The death receptor CD95 activates the cofilin pathway to stimulate tumour cell invasion

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The death receptor CD95 promotes apoptosis through well-defined signalling pathways. In colorectal cancer cells, CD95 primarily stimulates migration and invasion through pathways that are incompletely understood. Here, we identify a new CD95-activated tyrosine kinase pathway that is essential for CD95-stimulated tumour cell invasion. We show that CD95 promotes Tyr783 phosphorylation of phospholipase C- γ 1 through the platelet-derived growth factor receptor- β , resulting in ligand-stimulated phosphatidylinositol (4,5)-bisphosphate (PIP₂) hydrolysis. PIP₂ hydrolysis liberates the actin-severing protein cofilin from the plasma membrane to initiate cortical actin remodelling. Cofilin activation is required for CD95-stimulated formation of membrane protrusions and increased tumour cell invasion.

Keywords: CD95; colorectal; cancer; invasion; platelet-derived growth factor receptor; phospholipase

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INTRODUCTION

Death receptors on tumour cells can suppress metastasis formation by stimulating apoptosis (Smyth *et al*, 2001; Takeda *et al*, 2001; Koshkina *et al*, 2007; Yang *et al*, 2008). Although CD95 is best known for its ability to induce apoptosis, it can also function as a promoter of tumour and metastasis formation by stimulating tumour cell proliferation, survival and/or invasion (Barnhart *et al*, 2004; Peter *et al*, 2007; Kleber *et al*, 2008; Chen *et al*, 2010; Hoogwater *et al*, 2010; Nijkamp *et al*, 2010). We have recently shown that endogenous oncogenic Kirsten-RAS (KRAS)—one of the first genes to be mutated during colorectal cancer development—can transform death receptors into invasion-stimulating receptors (Hoogwater *et al*, 2010). The signal-transduction pathways that cause apoptosis downstream of activated CD95 have been well documented (Strasser *et al*, 2009). By contrast, less is

known about the factors that promote tumour cell invasion following CD95 stimulation. In many tumour cell lines, invasion is dependent on the actin-driven formation of membrane protrusions (Wang *et al*, 2006, 2007). In this report, we provide evidence that activated CD95 promotes the formation of cell protrusions through a new signalling pathway.

RESULTS AND DISCUSSION

To study invasion signalling pathways downstream of CD95, we used the *Kras*-mutant murine colorectal-cancer cell line C26. CD95 primarily signals motility and invasion in these cells (Hoogwater *et al*, 2010; Nijkamp *et al*, 2010; Fig 1A). Checkerboard analysis further showed that the CD95 ligand (CD95L) promotes directed—chemotaxis—rather than non-directed—chemokinesis—tumour cell migration (Fig 1B; supplementary Table S1 online). Real-time imaging showed that non-stimulated C26 cells spontaneously formed cell protrusions in a random manner with, on average, 1–2 protrusions per cell. On stimulation with CD95L, several protrusions increased to 4–6 per cell, and this increased the total cell by area by 35% over a period of 30 min (Fig 1C). Knockdown of the endogenous mutant *Kras*^{D12} allele mostly prevented CD95-induced formation of cell protrusions and invasion and allowed CD95 to induce apoptosis (Fig 1A; supplementary Fig S1 online). Stimulation of another murine colorectal-cancer cell line (MC38) with CD95L also resulted in increased migration, formation of cell protrusions and tumour cell invasion (supplementary Fig S2 online).

The polymerization of actin monomers into filamentous actin (F-actin) at the leading edge of migrating cells generates the forces that drive the formation of cell protrusions. Actin polymerization mainly occurs at one end of the filament, named the ‘barbed end’, and assembly of F-actin is controlled by regulation of several barbed ends. One of the key proteins in this process is the actin-binding protein cofilin, which can increase the number of barbed ends by severing existing F-actin (Ichetovkin *et al*, 2002) and by its intrinsic actin-nucleation activity (Andrianantoandro & Pollard, 2006). Cofilin is inactivated by LIM kinase 1 (LIMK1)-mediated phosphorylation on Ser3, which is located in the actin-binding domain. The phospho group on Ser3 inhibits cofilin binding to F-actin and makes it inactive (Moriyama *et al*, 1996; Arber *et al*, 1998; Yang *et al*, 1998; Bamburg, 1999; Bernard, 2007). We next

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Fig 1 | CD95 ligand stimulates rapid cofilin-dependent formation of cell protrusions and an increase in cell area. (A) C26 and C26 KRAS KD cells treated with CD95L (10 ng/ml) for 24 h and stained with propidium iodide. The apoptotic sub-G1 fraction was determined by FACS analysis (left panel). C26 and C26 KRAS KD cells were plated on matrigel-coated membranes in transwell chambers and were allowed to invade for 8 h in the presence or absence of CD95L (10 ng/ml; right panel). The bar graph shows fold increase relative to non-stimulated controls (= 1). Error bars represent s.e.m. on the basis of two independent experiments conducted in triplicate. (B) Checkerboard analysis in which CD95L (8 h) was added to the insert (top) or to the well (bottom) of the matrigel invasion chamber. All conditions were tested in three to five independent experiments. Numbers represent fold change of several invaded cells relative to control (no CD95L in either compartment), $\pm$ s.e.m. (P -value). Significance was tested using Student's t -test (unpaired; double sided). (C) C26 cells were transiently transfected with LIMK-GFP or GFP and were analysed by real-time imaging before and after stimulation with CD95L (10 ng/ml). Differential interference contrast images were used to determine several protrusions before and after stimulation. Inset shows C26 cells before (left panel) and after (middle panel) CD95L stimulation. The right image is a merge of binary threshold images at both time points. Yellow represents no change; red represents increased protrusion formation; and green represents membrane retraction. Numbers indicate new protrusions formed. Scale bar, 10 μ m. The generated images were also used to measure the total cell areas before and after stimulation (arrow) by using ImageJ software ($n = 4$). These values were plotted as the percentage of average cell area before stimulation (right panel). Asterisk indicates significance on the basis of P -values < 0.05 . (D) C26 and C26-LIMK1 cells were treated with CD95L (10 ng/ml) for 24 h and apoptosis and invasion were assessed as in A ($n = 3$). Asterisk indicates significance on the basis of P -values < 0.05 . CD95L, CD95 ligand; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein; KD, knockdown; LIMK, LIM kinase.

tested whether CD95-stimulated formation of cell protrusions was cofilin dependent by overexpressing LIMK1. As expected, LIMK1 transfection into C26 cells resulted in the phosphorylation (inactivation) of cofilin on Ser3 (pS3-cofilin; supplementary Fig S3 online). Cofilin inhibition by LIMK1 mostly prevented the formation of cell protrusions and the increase in cell area (Fig 1C), and reduced the invasive capacity of control and CD95L-stimulated C26 cells (Fig 1D). Instead, prolonged stimulation of C26-LIMK1 cells caused apoptosis (Fig 1D). Together, these data indicate that CD95 promotes the formation of cell protrusions in a

LIMK1-suppressible manner, suggesting that it is a cofilin-dependent phenomenon.

The activity of cofilin is negatively regulated by LIMK1-mediated Ser3 phosphorylation (Arber *et al*, 1998; Yang *et al*, 1998) and by binding to plasma membrane phosphatidylinositol (4,5)-bisphosphate (PIP₂; van Rhee *et al*, 2007, 2009). If CD95 activates cofilin by Ser3 dephosphorylation, one would expect cofilin phosphorylation to decrease in response to CD95L. However, pS3-cofilin levels in C26 cells are low due to KRAS/RAF1-mediated suppression of LIMK1 (Hoogwater *et al*, 2010) and were unaffected

Fig 2 | CD95 ligand stimulates phospholipase C-γ1 Tyr 783 phosphorylation and PIP₂ hydrolysis. (A) C26 cells were treated with CD95L (10 ng/ml) for the indicated time periods. Cell lysates were prepared and analysed for cofilin Ser3 phosphorylation (pS3-Cofilin), total cofilin, Tyr 783 phosphorylation of PLC-γ1 (pY783-PLC-γ1) and total PLC-γ1 by western blotting. (B) Cells were transiently transfected with mRFP-PH and analysed by real-time confocal microscopy. Images represent mRFP-PH in C26 cells before stimulation (left) and 15 min after stimulation with CD95L (10 ng/ml; middle). Scale bar, 5 μm. Plotting of total cell area (measured by differential interference contrast images) and PIP₂ hydrolysis (measured using mRFP-PH) over time shows that addition of CD95L (10 ng/ml; black bar) caused a simultaneous increase in cell area and PIP₂ hydrolysis (lower left panel). The average of five independent experiments is plotted ($n = 5$). The right lower panel shows a quantification of maximal CD95L-induced PIP₂ hydrolysis, relative to ionomycin-induced maximum translocation of the mRFP-PH probe (100%). Significance was tested using Student's *t*-test (unpaired; double sided); asterisk indicates $P < 0.05$. CD95L, CD95 ligand; mRFP-PH, PH domain fused to red fluorescent protein; PIP₂, phosphatidylinositol (4,5)-bisphosphate; PLC-γ1, phospholipase C-γ1.

by stimulation with CD95L (Fig 2A). Therefore, it seems unlikely that CD95 activates cofilin by promoting its dephosphorylation.

In breast cancer cells, epidermal growth factor (EGF) stimulation causes local phospholipase C-γ1 (PLC-γ1)-mediated PIP₂ hydrolysis that liberates cofilin from inhibition by PIP₂ (van Rhee et al, 2007, 2009). PLC-γ1 is activated by growth factors through phosphorylation of Tyr783, which increases its PIP₂ hydrolysis activity (Kim et al, 2000). Stimulation of C26 or MC38 cells with

CD95L caused an increase in Tyr783-PLC-γ1 phosphorylation without altering cofilin Ser3 phosphorylation (Fig 2A and 4C). PIP₂ hydrolysis can be detected by using the PLC-δ1 PH domain fused to red fluorescent protein (RFP; mRFP-PH) as a PIP₂ probe (Stauffer et al, 1998; Varnai & Balla, 1998; van Rhee et al, 2005). In resting C26 cells, mRFP-PH is bound to PIP₂ in the plasma membrane (Fig 2B, left panel). Stimulation with CD95L induced a rapid 40–50% translocation of mRFP-PH from the membrane to the cytosol (Fig 2B). Interestingly, PIP₂ hydrolysis following CD95L stimulation occurred concomitantly with the formation of cell protrusions and the increase in cell area (Fig 2B, lower left panel).

The results so far indicate that CD95L promotes cofilin activation to induce cell protrusions, possibly through PLC-γ1-mediated PIP₂ hydrolysis. To test the requirement for PLC in CD95L-stimulated formation of cell protrusions and invasion, C26 cells were treated with the PLC inhibitor U73122. As expected, U73122 inhibited CD95L-stimulated PIP₂ hydrolysis (Fig 3A). Strikingly, in U73122-treated cells, CD95L caused an increase in plasma membrane PIP₂ levels. This could indicate that PIP-5 kinase is activated following CD95L stimulation. Importantly, U73122 not only prevented PIP₂ hydrolysis, but also blocked CD95L-stimulated formation of cell protrusions and the increase in total cell area (Fig 3B). In addition, RNA interference-mediated suppression of PLC-γ1 significantly reduced the CD95L-stimulated increase in cell area (Fig 3B). Transwell assays further showed that U73122 treatment or PLC-γ1 knockdown reduced CD95L-stimulated invasion of C26 and MC38 cells (Fig 3C,D).

PLC-γ1 is a growth factor-responsive PLC that is activated by receptor and non-receptor tyrosine kinases, including Src family kinases (SFKs) and the platelet-derived growth factor receptor-β (PDGFR-β; Ronnstrand et al, 1992; Kim et al, 2000). Interestingly, SFKs are activated by CD95 and might have a role in both apoptosis and invasion signalling (Schlottmann et al, 1996; Kleber et al, 2008). However, the small molecule SFK inhibitor SU6656 (Blake et al, 2000) had no effect on CD95L-stimulated Y783-PLC-γ1 phosphorylation in C26 cells (data not shown). To search for candidate PLC-γ1 kinases, C26 cells were stimulated with EGF and PDGF. PDGF, but not EGF, effectively stimulated PLC-γ1 phosphorylation in C26 cells (Fig 4A). Interestingly, PLC-γ1 activation is required for PDGFR-induced cell migration (Kundra et al, 1994). CD95L caused a marked increase in phosphorylation of Tyr 1021 of the PDGFR-β, which mediates binding to and activation of PLC-γ1 (Fig 4B; Ronnstrand et al, 1992). To assess whether PDGFR-β activity might have a role in CD95L-stimulated PLC-γ1 phosphorylation and tumour cell invasion, cells were treated with AG1296, a specific PDGFR inhibitor, or sunitinib, a PDGFR/vascular endothelial growth factor receptor inhibitor. Both AG1296 and sunitinib blocked CD95L-stimulated phosphorylation of PLC-γ1 (Fig 4C). In addition, RNA interference-mediated suppression of PDGFR-β expression reduced CD95L-stimulated PLC-γ1 phosphorylation and cell-area enlargement (Fig 4D). Moreover, sunitinib, AG1296 and PDGFR-β-targeted small-interfering RNAs all prevented basal and CD95L-stimulated tumour cell invasion (Fig 4E,F). These results indicate that CD95L promotes PLC-γ1 Tyr 783-phosphorylation through the PDGFR-β.

In conclusion, our results identify a new CD95-initiated signalling pathway involving PDGFR-β-mediated PLC-γ1 activation and PIP₂ hydrolysis. Future work should address how CD95 stimulates PDGFR-β tyrosine phosphorylation and whether this requires PDGF binding to the receptor. Alternatively, a direct

Fig 3 | CD95 ligand-induced tumour cell invasion requires phospholipase C-γ1 activation. (A) C26 cells were transiently transfected with mRFP-PH. After 24 h, cells were either pretreated with U73122 (5 μM) or with vehicle (DMSO) for 1 h before stimulation with CD95L (10 ng/ml). PIP₂ hydrolysis was measured over time, as shown in Fig 2B. (B) C26 cells were either pretreated with U73122 (5 μM) or with vehicle (DMSO) for 1 h before stimulation with CD95L (10 ng/ml). Knockdown of PLC-γ1 was established by transfection of siRNAs 2 days before stimulation. Cells were analysed by real-time imaging. Several cell protrusions and the increase in cell area were measured as shown in Fig 1B. (C) Transwell invasion assays of C26 and MC38 cells treated with U73343 or U73122 were performed as in Fig 1A. Data are from two independent experiments conducted in triplicate. (D) Transwell invasion assays of C26 and MC38 cells transfected with control or PLC-γ1 siRNAs ($n=2$). Significance was tested using Student's t -test (unpaired; double-sided); asterisk indicates $P<0.05$. CD95L, CD95 ligand; DMSO, dimethyl sulphoxide; mRFP-PH, PH domain fused to red fluorescent protein; PIP₂, phosphatidylinositol (4,5)-bisphosphate; PLC-γ1, phospholipase C-γ1; siRNA, small-interfering RNA.

Fig 4 | For caption see next page.

Fig 4 | Platelet-derived growth factor receptor- β mediates CD95-stimulated phospholipase C- γ 1 phosphorylation and tumour cell invasion. (A) C26 cells were stimulated with PDGF or EGF and pY783-PLC- γ 1 was analysed by western blotting. (B) C26 cells were stimulated with CD95L and PDGFR- β phosphorylation on Tyr 1021 was assessed by western blotting. (C) C26 and MC38 cells were pretreated with sunitinib (2 μ M) or AG1296 (20 μ M) for 16 h and subsequently stimulated with CD95L for 5, 15 or 30 min. Cell lysates were prepared and analysed for pY783-PLC- γ 1 by western blotting. (D) C26 cells were transfected with siRNAs against PDGFR- β and stimulated for the indicated times with CD95L. Y783-PLC- γ 1 phosphorylation and PDGFR- β knockdown were analysed by western blotting. Cell area enlargement (right panel) was measured as shown in Fig 1A. (E) Transwell invasion assays of C26 and MC38 cells treated with sunitinib, AG1296 or vehicle (DMSO). Data are from two independent experiments ($n = 2$) conducted in triplicate. (F) Transwell invasion assay of C26 and MC38 cells transfected with control or PDGFR- β -targeting siRNAs ($n = 2$). Significance was tested using Student's *t*-test (unpaired; double sided), asterisk indicates $P < 0.05$. EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PDGFR- β , PDGF receptor- β ; PLC- γ 1, phospholipase C- γ 1; siRNA, small-interfering RNA.

interaction between activated CD95 and the PDGFR- β could recruit the latter into the CD95 signalling complex, which contains tyrosine kinase activity (Sancho-Martinez & Martin-Villalba, 2009). In this hypothesis, CD95 stimulation could cause PDGFR- β activation in a PDGF-independent manner.

Earlier work has implicated caspase 8 and ERK in CD95-stimulated tumour cell invasion (Barnhart *et al*, 2004). More recently, it was shown that CD95 activates tyrosine kinases (Yes and Syk) to promote the expression of matrix-degrading metalloproteinases, thereby facilitating tissue invasion of tumour cells and myeloid cells (Kleber *et al*, 2008; Letellier *et al*, 2010). This study shows that tyrosine kinase activation by CD95 also results in the rapid actin-driven formation of cell protrusions, a process that is essential for tumour cell invasion. Thus, CD95 can promote tumour cell invasion through distinct pathways. The specific tyrosine kinases involved and the dominance of each of these pathways in determining invasive behaviour is probably cell-type dependent.

We propose a model in which CD95 stimulates PDGFR- β -mediated PLC activation and PIP₂ hydrolysis. The consequent release of cofilin from the plasma membrane and the continued suppression of LIMK1 by KRAS/RAF1 together allow robust activation of the cofilin pathway. Cofilin-generated actin barbed-ends act as nucleation points for *de novo* actin polymerization. This process is driven by the small GTPases Rac and Cdc42, which are also activated following CD95 stimulation (Subauste *et al*, 2000; Ruan *et al*, 2008). Cofilin and Rac/Cdc42 cooperate to stimulate actin polymerization, which causes the formation of cell protrusions (van Rheenen *et al*, 2009). This process is essential for tumour cell invasion and, presumably, for metastasis formation.

METHODS

Real-time imaging. Cells were seeded in a Lab-Tek Chambered #1.0 Borosilicate Coverglass System (Nalge Nunc International, Rochester, NY, USA) and were mounted on a Zeiss Axiovert 200M microscope for live-cell imaging (5% CO₂; 37 °C) for 2–14 h. Phase-contrast images were captured every 2 min using a Photometrics Coolsnap CCD camera (Scientific, Tuscon, AZ, USA). Images were processed using the Metamorph software (Universal imaging, Downingtown, PA, USA). Several protrusions was scored by off-line analysis of the generated videos and plotted as means $\pm$ s.e.m. For real-time fluorescence imaging, cells were placed on a Leica SP5 inverted microscope equipped with a $\times$ 63 1.3NA glycerol objective. Cells were maintained in Leibovitz-15-buffered medium (Invitrogen, Breda, The Netherlands) at 37 °C in a climate chamber.

Invasion assay. For *in vitro* invasion assays, 24-well BioCoat matrigel invasion chambers (#354480, BD Biosciences, Alphen

aan den Rijn, The Netherlands) were used according to the manufacturer's protocol. Cells were maintained under serum-free conditions overnight. In the upper compartment, 5×10^4 cells per well were plated in 0.5 ml serum-free medium. The lower compartment contained 0.75 ml medium with 5% FCS. CD95L (10 ng/ml) was added to the lower compartment. Cells were pretreated overnight in both compartments with 1 μ M U73122 and U73343 or with 2 μ M sunitinib for 1 h before plating. Invasion chambers were incubated for 8 h. Cells in the upper compartment were removed with a cotton swab. Transmigrated cells were fixed in 3.7% formaldehyde, stained with 4,6-diamidino-2-phenylindole and counted by analysing microscopic images (5–6 fields per membrane). Data are expressed relative to control. All assays were performed in triplicate and were repeated twice.

Checkerboard analysis. Checkerboard analysis was performed by adding 0, 2 and 10 ng/ml to the wells (bottom) and/or to the inserts (top) of the matrigel invasion chambers. Invasion assays were carried out as described above.

PIP₂ hydrolysis assay. Cells grown on coverslips were transiently transfected with mRFP-PH. The coverslips were mounted on a SP5 Leica confocal microscope. Images of RFP-PH were collected every 20 s and stored on the computer. The images were imported and analysed using a custom-made visual basics program. Following a threshold step, background regions were determined and the background was measured and subtracted from the images. Cells were traced with a fixed threshold resulting in a binary image. Regions of the plasma membrane were determined by eroding the binary image with six pixels. The cytosol region was determined by eroding the binary image by another four pixels. Mean fluorescence level of the plasma membrane and cytosol region was measured and plotted over time. To calibrate the plasma membrane to cytosol translocation, 5 μ M ionomycin + 2 mM calcium was added to induce a full membrane-to-cytosol translocation. PIP₂ hydrolysis was expressed as the ratio of fluorescent intensity of the plasma membrane to the cytosol, as induced by CD95 compared with ionomycin and calcium.

Statistical analysis. Statistical differences between groups were analysed by an unpaired two-sided *t*-test. Data are expressed as mean $\pm$ s.e.m. A *P*-value of < 0.05 was considered statistically significant.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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