coated with Axl-Fc were seeded with equal numbers of cells. After 18 h, the cells were cleared within a defined area by scratching, photographed in phase-contrast, and allowed to migrate into the cleared area in the presence of IL-15. Unstimulated cells served as a control. However, the migratory properties of IL-15-stimulated and unstimulated cells did not differ (data not shown). These limited, initial data do not support a costimulatory role of IL-15 in the Axl-dependent mechanism(s) that mediate cell migration.

Since the other two members of the Axl RTK family, Tyro3 and Mer, share the same ligand with Axl, we also examined their potential interaction with IL-15Rα. Remarkably, the expression of Tyro3 and Mer at mRNA (Supplementary Figure 3A) and protein (Supplementary Figure 3B) levels was rather low in both cell lines, compared to Axl. Also, unlike Axl, surface staining of Tyro3 and Mer was more intense in parental L929 fibroblasts than in the TNFα-resistant subline (Supplementary Figure 3C). Importantly, in line with the two-site ELISA results, no associations between IL-15Rα and either of these two RTKs were observed in IP experiments (data not shown).

Thus, IL-15Rα specifically interacts with Axl and not other members of this RTK family. Contrary to current views on the trimeric structure of IL-15R (Fehninger and Caligiuri, 2001), Axl RTK may serve as an integral component of the functional IL-15R complex due to a constitutive physical association of the IL-15Rα chain with Axl even in the absence of ligand stimulation.

**The extracellular portion of Axl is essential for its association with IL-15Rα**

To better understand the molecular basis for this physical interaction, we co-transfected COS-7 cells with plasmids encoding the full-length IL-15Rα and one of two Axl mutant constructs, followed by IP with anti-IL-15Rα Abs (Figure 3). A chimeric protein (Bek-Axl) in which the extracellular domain of Axl was substituted with that of fibroblast growth factor receptor (Bek) (Bellosta et al, 1995) exhibited a dramatic decrease in the ability to associate with IL-15Rα in co-IP studies (Figure 3A). Conversely, Axl-DN, a dominant-negative mutant of Axl that lacks the kinase domain (Bellosta et al, 1995), still preserved the capacity to co-precipitate with IL-15Rα (Figure 3B). Next, we performed *in vitro* co-IP of IL-15Rα-Fc with different recombinant Axl proteins that contained either one of two IgG domains of Axl (Axl-Ig1) or Axl-Ig2), or both the domains (Axl-Ig1+2)). However, these proteins failed to precipitate with IL-15Rα-Fc (Supplementary Figure 4). These data suggest that the Axl and IL-15Rα association requires the intact extracellular domain of Axl RTK.

Next, we determined whether the IL-15-induced phosphorylation of IL-15Rα is mediated by Axl. COS-7 cells were co-transfected with IL-15Rα and Axl constructs, followed by cell stimulation with IL-15 and assessment of IL-15Rα phosphorylation. IL-15Rα phosphorylation was seen only in cells expressing both constructs, whereas transfection of IL-15Rα with an empty vector or Axl-DN had no effect (Figure 3C). This strongly suggests that the IL-15-induced phosphorylation of IL-15Rα is mediated by Axl RTK.

**IL-15 transactivates the Axl signaling pathway**

Axl stimulation by Gas6 suppresses apoptosis in NIH 3T3 fibroblasts by upregulating Bcl-xL through activation of the
PI3K/Akt pathway and NF-κB (Goruppi et al., 1997; Demarchi et al., 2001). Because IL-15 induced Axl phosphorylation, we compared the effects of IL-15 and Gas6 on L929 cells. Intriguingly, both IL-15 and Gas6 induced similar patterns of Axl phosphorylation within the first minutes of action (Figure 4A). IL-15 stimulation essentially mirrored Gas6-mediated signaling events, also resulting in the phosphorylation of PI3K and Akt. A modest phosphorylation of ERK1/2 was detected (Figure 4A), whereas the phosphorylation state of JNK and p38 kinase was unaffected (data not shown).

On analyzing Bcl-xL and Bcl-2 protein expression in L929 cells treated with IL-15 or Gas6 for 24 h, we found that IL-15 and Gas6 upregulated these proteins in L929 cells (Figure 4B). L929R fibroblasts exhibited high basal expression of these two proteins, which could not be upregulated further by IL-15 (data not shown). To explore whether the similarity in downstream signaling events in response to Gas6 or IL-15 stimulation reflected transactivation of the Axl signaling cascade by IL-15, we repeated our experiments in Axl-depleted cells. Transfection with Axl siRNA revealed that IL-15 failed to activate PI3K, Akt, or ERK1/2 in Axl-deficient cells (Figure 5). This suggests that IL-15 transactivates the Axl signaling pathway in L929 fibroblasts.
Gas6 does not affect IL-15-mediated Axl transactivation

Figure 6 Gas6 does not affect IL-15-mediated Axl transactivation. (A) Cells were incubated for 10 min with 100 ng/ml Axl-Fc or control IL-3R-Fc proteins and then IL-15 or Gas6 (100 ng/ml) was added for another 5 or 15 min. Axl phosphorylation was detected by WB. (B) L929 cells were cultured in the presence of warfarin (100 μg/ml) for 24 h and stimulated with IL-15 or Gas6. Axl phosphorylation was detected by WB. (C) Recombinant Gas6 or IL-15 (10 nM) was incubated with 10 nM of Axl-Fc, IL-15Rz-Fc, or control IL-3R-Fc proteins. Complexes were captured on protein A-agarose and analyzed by WB using anti-Gas6 (upper panel) or anti-IL-15 (lower panel) Abs.

Gas6 is not involved in the IL-15-mediated Axl transactivation

Next, we assessed the role of Gas6 in the IL-15-mediated transactivation of Axl under conditions that prevented endogenous Gas6 activity, using a chimeric Axl-Fc protein that captures Gas6 and inhibits its receptor binding (Costa et al., 1996). However, IL-15 still induced Axl phosphorylation in the absence of Axl-Fc (20–100 nM), whereas this chimeric protein abrogated Gas6-specific signaling in both L929 and L929R cells (Figure 6A). Importantly, IL-15 also stimulated the phosphorylation of Axl in L929 cells treated with warfarin (Figure 6B). Warfarin selectively inhibits the post-translational γ-carboxylation of Gas6, which is essential for its receptor binding and growth stimulation (Yanagita et al., 1999). These findings essentially rule out that endogenous Gas6 participates in the IL-15-mediated transactivation of Axl. To exclude a direct binding of IL-15 to Axl, we performed in vitro binding assays as described (Nagata et al., 1996), using purified recombinant proteins. However, no interactions other than the expected binding of IL-15 and Gas6 to their respective receptors were detected (Figure 6C).

IL-15 cross-modulates the Axl signaling pathway in mouse embryonic fibroblasts

To check whether the ability of IL-15 to stimulate the IL-15Rz-mediated activation of Axl was restricted to L929 fibrosarcoma cells, mouse embryonic fibroblasts (MEFs) obtained from wild-type (WT), IL-15Rz-deficient (IL-15Rz−/−) (Lodolce et al., 1998), or Axl-deficient mice (Axl−/−) were also examined (Bellotta et al., 1997). MEFs from WT and Axl−/− mice almost equally expressed IL-15Rz, while MEFs from WT and IL-15Rz−/− animals had equal expression of Axl by WB (Figure 7A) and FACS analysis (Figure 7B). All three types of MEFs had comparable levels of IL-2Rγ expression (Figure 7A). To test whether IL-15 signaling in these cells depends on the expression of Axl, WT, Axl−/−, or IL-15Rz−/− MEFs were stimulated with IL-15 or Gas6. Subsequently, Axl was precipitated from the cell lysates using anti-Axl Abs, and protein precipitates were analyzed by WB.

Indeed, both IL-15 and Gas6 stimulated the phosphorylation of Axl in MEFs from WT mice, and the patterns of phosphorylation of downstream signaling molecules closely resembled those observed in L929 cells, demonstrating the phosphorylation of PI3K/Akt and ERK and upregulation of Bcl-2 (and, to a lesser extent, of Bcl-xL) also in primary fibroblasts (Figure 7C and D). Anti-Axl Abs also co-precipitated the IL-15Rz chain in both non-stimulated and stimulated WT MEFs. This indicates that IL-15Rz and Axl are constitutively associated not only in immortalized but also in primary mouse fibroblasts (Figure 7C). IL-15, and to a lesser extent Gas6, induced IL-15Rz phosphorylation within the first 15 min in WT MEFs, whereas both failed to induce IL-15Rz phosphorylation in Axl−/− animals (Figure 7C, middle part). This supports the concept that IL-15Rz is directly phosphorylated by activated Axl RTK.

As expected, the IL-15-mediated activation of Axl signaling cascade was absent in MEFs of IL-15Rz−/− mice, while Gas6-induced signaling remained intact (Figure 7C, right part). This confirms that activation of the PI3K/Akt pathway in WT MEFs results from the IL-15Rz-mediated transactivation of Axl-specific downstream signaling. The fact that IL-15 failed to mediated Axl phosphorylation in the absence of IL-15Rz argues for the role of the ligated high-affinity cytokine receptor chain as the initiator of Axl transactivation, and further excludes a direct interaction of IL-15 with Axl.

Next, the binding affinity of IL-15R complex in the presence of absence of Axl expression was compared by 125I-IL-15-labeled ligand binding assays and Scatchard plot analysis. We utilized immobilized long fibroblasts obtained from Axl−/−, IL-15Rz−/−, and IL-2Rγ−/− (RAG−/−) mice. These experiments demonstrated that neither Axl nor IL-2Rγ expression affected binding affinities of IL-15Rz toward IL-15, which remained constant in all cell types tested (Kd ≈ 1011 pM), except cells from IL-15Rz−/− animals, which served here as a control (Supplementary Figure 5).

Since Gas6 supports survival of fibroblasts upon serum deprivation (Bellotta et al., 1997), we next examined whether IL-15 and Gas6 rescue MEFs from cell death induced by serum starvation. Gas6 and IL-15 were almost equipotent in their ability to support survival of MEFs from WT mice, but not Axl−/− mice, whereas Gas6 (but not IL-15) supported survival of IL-15Rz−/− MEFs (Figure 7E). Again, this argues for the use of a common signaling pathway by IL-15 and Gas6. In summary, these data suggest that IL-15 stimulation triggers transactivation of Axl, which in turn results in IL-15Rz phosphorylation, presumably by the activated tyrosine kinase domain of Axl. Although Gas6 can signal also in the absence of IL-15Rz, proper signal transduction through Axl is indispensable for IL-15-induced signaling in mouse primary fibroblasts.
IL-15Rα and Axl constitutively associate in murine dendritic cells

Next, we explored whether cross-communication between the IL-15Rα and Axl signaling pathways can also be demonstrated in additional primary cells. To this end, we tested whether IL-15 associates with Axl in murine dendritic cells (DCs). DCs express Axl at the mRNA and protein level, as well as IL-15Rα and IL-2Rγ of the IL-15R complex (data not shown). These cells also release a proteolytically processed, soluble form of Axl (Budagian et al., 2005). Here, we show that murine DCs also display constitutive association of IL-15Rα and Axl, as detected by co-IP (Figure 8A) and two-site ELISA (Figure 8B and C). No association between Axl and IL-2Rγ was seen, even upon IL-15 stimulation. However, neither IL-15 nor Gas6 were able to induce Axl, phospho-Pi3K, and Akt phosphorylation in murine DCs (data not shown). This indicates that the activation of Axl signaling pathway is cell-type-dependent.

Finally, we asked whether other cytokine receptors, which share the γc chain, may also associate with Axl upon stimulation with the corresponding ligand. DCs were stimulated with IL-2, IL-4, IL-7, IL-9, or IL-21 (DCs express high-affinity receptor subunits of these cytokines; data not shown). However, none of these cytokines induced Axl phosphorylation or the association of Axl with the high-affinity subunits of the relevant cognate receptors (Figure 8A and data not shown) or with the γc chain (Figure 8A). No phosphorylation of PI3K, Akt, and ERK1/2 was seen in response to these cytokines (data not shown). Therefore, among these cytokine receptors, only the high-affinity α chain of IL-15Rα associated with Axl RTK in murine DCs.

Discussion

Our study suggests a new model for how cross-communication between an RTK and a cytokine receptor can promote cell survival, and identifies Axl as a member of the RTK family that can integrate signaling from heterologous receptor systems (Figure 9). Specifically, we provide the first evidence that the Axl signaling pathway is essential for efficient IL-15-mediated protection of mouse fibroblasts from TNFα-induced cell death. IL-15 literally transduces the Axl signaling cascade by binding to IL-15Rα. Thus, the Axl signaling pathway is activated directly by Gas6 and indirectly by IL-15. Nevertheless, IL-15 induces rapid Axl phosphorylation even when Gas6-mediated Axl activation is inhibited. Therefore, Axl transactivation is likely mediated through mechanism(s) that do not involve the interaction of Axl with endogenous Gas6.

It is increasingly appreciated that the interaction of one ligand with its high-affinity cognate receptor can activate more than one signal transduction pathway, both on the...
in intracellular and the cell membrane level (Mera et al., 1999; Hirota et al., 2001).

Here, we reveal an unprecedented cooperation in signal transduction of a cytokine receptor and a transmembrane tyrosine kinase, adding yet another ‘odd couple’ to the ever-growing list of heterologous receptor interactions, as exemplified by the transactivation of EGF family receptors (¼ inter-receptor signal transmission between G-protein-coupled receptors and RTKs) (Gschwind et al., 2001). In addition, G-protein-coupled receptors engage in cross-talk with growth hormone, prolactin, integrins, and PDGF (Prenzel et al., 2000), while the growth factor receptor ErbB2 forms a complex with the gp130 subunit of the IL-6R complex, and is critical for IL-6-mediated MAPK activation (Qui et al., 1998).

That Axl can modulate the functions of other membrane receptors is further supported by a recent report that cross-talk between Axl and vascular endothelial growth factor receptor 2 regulates activation of an angiogenic program in endothelial cells (Gallicchio et al., 2005). Thus, like members of the EGFR family, Axl may serve as a key signaling element for the integration of multiple, changing environmental influences. ‘Promiscuous receptors liaisons’ of the kind revealed in the current study appear to be only the tip of an ‘iceberg’ of cross-signaling complexity, whose full functional significance under physiological and pathological conditions we can only but guess.

Although this remains to be validated in other cells besides murine DCs, our data suggest that the IL-15Rs/Axl interaction is specific to IL-15 and that IL-2 does not activate the Axl pathway. The specificity of the IL-15Rs/Axl interaction identified here is underscored by the finding that neither IL-2 nor other cytokines, which utilize the γc chain for signaling, induce the association of Axl with their high-affinity α receptors upon ligand stimulation. We also show that the observed cytokine receptor and RTK interaction is specific for Axl. In DCs, however, contrary to fibroblasts, IL-15 stimulation did not recruit IL-2Rγ to the IL-15Rα/Axl complex. This suggests cell type-specific differences in individual cross-

**Figure 8** Axl and IL-15Rα coassociate in mouse DCs. (A) DCs were stimulated with cytokines for 15 min. Axl was precipitated from the cell lysates and precipitates were analyzed by WB using anti-IL-4Rα (upper panel), anti-IL-2Rγ (second panel), and anti-IL-15Rα (third panel) Abs. IP using specific Abs served as a positive control. For loading control, blots were developed with anti-Axl Abs (lower panel). (B) Plates were coated with anti-Axl Abs, and biotinylated anti-Axl (specific) or anti-IL-15Rα (two-site) Abs were used as detection Abs. (C) Plates were coated with anti-IL-15Rα Abs, and biotinylated anti-IL-15Rα (specific) or anti-Axl (two-site) Abs served as detection Abs.

**Figure 9** Hypothetical model of cross-talk between IL-15 and Axl signaling pathways suggested by the current study.
transformation fibroblasts even in the absence of ligand upon malignant phenotype.

We also show that an RTK can constitutively associate with the high-affinity subunit of a cytokine receptor even in the absence of ligand stimulation. The promiscuous liaison between these two distinct receptor systems operates already at the cell membrane level, and Axl RTK seems to serve as an integral component of a functional IL-15 receptor complex. This may be particularly important for IL-15 effects on cells that do not express IL-2R (such as fibroblasts) and/or γc chains, and the ability of IL-15 to transactivate Axl may primarily refer to non-immune cells.

Although our pilot data do not support that the Axl and IL-15Rα interaction occurs in lipid rafts, this possibility remains to be excluded definitely. Interestingly, IL-15Rα, IL-2Rα, IL-2Rβ, and IL-2Rγ subunits can form supramolecular receptor clusters in lipid rafts, which may be critical in the cross-regulation of IL-15- versus IL-2-mediated responses (Vamosi et al., 2004). Perhaps, the Axl/IL-15Rα interaction segregates the IL-15Rα subunit from membrane microdomains, thereby excluding it from IL-2Rα and limiting its availability for other microdomain-associated molecules.

Axl and IL-15Rα associate and colocalize on the cell surface, while no physical interaction of IL-15 and Axl is observed. Considering that IL-15Rα can partially oligomerize upon ligand binding (Vamosi et al., 2004), it is tempting to speculate that IL-15 treatment physically affects the distribution of Axl on the cell membrane, leading to homophilic interactions between the extracellular domains of several RTK molecules. Our mapping analysis, which shows that the extracellular domain of Axl is required for the described IL-15Rα/Axl interaction, further supports this notion. Activated Axl then phosphorylates IL-15Rα and activates the PI3K/Akt signaling pathway. Since Syk kinase, which phosphorylates IL-15Rα (Bulanova et al., 2001), is absent in L929 cells (E Bulanova, unpublished observation), it can essentially be ruled out that Syk interacts with Axl through its SH2 domain and mediates IL-15Rα/Axl interaction in these cells. However, a transient participation of other molecule(s) in this process, especially in vivo and in other cell types than the ones studied here, cannot yet be excluded.

The L929 cell line has been commonly used to study TNFα cytotoxicity, since it is one of the few systems in which TNFα exerts its cytotoxic effects in the absence of inhibitors of de novo protein synthesis (Strelow et al., 2000). Given the exquisite sensitivity of L929 cells to TNFα-induced cell death, the newly acquired, IL-15-mediated resistance of L929 cells to TNFα underscores the capacity of tumor cells to activate protective mechanisms in response to negative selective pressure, which may result in the development of a more malignant phenotype in vivo. The observed high expression of Axl in L929 cells is in line with the ability of Axl to transform fibroblasts even in the absence of ligand upon engineered overexpression (O’Brien et al., 1991).

It is still unclear how TNFα kills L929 cells: apoptosis, necrosis, both (Humphreys and Wilson, 1999; Los et al., 2002), and/or ‘atypical’ apoptosis with membrane blebbing and unusual degradation of poly(ADP-ribose) polymerase in the absence of caspase activity (Strelow et al., 2000). Therefore, the analysis of caspase activity does not aid in the understanding of the exact cell death mode in this system. Our data support a key role for ceramide as the mediator of caspase-independent apoptosis in L929 cells (Strelow et al., 2000). Since Bcl-2 blocks the effects of ceramide on mitochondria (Lin et al., 2005), the Axl-mediated overexpression of Bcl-2 and Bcl-xL, identified here may counteract the proapoptotic effects of ceramide. It is still an open question whether Axl prevents generation of ceramide through inhibiting its production and/or accelerating its degradation.

Our data suggest that a two-pronged protective mechanism underlies the antiapoptotic properties of IL-15 on L929 fibroblasts: (a) the previously reported, immediate recruitment of TRAF2 to IL-15Rz, with subsequent NF-κB activation, by IL-15 (Bulfone-Paus et al., 1999); and (b) IL-15-mediated transactivation and upregulation of Axl, with subsequent activation of PI3K/Akt and upregulation of Bcl-2 and Bcl-xL (Figure 9). While these mechanisms likely cooperate to suppress apoptosis in L929 fibroblasts, only some of them may be operative in other cell types. However, the novel concept that IL-15 and Gas6 utilize a common signaling pathway and that Axl RTK represents yet another convergence point for signal integration and diversification between heterologous signaling systems (such as RTKs and cytokine receptors) (Figure 9) also applies at least to some primary cell types.

Materials and methods

**Cytokines, Abs, fusion proteins, and plasmids**

Recombinant human IL-15 and TNFα were purchased from TEBU; Gas6 and biotinylated Abs and Axl-Fc, IL-15Rz-Fc, and IL-3R-Fc proteins were from R&D Systems. Abs against IL-15Rz, Axl, Bek, IL-2Rγ, ERK, pERK, PI3K, Bcl-2, Bcl-xL, Akt, and phospho-Akt were purchased from Santa Cruz Biotechnology. Mouse anti-phosphotyrosine (anti-pTyr) and anti-IL-4Rα Abs were from BD PharMingen. IL-15-IgG2b fusion protein was produced as described previously (Bulfone-Paus et al., 1997b). Cloning of mouse Axl, Bek-Axl, Axl-cDNA was described elsewhere (Bellosta et al., 1995; Bulfone-Paus et al., 1999).

**Cell lines, culture, and transfection**

The parental L929 cell line was purchased from ECACC. The L929R cell line, a TNFα-resistant derivative of L929 cells, was established by using IL-15 and TNFα in combination. In brief, L929 cells were cultured over a 1-month period with 5 ng/ml TNFα and gradually decreasing IL-15 concentrations (20–0 ng/ml). After that time, cells started to proliferate again despite the continued presence of TNFα in the medium. Parental and derivative cells exhibited similar rates of proliferation and same morphological appearance, as assessed by [³H]thymidine incorporation and light microscopy (data not shown).

Bone marrow-derived DCs were generated as described (Brandt et al., 2003). L929, L929R, DCs, and COS-7 cells were maintained in RPMI-1640. MEFs from WT, Axl−/−, and IL-15Rz−/− mice were generated as described (Bellosta et al., 1997) and maintained in DMEM. Culture medium was supplemented with 10% fetal calf serum, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. COS-7 cells were transiently transfected using LipofectAMINE 2000 (Gibco-Invitrogen), harvested, and analyzed 48 h post-transfection.

**siRNA transfection**

SIRNAs were purchased from Eurogentec. Double-stranded siRNA for Axl or scrambled control was obtained by annealing the
respectively, two RNA oligonucleotides: Axl 5′-UAUACAGGUCCG-CAGAGG-dTdT-T3′ and 3′-dTdT-UAUGUGCUCCGGUCUCCU-5′; scrambled control 5′-CUGCGACUGUAAAUGUG-dTdT-T3′ and 3′-dTdT-GACGUAU-UACAGGUCCG-CAGAGG. Cells were transfected with jetSI-FluoF siRNA transfection reagent (Eurogentec), using 20 nM of double-stranded siRNA. At 48 h post-transfection, cells were analyzed for mRNA/protein expression by RT–PCR/WB. Transfection efficiency was about 80–90%, as detected by fluorescent microscopy.

**Two-site ELISA**
A 96-well plate (Greiner) was coated with 1 μg/ml of anti-Axl or anti-IL-15R Abs. Wells were blocked with 2% BSA in PBS. Cells were lysed with 1% NP-40 buffer. Lysates (50 μl/well) were added to the plate and incubated overnight at 4°C. Serial dilutions of murine recombinant Axl or IL-15R Abs were included to exclude contamination in all experiments.

**RT–PCR**
RNA was extracted using TRIZOL reagent (Invitrogen). cDNA was synthesized from 5 μg total RNA using random oligonucleotides and the SuperScriptII™ kit (Invitrogen). cDNA was amplified by standard PCR procedure as described (Bulfone-Paus et al., 1999). Negative controls consisted of isotype-matched Abs (BD Pharmingen). The percentage of apoptotic cells was evaluated by the ApoTag Annexin-V-FITC Apoptosis kit (BioSource) and FACS analysis was performed by FACS caliber (Becton Dickinson) using CELLQuest software.

**Ceramide quantitation**
Cells were left untreated or treated with 100 ng/ml TNFα alone or in combination with 20 μM of benzoyloxycarbonyl-Val-Ala-Asp(Ome)- fluoromethylketone (zVAD-fmk) for 4 h. Ceramide was quantified by the charring method following high-performance thin-layer chromatography as described (Jensen et al., 1999). Thin-layer chromatography plates were scanned and analyzed using the software package PC-BAS (Raytest).

**FACS analysis**
For analysis of surface receptors expression, L929 and L929R cells were stained with monoclonal Abs or incubated with IL-15-igG2b as described (Bulfone-Paus et al., 1999). Negative controls consisted of isotype-matched Abs (BD Pharmingen). The percentage of apoptotic cells was evaluated by the ApoTag Annexin-V-FITC Apoptosis kit (BioSource) and FACS analysis was performed by FACS caliber (Becton Dickinson) using CELLQuest software.

**Confocal microscopy**
Cells were fixed with 2% paraformaldehyde, permeabilized by 0.25% Triton X-100 and stained with 1:100 dilutions of Abs. Alexa Fluor-488 anti-goat IgG (H + L) and Alexa Fluor-546 anti-rabbit IgG (H + L) (Molecular Probes) at a dilution of 1:100 were used as secondary Abs. Nuclei were stained using TOTO-3 dye (Molecular Probes). The specimens were mounted in DABKO (1,4-diazabicyclo [2.2.2]octane) anti-fading solution and analyzed by scanning confocal microscopy (Leica TCS SP).

**Data analysis**
All experiments were performed in at least three independent assays, which yielded highly comparable results. Data are summarized as mean ± s.d. Statistical analysis of the results was performed by Student’s t-test for unpaired samples. A P-value of <0.05 was considered as statistically significant.

**Supplementary data**
Supplementary data are available at The EMBO Journal Online.

**Acknowledgements**
We are grateful to Dr T Sasaki for recombinant Axl proteins, Dr K Brand for the DCs generation, M Hein for excellent technical assistance, and Dr K Wiebauer for critical reading of the manuscript. This study was supported by a grant from DFG to SBP and DA (SFB415: A10/A4).

**References**

© 2005 European Molecular Biology Organization
Transactivation of Axl by IL-15 controls cell death

V Budagian et al


Mera A, Sug aM, Ando M, Suda T, Yamaguchi N (1999) Induction of cell shape changes through activation of the IL-3 common β chain by the RON receptor tyrosine kinase. *J Biol Chem* 274: 15766–15774


