

# Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293T and melanoma cells by CrkII and Rho-mediated signalling

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## Summary

Eph receptor tyrosine kinases and ephrins regulate morphogenesis in the developing embryo where they effect adhesion and motility of interacting cells. Although scarcely expressed in adult tissues, Eph receptors and ephrins are overexpressed in a range of tumours. In malignant melanoma, increased Eph and ephrin expression levels correlate with metastatic progression. We have examined cellular and biochemical responses of EphA3-expressing melanoma cell lines and human epithelial kidney 293T cells to stimulation with polymeric ephrin-A5 in solution and with surfaces of defined ephrin-A5 densities. Within minutes, rapid reorganisation of the actin and myosin cytoskeleton occurs through activation of RhoA, leading to the retraction of cellular protrusions, membrane blebbing and detachment, but not apoptosis.

These responses are inhibited by monomeric ephrin-A5, showing that receptor clustering is required for this EphA3 response. Furthermore, the adapter CrkII, which associates with tyrosine-phosphorylated EphA3 *in vitro*, is recruited *in vivo* to ephrin-A5-stimulated EphA3. Expression of an SH3-domain mutated CrkII ablates cell rounding, blebbing and detachment. Our results suggest that recruitment of CrkII and activation of Rho signalling are responsible for EphA3-mediated cell rounding, blebbing and de-adhesion, and that ephrin-A5-mediated receptor clustering and EphA3 tyrosine kinase activity are essential for this response.

Key words: Cell adhesion, Actin cytoskeleton, Eph receptor protein-tyrosine kinases, Melanoma, Metastasis

## Introduction

Eph receptor tyrosine kinases (RTKs) and their membrane-bound ephrin ligands are key regulators of cell movement during development (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999). Their interaction can lead to the activation of bidirectional signalling pathways in ligand- and receptor-expressing cells (Davy et al., 1999; Holland et al., 1998). Both receptor and ligand-initiated signals mediate repulsion, adhesion and de-adhesion mechanisms involved in the motility of adherent cells.

Previously we showed that Eph signalling is essential for directing the movement of cells during zebrafish embryogenesis, disruption of the EphA3 function leading to severe defects in gastrulation (Lackmann et al., 1998; Oates et al., 1999). The nature of these gastrulation defects and loss of cell adhesion in pre-gastrulation *Xenopus* embryos by activated EphA4 or ectopically expressed ephrin-B (Jones et al., 1998; Winning et al., 1996) suggests that Eph receptors and ephrins function by modulating cell adhesion and motility (Bruckner and Klein, 1998; Flanagan and Vanderhaeghen, 1998; Xu et al., 2000). This notion is supported by accumulating evidence suggesting that stimulated Eph receptors and ephrins modulate integrin function (Becker et al., 2000; Davy et al., 1999;

Huynh-Do et al., 1999; Jones et al., 1998; Wahl et al., 2000; Winning et al., 1996; Zisch et al., 2000; Zou et al., 1999).

Eph receptor activation leads to tyrosine phosphorylation of three major autophosphorylation sites, two of which are located in the highly conserved juxtamembrane region, and a third in the activation loop of the kinase domain. Together, these residues function to regulate kinase activity, their phosphorylation being required for full intrinsic enzyme activity (Binns et al., 2000; Zisch et al., 2000). The juxtamembrane tyrosines are also likely to be important for signal transfer, and were suggested as docking sites for known signalling molecules including Fyn, Src, Ras GTPase-activating protein (RasGAP), SHEP-1 and Nck (reviewed by Mellitzer et al., 2000). The involvement of most of these molecules in the organisation of the cytoskeleton (Schoenwaelder and Burridge, 1999; Schlaepfer and Hunter, 1998) supports an emerging concept from functional experiments of a direct communication between activated Eph receptors and signalling pathways regulating cell adhesion and plasticity of the actin cytoskeleton (Schmucker, 2001).

The reorganisation of cell morphology during adhesion and detachment involves changes in focal adhesion complexes and in the actin/myosin cytoskeleton and is regulated principally

by signals emanating from clustered cell adhesion receptors. Members of the Ras/Rho families of GTPases are essential in these pathways (reviewed by Hall, 1998; Schoenwaelder and Burridge, 1999), as they relay signals from various growth factor or integrin-derived pathways to effect characteristic changes to the cell morphology. The Crk family of adapter proteins (Matsuda et al., 1992) are also intimately involved in signal-transduction processes regulating integrin-dependent cell adhesion (Vuori et al., 1996; Zvara et al., 2001) and migration (Arai et al., 1999; Klemke et al., 1998; Petit et al., 2000). Via its src homology, SH2 and SH3 domains, Crk interacts with downstream signalling molecules including c-Abl, p130<sup>CAS</sup> (Crk-associated substrate), paxillin, Cbl, C3G and DOCK 180 (reviewed by Feller et al., 1998).

Although the integrin-mediated interaction with the extracellular matrix is essential for migration and survival of adherent cells, the deregulated adhesion or loss of adhesion dependence is a hallmark of tumour progression and metastasis (Giancotti and Ruoslahti, 1999). It is now well established that tumour progression in human cutaneous melanoma correlates with integrin expression and function (Johnson, 1999; Seftor et al., 1999). Intriguingly, several Eph receptors and ephrins are overexpressed in tumour specimens and tumour cell lines (reviewed by Dodelet and Pasquale, 2000). Moreover, increased expression levels of EphA2, EphA3, ephrin-A1 and ephrin-B2 correlate with melanoma tumorigenesis and metastatic progression (Chiari et al., 2000; Easty et al., 1995; Easty et al., 1999; Vogt et al., 1998). Given their essential function in regulating cell motility and adhesion during normal development, it is possible that the prometastatic effects of Eph/ephrin overexpression in tumours are due to changes in cell adhesion and motility that facilitate tumour cell dislodgement and invasion. To test this notion we studied the responses of EphA3-expressing melanoma cell lines and EphA3-transfected 293T cells (human kidney epithelial 293T cells) to ephrin-A5 stimulation and its impact on changes in cell adhesion and actin cytoskeletal organization.

We show that EphA3 activation induces retraction of cell protrusions, cell rounding, membrane blebbing and de-adhesion, and that ephrin-A5-mediated receptor clustering and the tyrosine kinase activity of EphA3 are essential for this response. By expression cloning we identify CrkII as a cytoplasmic ligand of activated EphA3 and show that CrkII signalling is crucial for ephrin-A5-mediated cell rounding. EphA3 activation also results in transient activation of RhoA, and treatment of cells with the Rho inhibitor C3-transferase or the ROCK inhibitor Y-27623 abrogate ephrin-A5-mediated cell rounding and membrane blebbing, respectively. A dominant-negative, SH3 mutant CrkII abrogates both ephrin-A5-induced cell morphological changes and RhoA activation. Our findings suggest a pathway where ephrin-A5-induced recruitment of CrkII to EphA3 and a rapid, transient increase in activated RhoA result in cell rounding, membrane blebbing and detachment.

## Materials and Methods

### Expression constructs and reagents

The cloning of full-length EphA3 (Wicks et al., 1992) into pEFBos (Nicola et al., 1996) has been described previously. Mutants of full-

length EphA3 in pEFBos were prepared using the QuickChange mutagenesis system (Stratagene). Plasmid, pEGFP(enhanced green fluorescent protein)-actin was from Clontech, and myc-tagged Crk II expression constructs pCAGGS Crk and pCAGGS Crk SH3\* were a generous gift from M. Matsuda (International Medical Centre, Japan). For the expression vector encoding the EphA3 intracellular domain (EphA3 ID), residues 583-983 of the EphA3 receptor were cloned into the pET32a expression vector (Novagen) between the *EcoRI* and *XhoI* sites. The expression vector encoding TC45PTP as a GST fusion protein (Tiganis et al., 1999) was a gift from T. Tiganis (Monash University, Victoria, Australia). The RhoA-binding domain (RBD) of Rhotekin was a kind gift from K. Burridge (UNC, Chapel Hill, NC).

Expression plasmids encoding fusion proteins in which either the extracellular domains of ephrin-A5 or EphA3 were fused to the hinge and Fc region of human IgG1 (gift from D. Cerretti, Immunex Corp., Seattle, WA) were used to transfect Chinese Hamster Ovary (CHO) cells. EphA3-Fc and Ephrin-A5 Fc (fusion protein between the Fc portion of human IgG and the C-terminus of ephrin-A5) were purified from cell culture supernatants by protein-A affinity chromatography. Flag-tagged monomeric ephrin-A5 was purified to homogeneity from transfected CHO cell supernatants as described previously (Lackmann et al., 1997). To facilitate formation of clustered ephrin-A5, ephrin-A5 Fc was incubated (20 minutes) at 1/10 molar ratio with anti-human Fc antibody (Jackson Laboratories).

A native EphA3-specific (clone IIIA4) monoclonal antibody (Mab) and affinity-purified rabbit polyclonal antibodies have been described previously (Boyd et al., 1992; Lackmann et al., 1997). Anti-myc 9E10 antibodies were a generous gift from E. Stanley (WEHI, Melbourne, Australia). Additional antibodies and reagents were purchased from Transduction Laboratories (focal adhesion kinase, Crk and p130<sup>CAS</sup>), New England Biolabs and Upstate Biotechnology (phosphotyrosine), Santa Cruz (RhoA), BD Pharmingen (annexinV-FITC), and Sigma ( $\alpha$ -tubulin), HRP-labelled secondary antibodies from Jackson laboratories (anti-mouse) and Bio-Rad (anti-rat), Alexa-labelled antibodies and rhodamine-phalloidin from Molecular Probes.

### Cell culture and microscopy

The AO2 and AO9 melanoma cells lines were obtained as clones of adherent cells derived from mechanically dispersed deposits of secondary metastatic melanomas (M. D., C. W. Schmidt, M. O'Rourke, unpublished). LiBr secondary malignant melanoma cells were described previously (Coates and Crawford, 1977). Melanoma cells were cultured in RPMI, 10% iron-fortified fetal bovine serum. Human kidney epithelial (HEK) 293T cells were maintained in DME, 10% fetal calf serum (FCS). Transient transfection with the various expression constructs was carried out using Fugene 6 (Roche Biochemicals).

To study stimulation *in situ*, melanoma or 293T cells were seeded onto fibronectin-coated round glass coverslips in 6-well dishes. 293T cells were transfected with the various expression constructs or the w/t EphA3 or pEGFP-actin, or both, using Fugene. Before examination, the cells were kept in DME, 1% FCS for at least 3 hours. For analysis on a Bio-Rad 1024 confocal microscope, coverslips were placed in Sykes Moore chambers (Sykes and Moore, 1959) and images taken every minute, 10 minutes before and 50 minutes after the addition of ligand or control solution.

For immunocytochemistry, cells on coverslips were washed with PBS, fixed with 4% para-formaldehyde in PBS for 10 minutes and permeabilised with 0.5% Triton X100 for 5 minutes before blocking for 30 minutes in PBS/0.2% BSA. Cells were incubated with relevant antibodies as indicated in figure legends, washed with PBS and incubated with Alexa-labelled secondary antibodies. For actin staining, permeabilised cells were stained with rhodamine-labelled phalloidin (0.1  $\mu$ g/ml).

### Adhesion assays

Ephrin-coated surfaces were prepared by immobilising ephrin-A5 Fc onto 'Reacti-Bind' Protein A coated plates (Pierce). Wells were washed with PBS/0.1% Tween 20 (PBST) before incubating overnight at 4°C with ephrin-A5 Fc in PBST/0.1% BSA at concentrations as indicated. Nonbound ephrin-A5 Fc was recovered for analysis and the plate was washed extensively before seeding cells. The density of bound ephrin-A5 Fc was calculated using Biacore analysis (Biacore AB) by estimating the ephrin-A5 concentration in nonbound fractions with a sensor chip containing EphA3 extracellular domain (Lackmann et al., 1997). The ability of Protein-A-tethered ephrin-A5 to bind EphA3 was confirmed by applying a constant concentration of the soluble EphA3 extracellular domain to wells of increasing ephrin-A5 densities and estimating the fraction of nonbound EphA3 by Biacore analysis as described (Lackmann et al., 1998).

Before seeding cells, residual Fc binding sites in wells were blocked by incubation with culture media containing 10% FCS. Cells were seeded ( $3 \times 10^4$  cells/well) onto the plates and allowed to adhere for 6 hours before fixation in 4% paraformaldehyde for microscopy. Adherent cells were stained with 0.5% crystal violet, solubilised in 0.1% SDS, and quantified by measuring the OD at 570 nm of individual wells in a microtiter plate reader. Each value represents the mean and s.d. from absorbance readings of triplicate wells made in parallel.

Melanoma cells cultured in 96-well plates were stimulated with preclustered ephrin-A5. Following PBS washes, adherent cells were fixed with 4% paraformaldehyde and quantified by crystal violet staining as described above.

### Cell viability and apoptosis assays

Cell viability was assessed by vital dye (0.5% trypan blue) exclusion and apoptotic cells quantified by flow cytometric analysis using established methods, including annexinV-FITC cell membrane staining and examination of nuclear condensation as described (Vermes et al., 2000). Briefly, combined adherent and detached cells from cultures of adherent cells, treated for 4 hours or as indicated with preclustered (see below) ephrin-A5 Fc or with staurosporin (Sigma), were washed once with PBS before incubation either in  $\text{Ca}^{2+}$ -containing buffer with 1  $\mu\text{g}/\text{ml}$  FITC-annexin and 1  $\mu\text{g}/\text{ml}$  propidium iodide (PI), or after permeabilisation in 0.2% Triton-X100/0.1% sodium acetate, pH 6.0 with 50  $\mu\text{g}/\text{ml}$  PI. In the latter assay, apoptotic cells are characterised by a PI staining intensity of their nuclei that is lower than that of normal cells. Viable and apoptotic cells were quantified by flow cytometry.

### Immunoprecipitation and western blotting

Cells were serum starved for 4 hours in DME/1% FCS. After 10 minutes stimulation with preclustered ephrin-A5 (1.5  $\mu\text{g}/\text{ml}$  or as indicated in figure legends), cells were lysed in 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X100, 1 mM  $\text{NaVO}_4$ , 10 mM NaF and protease inhibitors (TBS-Tx100). Lysates were immunoprecipitated with IIIA4 Mab coupled to Trisacryl (1 hour at room temperature or overnight at 4°C) (Boyd et al., 1992) or with 5  $\mu\text{g}/\text{ml}$  anti-CrkII Mab and protein A Sepharose (Pharmacia). Washed (TBS-Tx100) immunoprecipitates were analysed by western analysis with appropriate antibodies. Blots were visualised using an ECL substrate (Pierce).

### Assay for GTP-bound RhoA and ADP-ribosylation of RhoA by C3-exoenzyme

RBD was expressed as GST fusion proteins in BL21 cells. The levels of GTP-bound RhoA in cell lysates were measured as previously described (Schoenwaelder et al., 2000), according to Ren et al. (Ren et al., 1999), except that cells were lysed in TBS-Tx100, and the NaCl and  $\text{MgCl}_2$  concentrations were adjusted to 500 mM and 10 mM,

respectively, before the addition of the beads. ADP-ribosylation of RhoA in cell extracts was analysed as described previously (Leng et al., 1998). Briefly, EphA3-293T cells (60-70% confluent), after 15-20 hours incubation with 10  $\mu\text{g}/\text{ml}$  C3 exoenzyme, were rinsed with PBS and lysed on ice in buffer containing 1.5% Triton X-100, 0.8% DOC, 0.2% SDS, 145 mM NaCl, 20 mM Hepes, pH 7.4, 3 mM EGTA, 2 mM  $\text{MgCl}_2$ , 25  $\mu\text{g}/\text{ml}$  PMSF, 5  $\mu\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  aprotinin. Control cells were supplemented with an appropriate amount of C3 before washing and lysis. In vitro ADP-ribosylation was performed immediately at 30°C for 60 minutes on 50  $\mu\text{g}$  samples of total cell lysate by the addition of 200  $\mu\text{M}$  GTP, 10  $\mu\text{M}$   $\text{NAD}^+$ , 10  $\mu\text{g}/\text{ml}$  C3 exoenzyme and 0.5-2.5  $\mu\text{Ci}/\text{assay}$   $^{32}\text{P}$   $\text{NAD}^+$ . Reactions were terminated by the addition of sample buffer, boiled and analysed on SDS-PAGE, followed by autoradiography.

### Bacterial expression of EphA3 ID and protein expression library screen

EphA3 ID constructs were transformed into *Escherichia coli* BL21(DE3). After overnight induction at 16°C, cell pellets were sonicated in phosphate buffer pH 8.0, 300 mM NaCl, 10 mM imidazole, pH 8, 10 mM  $\beta$ -mercaptoethanol and protease inhibitors (EDTA-free 'Complete', Roche Biochemicals), cleared lysates applied to Ni-NTA Fast Flow resin (Qiagen) and bound protein eluted with 500 mM imidazole in the above buffer. Thioredoxin and His Tags were cleaved from the eluted protein in 10 mM Tris, 150 mM NaCl, 2mM  $\text{CaCl}_2$  with enterokinase (Novagen) for 8 hours. Impurities and thioredoxin/His tags were removed by re-application to Ni NTA, the EphA3 ID remaining in the unbound fraction. All experiments were performed with enterokinase cleaved EphA3 ID. A 16 day mouse embryo cDNA library in  $\lambda\text{Exlox}$  (Novagen) was used to identify potential signalling partners of EphA3. Approximately  $2 \times 10^5$  independent clones from the library were screened according to the manufacturer's instructions. Briefly, cDNA clones were immobilised on nitrocellulose filters (Osmonics), which were washed and blocked with PBST/1% BSA and incubated with 1  $\mu\text{g}/\text{ml}$  phosphorylated EphA3 ID in PBS/0.1% Triton X100/1mM  $\text{NaVO}_4$ . Clones that bound EphA3 ID were identified by incubation with 4G10 anti-phosphotyrosine (PY) antibody and HRP-conjugated anti-mouse antibody, visualised with ECL (Amersham Pharmacia Biotech). Positive clones were plaque purified and their cDNA inserts rescued by auto-subcloning in pExlox. The cDNA inserts were sequenced and the proteins identified using BLAST 2.0 (Altschul et al., 1997).

### Mapping of EphA3 derived phosphopeptides and phosphopeptide synthesis

EphA3 ID was dephosphorylated by the addition of TC45PTP linked to glutathione Sepharose overnight at 4°C. Phosphorylated and dephosphorylated EphA3 ID were purified by reverse phase HPLC and recovered proteins digested with TPCK-treated trypsin (Roche Biochemicals). Tryptic peptides were fractionated by C18 reverse phase HPLC (2.1x250 mm, Vydac) with a 90 minute gradient of 60%  $\text{CH}_3\text{CN}$  in 0.1% trifluoroacetic acid and individual peptides recovered manually according to their 214 and 280 nm absorbances. Tyrosine phosphopeptides, identified by their altered retention were analysed by MALDI/MS as described (Reid et al., 1998).

Tyrosine phosphorylated (P) peptides corresponding to Lys<sub>589</sub>-Glu<sub>611</sub> of the juxtamembrane domain and to Asp<sub>773</sub>-Ile<sub>788</sub> of the activation loop of EphA3 were synthesised by solid-phase chemistry, and purity (>98%) and integrity confirmed by electro-spray mass spectrometry (Meutermaans and Alewood, 1996):

Y596P: KLPGLR<sub>TY</sub>(PO<sub>4</sub>)VDPHTYEDPTQAVHE

Y602P: KLPGLR<sub>TY</sub>VDPHTY(PO<sub>4</sub>)EDPTQAVHE

Y596, 602P: KLPGLR<sub>TY</sub>(PO<sub>4</sub>)VDPHTY(PO<sub>4</sub>)EDPTQAVHE

Y779P: DDPEAA<sub>Y</sub>(PO<sub>4</sub>)TTRGGKIPI.

## Results

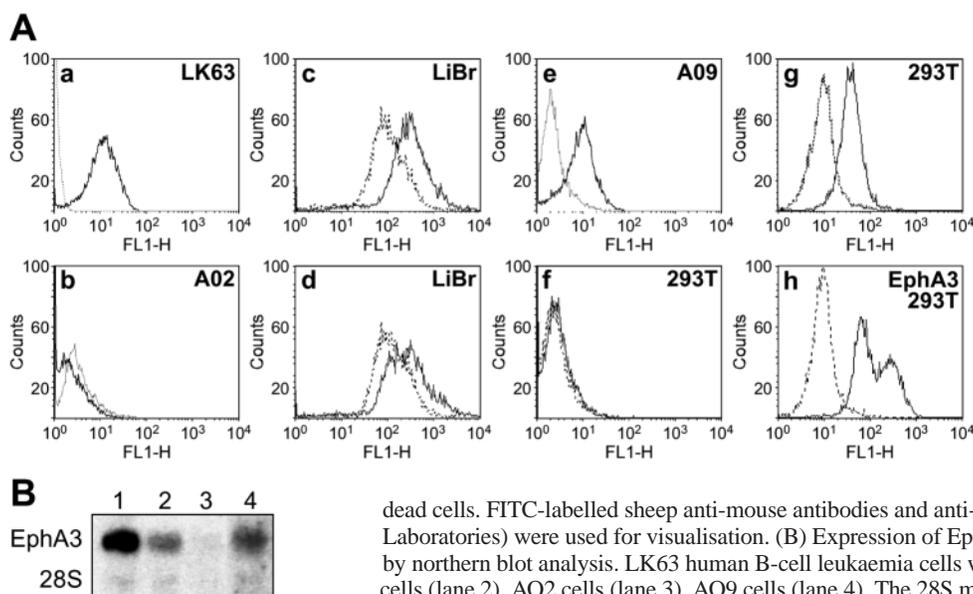
### Stimulation with ephrin-A5 causes retraction of cell processes and de-adhesion of EphA3-expressing melanoma cell lines

Tumour-specific EphA3 expression has been documented in human malignant melanoma and elevated EphA3 levels have been found in 72% of human melanoma lines (Chiari et al., 2000). By comparing the expression levels of EphA3 in several human melanoma cells we selected two recently isolated lines, AO2 and AO9 (M. D. and A. W. B., unpublished), and the previously established LiBr cell line (Coates and Crawford, 1977) for further examination. Using the anti-EphA3 Mab IIIA4 for flow cytometric analysis indicated significant EphA3 expression levels on LiBr and AO9 cells (Fig. 1A,c,e), whereas no immunoreactivity was detected on AO2 melanoma cells (Fig. 1A,b). Using a chimeric EphA3 Fc fusion protein for detection, we also observed significant expression of ephrin-A on LiBr cells (Fig. 1A,d), but not on AO2, AO9 (not shown) or 293T cells (Fig. 1A,f). Northern blot analysis confirmed matching levels of EphA3 mRNA in these cells (Fig. 1B). Before treatment with preclustered ephrin-A5 Fc, the majority of cells from all three lines formed flat, +/- spindle-shaped, adherent cell bodies (Fig. 2A) with extensive, dendritic processes containing actin stress fibres (Fig. 2B). Some rounded melanoma cells are routinely found in less-strongly adherent melanoma cell lines, including LiBr cultures. Within 10-30 minutes after stimulation with clustered ephrin-A5 we observed retraction of cell processes and cell rounding in previously elongated, spindle-shaped cells (Fig. 2A,B). These changes in cell morphology were accompanied by redistribution of polymerised actin into dense cortical actin rings (Fig. 2B). In AO9 cells, which were more spread-out and normally grow as tightly attached monolayer cultures (M. D. and S. K., unpublished), this effect was less pronounced (Fig. 2A). By 30 minutes only 65% of the starting LiBr population remained attached to the tissue culture surface, whereas AO9 cells were at least weakly adherent throughout the experiment (Fig. 2A,C). AO2 cultures, by comparison, showed no change in morphology throughout the experiment (Fig. 2A). To show that rounding and detachment were caused by ephrin-A5 Fc

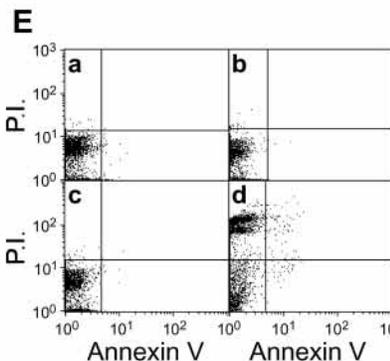
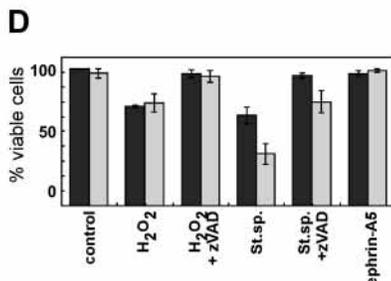
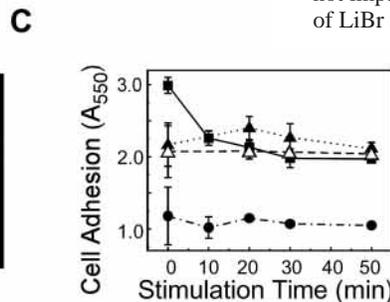
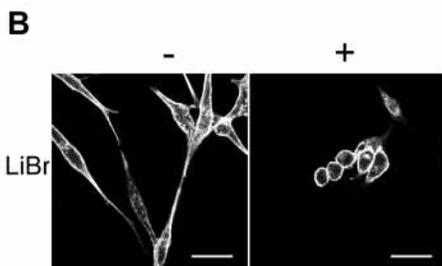
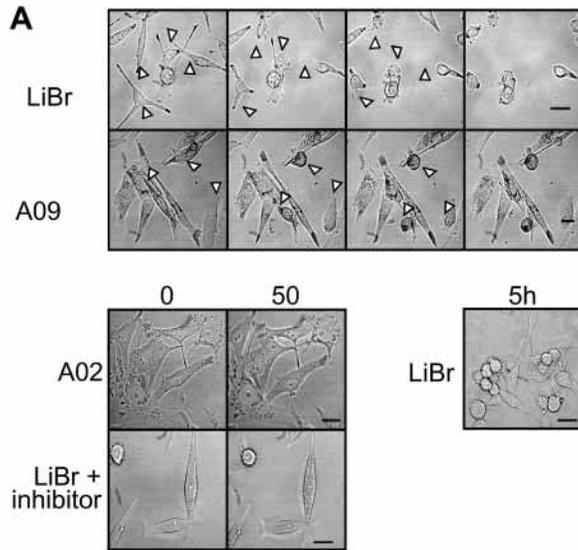
clustering of EphA3, LiBr cells were pre-incubated with soluble, monomeric ephrin-A5 to block available ligand binding sites. This pretreatment abrogated cell rounding and detachment, indicating that EphA3 clustering was required for the observed response (Fig. 2A,C). In addition to changes in cell shape and adhesion, extensive, dynamic membrane blebbing was observed within 10 minutes of ephrin-A5 challenge and persisted for at least 5 hours (Fig. 2A). Assessment of cell viability by vital dye exclusion and annexin binding to the plasma membrane showed that these changes to cell morphology, even after extended treatment with ephrin-A5, are not due to apoptosis (Fig. 2D,E).

### Ephrin-A5-mediated morphological changes during de-adhesion are differentially affected by mutation of tyrosines within the EphA3 juxtamembrane and activation loop domains

To examine the molecular mechanism of ephrin-A5-mediated cell-morphological changes in more detail, we expressed w/t or mutant EphA3 in 293T cells. Although we could not detect endogenous ephrin-A in these cells (Fig. 1A,f), they do contain some endogenous EphA3 (Fig. 1A,g) (Dottori et al., 1999), suggesting them as an appropriate model system for our studies. 293T cells do not display distinct actin stress fibres, but staining of permeabilised cells with rhodamine-phalloidin and an anti- $\alpha$ -tubulin antibody displays complex actin (Fig. 3A-C) and microtubule networks (Fig. 3E), with distinct fibre bundles extending into cell protrusions and lamellipodia. Following exposure to ephrin-A5, nontransfected 293T cells do not change their shape or gross morphology, and changes to the actin cytoskeleton are unremarkable (Fig. 3B). By contrast, treatment of w/t EphA3-transfected 293T cells with ephrin-A5 results in a dramatic contraction of the actin and microtubule cytoskeleton into dense fibre bundles surrounding cell nuclei (Fig. 3D,F). Parallel staining of permeabilised cells with an anti-phosphotyrosine Mab to outline focal adhesion complexes suggests that cell contraction is accompanied by loss of focal contacts and focal adhesions from the cell periphery (Fig. 3G,H). Furthermore, examination of vertical sections suggests that rounding and contraction of the cell body is accompanied by a



**Fig. 1.** Expression levels of EphA3 and ephrin-A on various melanoma cell lines and 293T cells. (A) LK63 human B-cell leukaemia cells, melanoma cells and 293T cells as indicated were analysed for cell-surface expression of EphA3 (a-c,e,g,h) with IIIA4 anti-EphA3 monoclonal antibody (solid lines) or an IgG1 isotype matched nonrelevant control antibody (broken lines), and of ephrin-A5 (d,f) with an EphA3-human Fc fusion protein (solid lines) or with EphA1-human Fc fusion protein (broken lines). Analysed cell populations were gated to exclude propidium iodide (PI)-positive dead cells. FITC-labelled sheep anti-mouse antibodies and anti-human Fc antibodies (both Jackson Laboratories) were used for visualisation. (B) Expression of EphA3 in melanoma cell lines was examined by northern blot analysis. LK63 human B-cell leukaemia cells were used as positive control (lane 1); LiBr cells (lane 2), AO2 cells (lane 3), AO9 cells (lane 4). The 28S mRNA is shown as loading control.



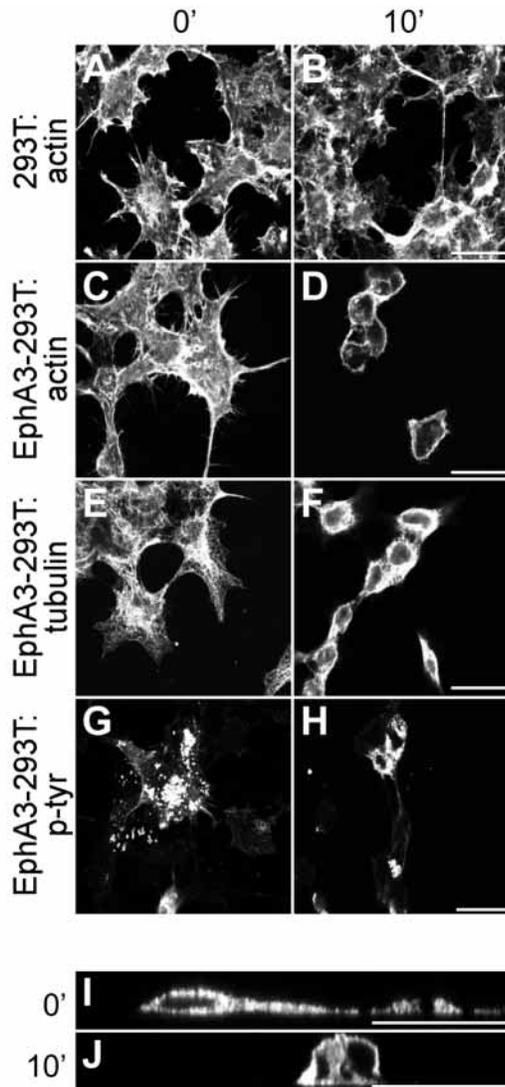
**Fig. 2.** Melanoma cell rounding, de-adhesion and membrane blebbing induced by clustered ephrin-A5 Fc is inhibited by soluble, monomeric ephrin-A5. (A) LiBr, AO9 and AO2 melanoma cells on fibronectin-coated glass coverslips were stimulated with 10 nM clustered ephrin-A5 Fc. Soluble, monomeric ephrin-A5 was added as inhibitor (+ inhibitor) to a parallel LiBr culture at 100-fold molar excess. Selected pictures of representative microscopic images taken during a 50 minute time course are shown. Arrowheads denote cells that retract their processes and round up during stimulation. Bar, 20  $\mu$ m. A supplemental video of the AO9 time course is available at: [http://www.ludwig.edu.au/addinfo/Figure2\\_AO9.mpg](http://www.ludwig.edu.au/addinfo/Figure2_AO9.mpg). (B) LiBr melanoma cells on fibronectin-coated glass coverslips before (-) or after (+) 10 minute ephrin-A5 stimulation were fixed, permeabilised and stained with rhodamine-phalloidin to visualise polymerised actin. Bar, 20  $\mu$ m. (C) Melanoma cells on fibronectin-coated 96-well plates were treated as described in (A) and at indicated times cells were fixed and stained with crystal violet. Values represent mean  $A_{550}$  absorbances from quadruplicate wells  $\pm$ s.d.: ■, LiBr cells; ▲, AO9 cells; △, LiBr cells with inhibitor; ●, AO2 cells. (D) Ephrin-A5 treatment does not impair cell viability in LiBr or EphA3 293T cells. Cultures of LiBr (grey bars) or EphA3 293T (black bars) cells in 24-well

plates were treated with preclustered human IgG (control) or ephrin-A5 for 2 hours at 37°C, detached by EDTA treatment and cell viability in quadruplicate samples determined by trypan blue exclusion assay. To induce apoptosis, cells in parallel wells were treated for 4 hours with 1  $\mu$ M staurosporin (St.sp.) or 0.5 mM H<sub>2</sub>O<sub>2</sub>. Control cells in parallel wells were treated for 30 minutes with the caspase inhibitor zVAD-fmk (0.1 mM) before addition of the apoptotic reagent. (E) Ephrin-A5 stimulation of LiBr cells does not result in increased numbers of apoptotic cells. LiBr monolayer cultures were treated for 2 hours at 37°C with (a), 1.5  $\mu$ g/ml or (b), 7.5  $\mu$ g/ml pre-clustered ephrin-A5 Fc; (c) 7.5  $\mu$ g/ml preclustered control IgG; and (d) 1  $\mu$ M staurosporin. Cells in the supernatant and detached by trypsin/versene treatment were combined, washed in Annexin V binding buffer and analysed for binding of Annexin V to the outer plasma membrane (apoptosis) and propidium iodide (PI) uptake (necrosis) by flow cytometry. Viable cells (lower left quadrant) are negative for Annexin V and PI, apoptotic cells (upper left quadrant) are positive for Annexin V, necrotic cells (upper right quadrant) are positive for Annexin V and PI.

substantial gain in cell height and is not due to a net loss in cell volume (Fig. 3I,J).

We addressed the dependence of these cellular effects on EphA3 kinase activity and tyrosine phosphorylation within the EphA3 cytoplasmic domain by examining the effects of phenylalanine mutants of the conserved juxtamembrane tyrosines, Y596 and Y602, and of Y779, which is located within the activation loop of the kinase. Immunoblot analysis of parallel cell lysates indicated that these three tyrosine residues correspond to the major EphA3 phosphorylation sites of the receptor and that w/t and mutant receptors were expressed at comparable levels in transfected cells (see below, Fig. 6E). Cotransfection with an enhanced green fluorescent actin fusion protein (EGFP-actin) enabled the examination, by time-lapse fluorescence microscopy, of cell morphology changes resulting from ephrin-A5 stimulation of w/t or mutant EphA3-transfected cells. Expression of EGFP-actin on its own had no effect on the

cell morphology or the expression levels of endogenous actin in transfected cells (Ballestrem et al., 1998). In agreement, our 293T cells did not change their shape or actin cytoskeleton upon ligand stimulation (Fig. 4B, EGFP-actin). In the absence of ephrin-A5 stimulation w/t EphA3-transfected cells were flat, and spread out on the fibronectin-coated glass slide (Fig. 4, w/t, 0 minutes). They started to retract their extensions and lamellipodia 5-10 minutes after ligand addition (Fig. 4, w/t, 10). Concurrently, dynamic blebbing of the cell membrane occurred with increasing intensity and by 50 minutes many of the cells revealed membrane blebs as indicated by globular, actin-containing membrane protrusions surrounding the cells (Fig. 4A, w/t, 50). At this time, transfected cells started to detach, and by 50 minutes more than 50% of cells within the selected, representative field had moved out of the focal plane. Again, this response was not due to apoptosis (Fig. 2D). Importantly, addition of soluble, monomeric ephrin-A5 blocks the response



**Fig. 3.** Ephrin-A5-induced cell rounding involves contraction of the actin and microtubule network and loss of peripheral focal contacts. Parental (A,B) or EphA3-293T cells (C-J) were grown on fibronectin-coated glass coverslips. Following stimulation with ephrin-A5 fixed and permeabilised cells were incubated with rhodamine-phalloidin (A-D), or primary antibodies against  $\alpha$ -tubulin (E,F) and phospho-tyrosine (G-J) and Alexa-labelled secondary antibodies to visualise microtubule and focal complexes, respectively. Images were taken with a confocal fluorescence microscope; images in I and J represent vertical sections of individual cells illustrated in C and D, respectively. Bar, 20  $\mu$ m.

to ephrin-A5 Fc and cells did not change their morphology throughout the time course of the experiment (Fig. 4B, w/t + Inhibitor).

Mutation of either of the juxtamembrane tyrosines, Y559 or Y602, retarded the onset of cell rounding to about 20 minutes after stimulation. Although pronounced membrane blebbing was still apparent in Y559 EphA3-expressing cells after 50 minutes (Fig. 4A, Y559F, 50), blebbing was abrogated in Y602 EphA3-expressing cells (Fig. 4B, Y602F, 50). Mutation of both tyrosines together, or of the EphA3 activation loop tyrosine, Y779, profoundly affected the cellular responses to ephrin-A5 (Fig.

4B), the cells showing no alterations to their shape or actin distribution throughout the experiment. Transfection of cells with EphA3 harbouring mutations in all three tyrosines (3 $\times$ YF) affected the cell morphology independent of ephrin-A5 stimulation (Fig. 4B, 3YF). The cells, which display thin, dendrite-like processes remained adherent and did not significantly change their morphology during the time course of the experiment.

#### Surface-bound Ephrin-A5 Fc impairs adhesion and spreading in 293T cells expressing wild-type but not mutant EphA3

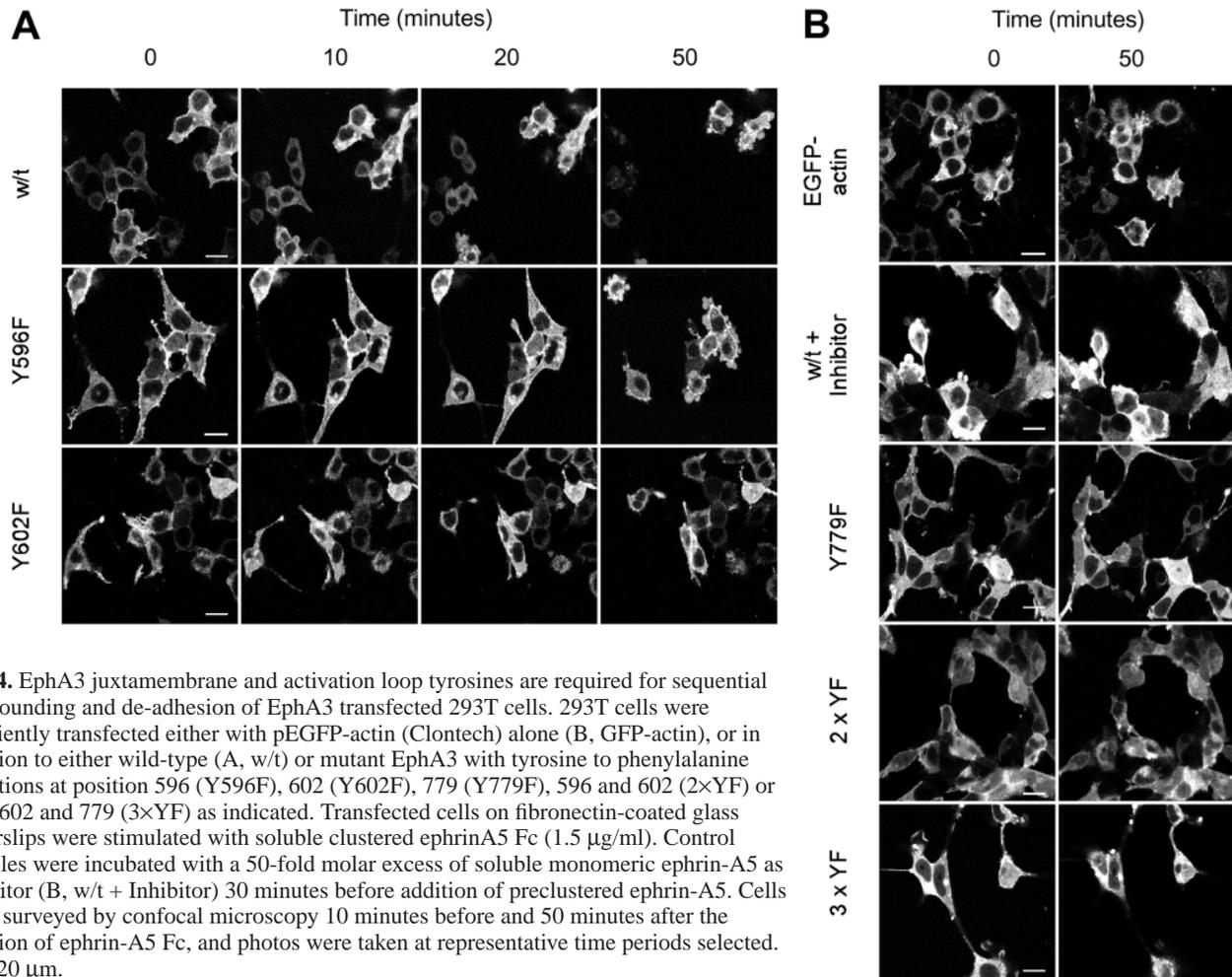
We sought to examine whether EphA3 activation by surface-bound ephrin-A5 would affect the re-adhesion of detached 293T cells. To mimic activation of EphA3 by its cell membrane-bound ligand in vitro, chimeric ephrin-A5 Fc was coupled to protein-A-coated 96-well plates via its C-terminal Fc domain. Biacore analysis of the nonbound fractions with an EphA3-coated sensor chip suggested a surface capacity of 0.2  $\mu$ g ephrin-A5 Fc/well. We also applied the soluble EphA3 extracellular domain to the ephrin-A5-coated surface, and by measuring the nonbound EphA3 in the supernatant confirmed that the immobilised ephrin-A5 is in a biologically active form, capable of interacting with the receptor (Fig. 5A). Although exposure of 293T cells, transfected with EGFP-actin, to ephrin-A5 had no measurable effect on adhesion, w/t EphA3-expressing cells remained round and failed to adhere to surfaces of increasing coating density of ephrin-A5 (Fig. 5B,C). The effect was noticeable even at the lowest ephrin-A5 density tested (8 pg/mm<sup>2</sup>). At a density of 0.18 ng/mm<sup>2</sup>, corresponding to an ephrin-A5 Fc concentration of 2 nM, only approximately 50% of the cells were able to attach.

Mutation to phenylalanine of single EphA3 juxtamembrane tyrosines Y596 or Y602 reduced the number of nonadherent cells. The effect of the Y602F mutation was more potent than the Y596 mutation, with more cells adhering to the ephrin-A5 surface. Mutation of both juxtamembrane tyrosines or the activation loop tyrosine Y779 or of all three tyrosines (Y596, Y602 and Y779) essentially ablated the response to ephrin-A5 surfaces and no significant effect of ephrin-A5 on 293T cell adhesion was observed (Fig. 5C). Thus, at an ephrin-A5 Fc surface density of 1.5 ng/mm<sup>2</sup>, only 30-40% of w/t EphA3-expressing cells attached to the wells, whereas 90-95% of the mutant Y779 EphA3 or Y596/Y602 EphA3-expressing cells were adherent at this ephrin-A5 concentration.

Taken together, these observations suggest that ephrin-A5-triggered mechanisms, causing cell detachment or repulsion, are dependent on EphA3 kinase activity and tyrosine phosphorylation, but are independent from prior engagement of cell adhesion mechanisms.

#### Identification of Crk as an interaction partner for activated EphA3

To identify cytosolic proteins downstream from activated EphA3, we screened a 16 day mouse embryo cDNA expression library using recombinant phosphorylated EphA3 intracellular domain (ID) as the probe. The soluble, tyrosine phosphorylated fusion protein was isolated from *E. coli* after overexpression of a cDNA encoding the intracellular portion of EphA3 fused to a cleavable thioredoxin and His tag. Following dephosphorylation



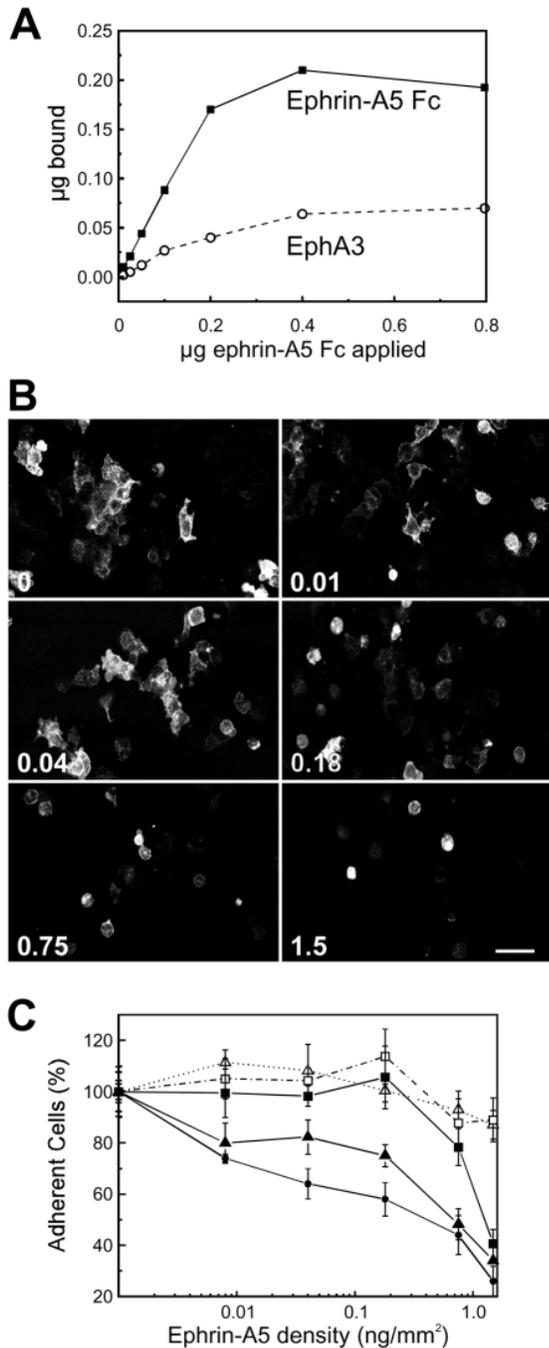
**Fig. 4.** EphA3 juxtamembrane and activation loop tyrosines are required for sequential cell rounding and de-adhesion of EphA3 transfected 293T cells. 293T cells were transiently transfected either with pEGFP-actin (Clontech) alone (B, GFP-actin), or in addition to either wild-type (A, w/t) or mutant EphA3 with tyrosine to phenylalanine mutations at position 596 (Y596F), 602 (Y602F), 779 (Y779F), 596 and 602 (2×YF) or 596, 602 and 779 (3×YF) as indicated. Transfected cells on fibronectin-coated glass coverslips were stimulated with soluble clustered ephrinA5 Fc (1.5  $\mu\text{g/ml}$ ). Control samples were incubated with a 50-fold molar excess of soluble monomeric ephrin-A5 as inhibitor (B, w/t + Inhibitor) 30 minutes before addition of preclustered ephrin-A5. Cells were surveyed by confocal microscopy 10 minutes before and 50 minutes after the addition of ephrin-A5 Fc, and photos were taken at representative time periods selected. Bar, 20  $\mu\text{m}$ .

with TC45 PTP (Tiganis et al., 1999), the purified EphA3 ID was able to rephosphorylate tyrosine residues upon the addition of ATP and  $\text{Mg}^{2+}$ , indicating that the kinase was functional (Fig. 6A). Analysis by mass spectrometry of tryptic peptides of the phosphorylated and rephosphorylated EphA3 ID unambiguously confirmed that the juxtamembrane tyrosines Y596, Y602 and the kinase activation loop tyrosine 779 were phosphorylated in the recombinant protein (M. L., unpublished data). Screening of approximately  $2 \times 10^5$  plaques yielded a single clone corresponding to the adapter protein CrkII (Matsuda et al., 1992).

#### Ephrin-A5-induced recruitment of Crk in 293T cells depends on EphA3 kinase activity

In lysates from LiBr melanoma cells and from EphA3-transfected 293T cells, Crk is associated with tyrosine-phosphorylated EphA3 (Fig. 6B-F). Immunoprecipitation of whole-cell lysates suggested that LiBr melanoma cells contain discernible levels of endogenous Crk and EphA3 (Fig. 6B, bottom panels). Endogenous EphA3 was apparent in anti-Crk immunoprecipitates from ephrin-A5-stimulated LiBr cells, but was undetectable in lysates of nonstimulated cells (Fig. 6C, top panel). Furthermore, exposure of EphA3-expressing 293T cells to increasing concentrations of surface-bound ephrin-A5 resulted in association of CrkII with the receptor, readily

noticeable at surface concentrations above 0.18  $\text{ng/mm}^2$ , equivalent to 2 nM ephrin-A5. By comparison, a slightly reduced level of EphA3 was detected in anti-Crk immunoprecipitates of cells that had been stimulated with a comparable concentration (10 nM) of preclustered ephrin-A5 Fc in solution (Fig. 6C). The association of EphA3 with Crk was apparent in stably transfected 293T cells within 1 minute of ephrin-A5 Fc addition and increased to maximal levels 30 minutes after stimulation (Fig. 6D). In agreement with previous studies indicating ligand-independent autophosphorylation of EphA3 at high receptor density (Lackmann et al., 1998), anti-PY western blots of anti-EphA3 immunoprecipitates from transiently transfected 293T cells revealed substantial receptor autophosphorylation in the absence of ligand (Fig. 6E, middle panel). Phenylalanine mutation of the EphA3 tyrosine Y779 (activation loop) reduced the phosphotyrosine level, whereas mutation of the juxtamembrane tyrosines Y596 and Y602 had little effect. Mutation of both (2×YF) or of all three tyrosines (3×YF) reduced the anti-PY signal to background levels (Fig. 6E, middle panel). Western blot analysis of anti-Crk immunoprecipitates from 293T cell lysates suggested association of EphA3 with CrkII in ephrin-A5-stimulated, EphA3-transfected cells. Interestingly, a comparable amount of EphA3 coprecipitated in Y596 and Y602 mutant EphA3-expressing cells was, in both cases, at higher levels than in



nonstimulated cells (Fig. 6E). By contrast, Crk association with the receptor was not evident in samples from Y779F, 2×YF or 3×YF mutant EphA3-expressing cells. The sequence motifs encompassing the EphA3 juxtamembrane tyrosines accommodate potential YXXP Crk SH2 domain docking sites (Birge et al., 1993). We further examined whether these motifs are involved in CrkII binding: A Sepharose-tethered 2×Y phosphopeptide, but not the nonphosphorylated counterpart, was able to bind CrkII from EphA3-transfected 293T lysates (Fig. 6F, left panel). However, no significant effect was observed when phosphopeptides corresponding to the juxtamembrane or activation loop sequences were added to CrkII immunoprecipitation reactions (Fig. 6F, right panel).

**Fig. 5.** Dose-dependent inhibition of cell adhesion and spreading by surface-bound ephrin-A5. (A) Capacity of Protein A-coated 96-well plates for Ephrin-A5 Fc (■) and of Protein A-bound ephrin-A5 for EphA3 (○). Ephrin-A5 Fc was applied at indicated concentrations and bound protein in the supernatant was estimated by Biacore analysis. To confirm the competence of tethered ephrin-A5 to interact with the receptor, the nonbound fraction of EphA3 (2 µg/ml) after incubation on ephrin-A5-coated wells was determined by Biacore analysis. The amount of bound EphA3 estimated from this assay is shown (○). (B) 293T cells, transiently transfected with w/t or mutant EphA3, and pEGFP-actin were plated ( $5 \times 10^4$  cells/well) onto wells coated with ephrin-A5-Fc at the indicated densities (ng/mm<sup>2</sup>). After 5 hours, adherent cells were fixed with 4% paraformaldehyde and examined by fluorescence microscopy. Sections of w/t EphA3-transfected cells are shown. Bar, 20 µm. (C) Following microscopy, adherent cells were quantified using crystal violet staining. Cell attachment is expressed as a percentage (mean±s.d. from three independent assays) relative to adhesion seen on non-ephrin-A5-coated wells; ●, w/t EphA3; ▲, Y596F EphA3; ■, Y602F EphA3; □, Y 779F EphA3; △, Y596F+Y602F EphA3.

#### A dominant-negative Crk SH3 domain mutant abrogates ephrin-A5-mediated cell rounding and actin cytoskeletal changes

To assess the functional relevance of ephrin-A5-induced recruitment of Crk to EphA3, we monitored actin-cytoskeletal changes in EphA3-overexpressing 293T cells, which were transfected with either w/t Crk, a dominant-negative Crk SH3 domain mutant (SH3\* Crk), or both expression constructs. Overexpression of w/t Crk induces membrane spreading and membrane ruffling (Dolfi et al., 1998) by recruitment of DOCK180 to the Crk/p130<sup>CAS</sup> complex (Kiyokawa et al., 1998), whereas SH3 domain mutated Crk causes abrogation of membrane ruffling and spreading (Nakashima et al., 1999). In agreement, untreated w/t Crk-transfected EphA3-293T cells reveal extensive spreading and ruffling, whereas SH3\* Crk-transfected cells lack any signs of ruffling (Fig. 7, -) and display distinct, dendrite-like actin-rich cell processes. Treatment with preclustered ephrin-A5 Fc leads to rounding, contraction of the actin cytoskeleton and loss of membrane ruffles at comparable levels (Fig. 7, +) in w/t-Crk overexpressing and control cells. By contrast, SH3\*Crk-expressing cells appear not to respond to ephrin-A5 treatment and do not change their morphology during the experiment. Cotransfection of SH3\* Crk together with w/t CrkII rescues the SH3\* phenotype, and membrane ruffling and spreading of the cells resembles that of w/t CrkII-transfected cells. Importantly, these cells regain their reactivity to ephrin-A5 stimulation and respond with pronounced cell rounding and contraction of the actin cytoskeleton. Staining of transiently transfected cells with anti-myc Mab confirms the expression of myc-tagged Crk constructs and suggests that, following ephrin-A5 stimulation, Crk colocalises with the cortical actin ring. By contrast, nonstimulated (not shown) or SH3\* Crk-transfected cells display diffuse cytoplasmic anti-myc (Crk) staining (Fig. 7, +, anti-myc).

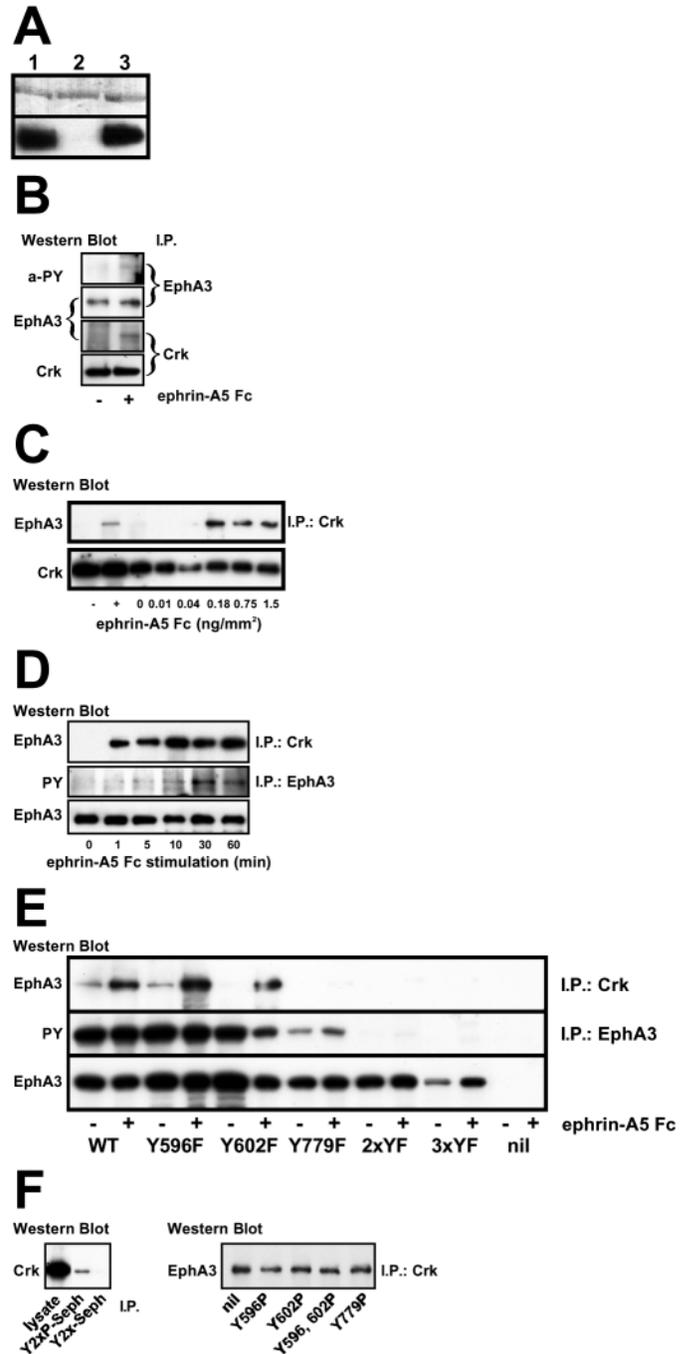
#### Modulation of Rho activity following EphA3 activation relies on a functional Crk SH3 domain

The distinctive effects of ephrin stimulation on the cytoskeleton prompted us to examine the involvement of RhoA as a downstream effector molecule. We examined cell lysates

**Fig. 6.** Crk rapidly binds the phosphorylated EphA3 ID and is able to distinguish between ligand-independent and ligand-mediated receptor phosphorylation. (A) Coomassie-stained SDS PAGE (top) and anti-PY western blot (bottom) illustrating the phosphorylated EphA3 ID as purified from the *E. coli* (1), dephosphorylated by TC45Pase (2) and rephosphorylated by the addition of ATP and Mg<sup>2+</sup> (3). (B) Ephrin-A5-dependent association of Crk with endogenous EphA3 in LiBr melanoma cells. Adherent cultures of LiBr cells were treated for 10 minutes with preclustered ephrin-A5 Fc (+) or left untreated (-). Endogenous Crk in cell lysates was immunoprecipitated with anti-CrkII antibody and western blots probed with the same antibody to detect total CrkII or with anti-EphA3 antibodies to detect CrkII-associated EphA3. Parallel samples were immunoprecipitated with anti-EphA3 Mab and western blots probed for EphA3 and phosphotyrosine as indicated. (C) Dose-response of ephrin-A5 stimulated CrkII recruitment to EphA3. EphA3-transfected 293T cells were transferred to tissue culture wells containing surface-bound ephrin-A5 Fc at the indicated concentrations for 10 minutes, and endogenous CrkII in the cell lysates was immunoprecipitated with anti-Crk antibody. In parallel samples, the cells left in suspension were left untreated (-) or stimulated with 10 nM preclustered ephrin-A5 in solution (+). Blots of whole-cell lysates and of precipitated proteins were probed with anti-EphA3 and anti-CrkII antibodies as indicated. (D) Time course of CrkII binding to EphA3. EphA3 was (co) immunoprecipitated from lysates of EphA3 293T cells, which had been treated for the indicated times with 1.5 µg/ml of preclustered ephrin-A5 Fc, and analysed by western blot with anti-EphA3 and anti-phosphotyrosine antibodies as indicated. (E) 293T cells were left nontransfected (nil) or were transiently transfected with cDNAs encoding single, double (2x) or triple (3x) Tyr (Y)-Phe(F) mutants of EphA3. Anti-EphA3 immunoprecipitates from lysates of cells stimulated for 10 minutes with preclustered ephrin-A5 Fc (+) or left untreated (-), were analysed by western blot probed with anti-PY antibody. As loading control the total lysate was analysed by anti EphA3 western blot. Endogenously expressed Crk was immunoprecipitated from the same lysates and analysed by immunoblot for associated EphA3. (F) Crk was extracted from lysates of Crk-transfected 293T cells using Sepharose-coupled phosphopeptide Y596, 602P (Y2xP-Seph) or the nonphosphorylated peptide as control (Y2x-Seph) and analysed by western blot using the anti-Crk monoclonal antibody (left panel). The CrkII band in the crude cell lysate is shown in a parallel lane. Lysates from ephrin-A5 Fc stimulated cells were immunoprecipitated with anti-Crk antibody in the absence (nil) or presence of 50 µM of EphA3-derived phosphopeptides as indicated in the figure (right panel). Immunoprecipitates were analysed by western blots probed with antibodies to EphA3.

of parental or EphA3 293T cells for levels of activated Rho, precipitated with the recombinant RhoA binding domain (RBD) of rhotekin (Ren et al., 1999). Ephrin-A5 Fc stimulation consistently resulted in a transient increase in RBD-bound, active Rho to maximal levels within 5 minutes (Fig. 8A). No significant changes in the level of RBD-bound Rho were observed in the parental 293T cells (Fig. 8B). Activation in suspended EphA3 293T cells occurred with very similar kinetics, although peak levels of active Rho were observed slightly earlier than in adherent cells, 3 minutes after stimulation (Fig. 8C). The rise in active Rho followed a decline to basal levels 10 minutes after ephrin-A5 addition. Importantly, the rise in active Rho was not seen in ephrin-A5-stimulated EphA3 293T cells expressing dominant-negative SH3\* Crk (Fig. 8E), whereas expression of w/t Crk did not affect the kinetics of Rho activation (Fig. 8D).

To assess the involvement of RhoA functionally, we treated EphA3-293T cells either with recombinant *Clostridium*

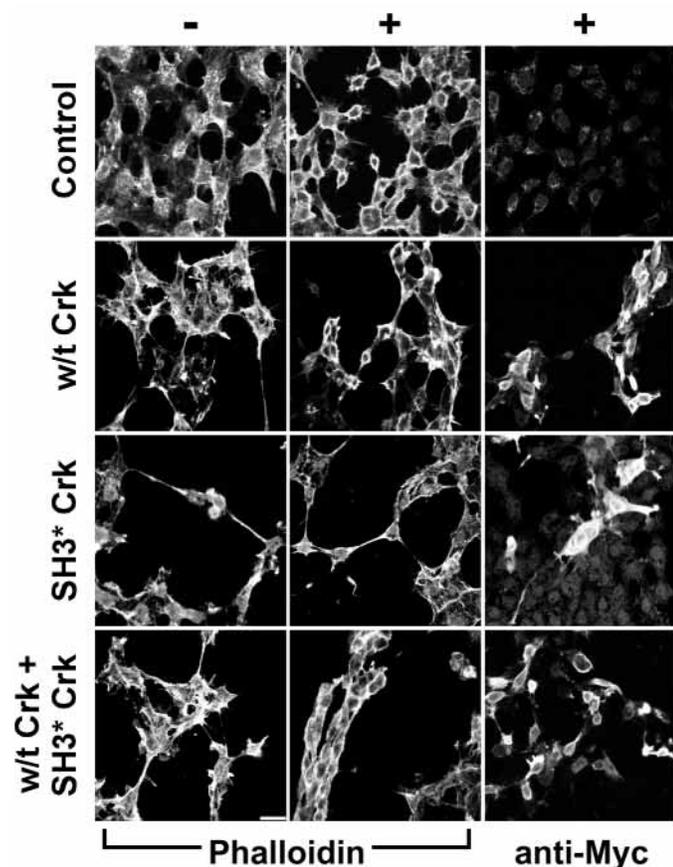


*botulinum* C3 toxin (Fig. 9A-E), which ribosylates and specifically inhibits Rho activity (Leng et al., 1998), or with Y-27632 (Fig. 9F), a specific Rho kinase inhibitor (Uehata et al., 1997), previously shown to inhibit ROCK-dependent membrane blebbing (Coleman et al., 2001). We adjusted the concentration of C3 to produce modest actin cytoskeletal changes without causing EphA3-293T cell rounding (Fig. 9C). Exposure of cells to this concentration of C3 reduced the level of active Rho significantly, as indicated by 70% reduced levels of Rho available for radioactive ADP ribosylation (Fig. 9E). C3 treatment under these conditions completely abrogated ephrin-A5-mediated cell rounding (Fig. 9D), thus supporting the involvement of Rho in this response. To examine the role

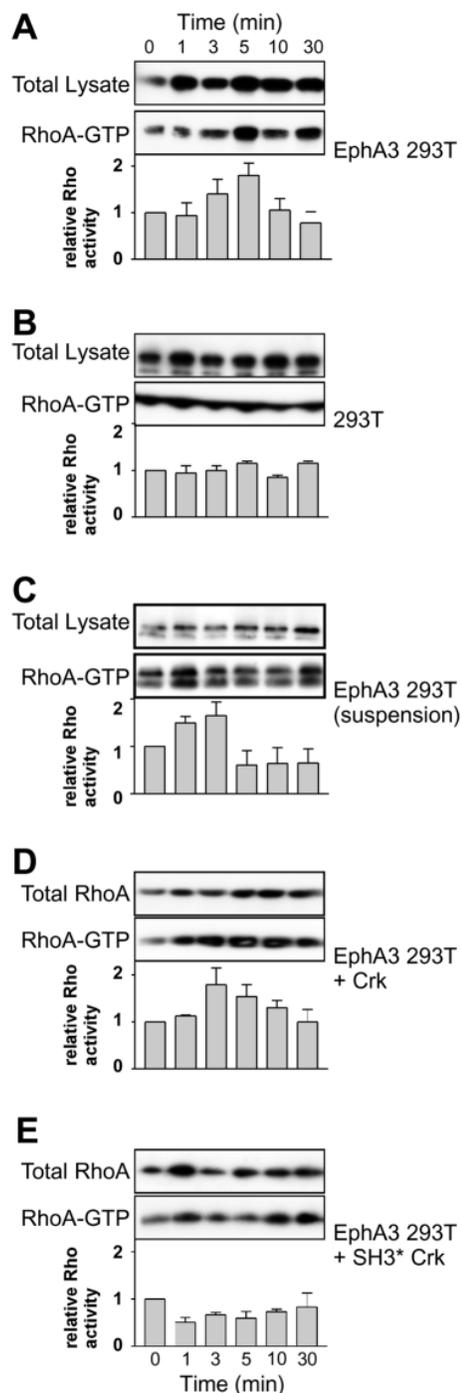
of ROCK, a downstream effector of active RhoA (Watanabe et al., 1999), we exploited time-lapse microscopy to monitor ephrin-A5-induced membrane blebbing in parental or EphA3-293T cells that were treated with the ROCK inhibitor Y-27632. Treatment of EphA3 293T cells with the Rho kinase inhibitor Y-27632 also completely abrogated ephrin-A5-induced membrane blebbing (Fig. 9F). Interestingly, this inhibition of ROCK activity had little effect on cell rounding (see <http://www.ludwig.edu.au/addinfo/Figure9Finhib.mpg>). For comparison we treated EphA3 293T cells with LY 294002, to inhibit phosphoinositide-3-kinase, which is involved in signalling pathways that effect cytoskeletal changes (Hall, 1998; Rodriguez-Viciana et al., 1997), and recently shown to modulate integrin activity downstream of EphA8 (Gu and Park, 2001). Although LY 294002 treatment inhibited ephrin-triggered membrane blebbing, the effect was less pronounced than inhibition by Y-27632 (Fig. 9F).

## Discussion

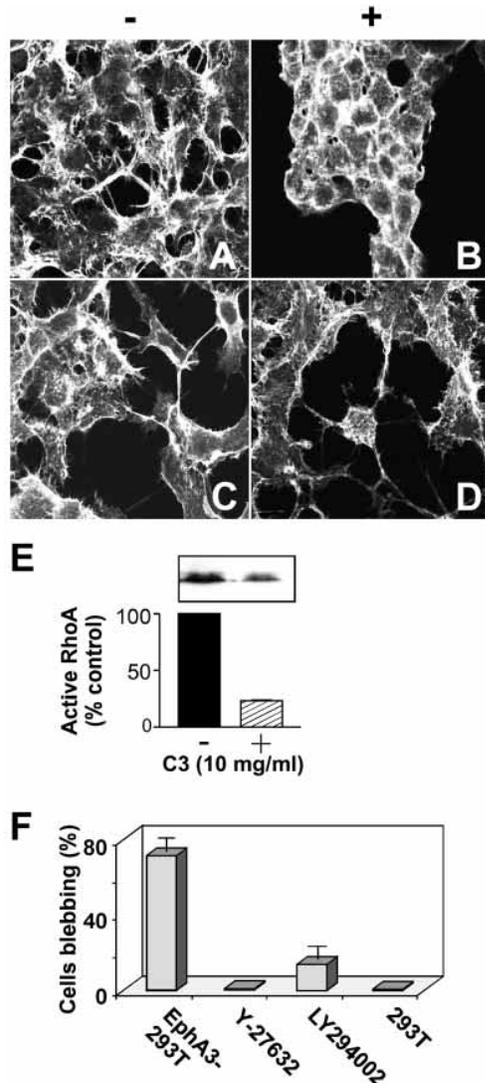
Eph or ephrin overexpression in tumours such as malignant melanoma (Chiari et al., 2000; Easty et al., 1995) could



**Fig. 7.** Dominant-negative SH3\* CrkII abrogates ephrin-A5-mediated cytoskeletal changes. EphA3 293T cells (control) were transiently transfected with myc-tagged, w/t CrkII, dominant-negative SH3\* CrkII or both w/t and SH3\* CrkII as indicated. Parallel cultures of stimulated (+) or nonstimulated (-) cells were fixed in 4% PFA. Cells were stained with rhodamine-phalloidin (Phalloidin) or anti-Myc antibody (anti-Myc) to monitor the actin cytoskeleton or CrkII derived proteins, respectively.



**Fig. 8.** Ephrin-A5 stimulation of EphA3-expressing 293T cells mediates a rapid increase of active Rho A. The levels of active RhoA in parental (B) or EphA3-293T cells (A,C-E) transfected with w/t Crk (D) or SH3\* Crk (E) as indicated, and which had been treated as adherent (A,B,D) or suspension (C) cultures with preclustered ephrin-A5, were analysed at the indicated times by precipitating GTP-bound RhoA with GST-RBD. Precipitated proteins and aliquots of the total lysates were analysed by immunoblotting with an anti-RhoA Mab. The intensity of bands representing active RhoA (RhoA-GTP) was normalised by comparison with Rho levels in corresponding total lysates using densitometry, and is indicated relative to baseline levels (given the arbitrary value '1') in the absence of ephrin-A5 stimulation. Each result represents the mean and s.d. from a minimum of three independent experiments.



**Fig. 9.** Inhibition of Rho and ROCK activities abrogate ephrin-A5-mediated cell rounding and membrane blebbing. EphA3 293T cells (A-D) were treated with 10  $\mu$ M C3 transferase exoenzyme overnight (C,D) and control cells left untreated (A,B). Parallel cultures of stimulated (+) or nonstimulated (-) cells were fixed in 4% PFA and stained with rhodamin-phalloidin. (E) Treatment with C3 transferase reduces RhoA available for ribosylation. Lysates from EphA3 293T cells with (+) or without (-) overnight C3 transferase treatment were used for ADP ribosylation assays in the presence of radioactive  $^{32}$ P-NAD. RhoA-associated radioactivity was quantified after SDS-PAGE and autoradiography (top) by densitometry. Mean and s.d. from two independent experiments are shown (bottom). (F) Cell blebbing in EphA3-293T cells, left untreated or after pretreatment with 10  $\mu$ M ROCK inhibitor Y-27632 or 10  $\mu$ M PI3 kinase inhibitor LY 294002 (2 hours), was monitored by time-lapse microscopy. As control, blebbing in parental nontransfected 293T cells is shown. Representative fields containing approximately 100 cells were monitored for 10 minutes before and 50 minutes after stimulation. Total and blebbing cells were counted by an observer blinded to experimental conditions. Values represent mean percentages and s.d. (three independent experiments) of cells that commenced blebbing following addition of ephrin-A5. Supplemental videos of time courses illustrating responses of EphA3-293T cells without or with Y-27632 treatment are available at: <http://www.ludwig.edu.au/addinfo/Figure9F.mpg> and at: <http://www.ludwig.edu.au/addinfo/Figure9Finhib.mpg>, respectively.

indicate relevance in tumour progression and metastasis (Dodelet and Pasquale, 2000; Easty et al., 1999; Vogt et al., 1998). Melanomas derive from neural crest-derived skin melanocytes, and although Eph signalling is crucial for neural crest migration (Krull, 1998; Wang and Anderson, 1997), Eph receptors are not expressed in mature melanocytes (Chiari et al., 2000; Easty et al., 1999). The notion that unscheduled expression of Eph RTKs and ephrins in melanomas may contribute to the malignant phenotype encouraged us to study responses of EphA3 expressing and nonexpressing melanoma cells to ephrin-A5 stimulation.

It is well established that signals from within the cells and cross-talk with RTKs, including Eph receptors (Schmucker, 2001), modulate the activity and avidity of adhesion receptors and thus the adhesive properties of cultured cells (Gumbiner, 1996). We show that, in EphA3-expressing adherent melanoma cell lines and in EphA3-overexpressing 293T cells, ephrin-A5-induced receptor activation promotes sequential retraction of cell processes, cell rounding, membrane blebbing and detachment. Functional and biochemical analysis of the underlying mechanisms reveals that these morphological changes are mediated through recruitment of CrkII to ligand-activated EphA3 and a concurrent, transient activation of RhoA. A rapid condensation of the actin cytoskeleton and microtubule network and a concurrent loss or re-distribution, or both, of focal contacts from the cell periphery (Fig. 3D-J) suggests that, like other Eph-like RTKs (Miao et al., 2000; Schmucker, 2001), ephrin-A5-activated EphA3 modulates focal adhesion signalling pathways. Although these cellular responses are reminiscent of the morphological events occurring during apoptosis (Jacobson et al., 1997), our experiments show that activation of EphA3 does not affect cell viability (Fig. 2D). In contrast to apoptotic blebs, which are static and often detach from the cell surface, ephrin-A5 triggers highly dynamic, spherical membrane protrusions that do not lead to cell fragmentation. Membrane blebbing is observed frequently in living cells during mitosis, cell spreading and migration and results from increases in the intracellular pressure and extensive changes to the actin cytoskeleton (Hagmann et al., 1999). Several recent reports suggest that increased contractility of the actomyosin network, due to increased phosphorylation of myosin light chains by myosin light chain kinase (Mills et al., 1998) and ROCK (Coleman et al., 2001), are essential in both apoptotic and nonapoptotic blebbing (Hagmann et al., 1999).

Tissue culture surfaces decorated with ephrin-A5 at defined densities allowed us to examine how activation of EphA3 effects the adhesion of detached cells. At 0.18 ng ephrin-A5/mm<sup>2</sup> (~2 nM), 50% of the cells remained in suspension (Fig. 5A,B). This concentration agrees well with the low nanomolar affinity of the ephrin-A5/EphA3 interaction (Lackmann et al., 1997), showing that these surfaces provide a physiologically relevant model of EphA3 stimulation. With decreasing ephrin-A5 density, w/t cells were able to adhere and gradually spread out (Fig. 5B). Importantly, Hattori et al. showed recently that the high-affinity ephrin-A2/EphA3 interaction triggers a specific ADAM metalloprotease-catalysed ephrin-cleavage at sites of cell-cell contact, which allows cell repulsion to occur (Hattori et al., 2000). Increased adhesion of several cell lines to ephrin-B Fc surfaces, prepared by passive adsorption onto nitrocellulose, has been linked to

EphB1-mediated integrin activation (Becker et al., 2000; Stein et al., 1998a), but this may result from mechanical tethering through EphB/ephrinB interactions (Miao et al., 2000). Some of the apparent differences in these reports may also relate to the different ephrin-coupling strategies yielding different densities of functional ephrin.

Two conserved tyrosines in the juxtamembrane region of Eph receptors reportedly function as SH2-domain docking sites for known signalling molecules including Fyn (Ellis et al., 1996), Src (Zisch et al., 2000), RasGAP, (Holland et al., 1997), SHEP-1 (Dodelet et al., 1999) and Nck (Stein et al., 1998b), but little is known about the functional consequences of these interactions. Together with the activation-loop tyrosine, these residues also function to regulate kinase activity, and their phosphorylation is required for full enzyme activity (Binns et al., 2000; Zisch et al., 2000). In agreement, we assigned these tyrosines (EphA3 Y596, Y602 and Y779) as the prominent autophosphorylation sites of EphA3 (M. L., unpublished). Exposure of EphA3-expressing w/t or mutant cells (Y596F, Y602F, Y779F, 2×YF and 3×YF) to either surface-tethered or to clustered ephrin-A5 in solution confirmed that the cellular response to EphA3 activation is critically dependent on their phosphorylation and a resultant functional EphA3 kinase (Figs 3, 4; Fig. 5C; Fig. 6E). Phenylalanine substitutions of individual juxtamembrane tyrosines reduced ephrin-A5 mediated repulsion (Fig. 5C) and delayed cell rounding, whereas mutation of the activation-loop tyrosine, of both juxtamembrane tyrosines, or of all three tyrosines together ablated ephrin-A5-induced cell rounding and repulsion and reduced the kinase activity to background.

Analysis of the signalling events following EphA3 activation resulted in two key observations: a dose-dependent recruitment of Crk to ligand-stimulated EphA3 and a rapid modulation of Rho activity. We initially identified Crk as a binding partner for phosphorylated EphA3, by probing a cDNA expression library with recombinant EphA3 ID. We confirmed this interaction by coprecipitation of EphA3 together with endogenous Crk in immunoprecipitates from ephrin-A5-stimulated LiBr melanoma cells (Fig. 6B), and from ligand-stimulated EphA3 293T cells (Fig. 6C-F). Binding of the recombinant Crk SH2 domain to the EphB3 juxtamembrane PY motif containing two consecutive 'YXXP' Crk SH2 domain binding sites has been reported (Hock et al., 1998). In agreement, we extracted CrkII from 293T cell lysates using a phosphopeptide comprising the corresponding EphA3 sequence, and site-directed mutagenesis of both of these consensus Crk binding sites together ablated the EphA3/Crk interaction. Interestingly, addition of excess juxtamembrane phosphopeptide to the immunoprecipitation reaction did not significantly reduce the amount of coprecipitated EphA3. We suggest two alternative explanations for these observations. Either the inability to disrupt the CrkII/EphA3 interaction by peptide competition indicates that stable CrkII recruitment occurs indirectly via an as yet unidentified component. This would imply that the interaction observed during our expression cloning strategy and the CrkII 'pull down' with Sepharose-tethered 2×Y phosphopeptide are due to the fortuitous use of the CrkII consensus sequence. Alternatively, CrkII binding via an SH2 interaction may require a defined configuration of ligand-induced EphA3 clusters, which cannot be disrupted by a short consensus phosphopeptide. Importantly, in EphA3-transfected 293T cells, CrkII binds prominently only to the ligand activated, and not to the autophosphorylated receptor (Fig. 6B-E). Furthermore, the

increase of EphA3-associated CrkII during ephrin-A5 stimulation closely parallels the cellular response monitored by time-lapse microscopy (Fig. 4; Fig. 6D). Perhaps in a cellular context CrkII is recruited to an EphA3 docking site that is functional only at a critical density or configuration of the EphA3 aggregate, which, in turn is determined by the concentration or density of preclustered or membrane-anchored ephrin-A5. This notion is in agreement with an earlier report showing that the composition of EphB1 signalling complexes and resulting cellular responses depend on the oligomeric state of the interacting ephrin-B1 (Stein et al., 1998a). Importantly, our experiments show that recruitment of Crk to EphA3 is accompanied by a concurrent, transient increase in active RhoA. Both events are essential in ephrin-A5-induced rounding and detachment of EphA3-bearing cells and are not reliant on cell adhesion. An increasing body of evidence implicates CrkII and its association with p130<sup>CAS</sup> in signal-transduction processes that regulate cytoskeletal rearrangements during cell adhesion and migration (Arai et al., 1999; Cary et al., 1998; Klemke et al., 1998). In support of our observations, a role for the Crk/p130<sup>CAS</sup> complex in regulating cell spreading was reported (Vuori et al., 1996) and Crk-induced membrane ruffling, cell spreading and Jun N-terminal kinase activation is blocked by a dominant-negative form of Rac. This suggests a linear pathway, where recruitment/activation of Rho-family GTPases to the Crk/p130<sup>CAS</sup> complex leads to JNK activation (Dolfi et al., 1998). Increasing evidence implies Eph receptor signalling upstream of this pathway, including EphB2, which modulates the activity of Erk2 and JNK1 (Zisch et al., 2000), and EphB1, which recruits a complex containing Nck, p62<sup>dock</sup> and RasGAP and mediates JNK activation (Holland et al., 1997; Stein et al., 1998c). The guanine nucleotide exchange factor (GEF) ephexin, which has GEF activity for Rho, Rac and Cdc42, and in retinal ganglion cells is constitutively associated with EphA4, provides further evidence for communication from Eph RTKs via RhoA to the cytoskeleton (Shamah et al., 2001). Our observation, that overexpression of SH3-domain mutated, dominant-negative CrkII affects ephrin-A5 induced RhoA activation, could indicate that CrkII acts upstream of Rho as a molecular link to effect EphA3-induced contraction of the cytoskeleton. Supporting this notion, it was shown recently that activation of Rho and Rho kinase is responsible for ephrin-A5-induced growth cone collapse (Wahl et al., 2000).

Adhesion and de-adhesion of cells to the substratum is regulated by a tightly controlled interchange of signals from several extracellular and intracellular origins, and their deregulation or uncoupling has been correlated with tumorigenesis and metastasis (Giancotti, 1997). Although an essential role of Eph RTKs in modulating actin dynamics, in particular in growing axons, is now well recognised (Schmucker, 2001), our observations indicate that activated EphA3 triggers CrkII- and Rho-mediated cytoskeletal changes and detachment, but not apoptosis. As the unscheduled expression of EphA3 and ephrins correlate with melanoma progression, we speculate that this response to EphA3 activation may be one of the initiating events leading to melanoma metastasis. It is noteworthy that, although ephrin-A5-induced detachment was not observed in EphA3-negative melanoma cells, the intensity of the response varied between EphA3-positive, strongly adherent and less strongly adherent melanoma cell lines. Interestingly, some of the less adherent LiBr cells were routinely rounded before stimulation with

exogenous ephrin, indicating potential autocrine EphA3 activation by endogenous ephrins. In support of this notion we found comparable cell-surface expression levels of EphA3 and ephrin-A on these cells.

Both CrkII and RhoA have central roles in the regulation of the dynamic actin signalling network. Reagents that modulate GTPase activities and affect the formation of CrkII signalling complexes induce the disassembly of focal adhesions and loss of cell substratum contact (Klemke et al., 1998; Escalante et al., 2000; Ren et al., 1999). Consistent with this concept, results presented in this study imply that recruitment of Crk to the EphA3 receptor and transient activation of Rho facilitate ephrin-A5-induced cell rounding, membrane blebbing and de-adhesion. CrkII and RhoA are candidates for mediating the communication between the ligand-activated EphA3 receptor and the actin cytoskeleton.

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