The EMBO Journal Vol.18 No.8 pp.2165–2173, 1999

Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins

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Receptors of the Eph family and their ligands (ephrins) mediate developmental vascular assembly and direct axonal guidance. Migrating cell processes identify appropriate targets within migratory fields based on topographically displayed ephrin gradients. Here, EphB1 regulated cell attachment by discriminating the density at which ephrin-B1 was displayed on a reconstituted surface. EphB1–ephrin-B1 engagement did not promote cell attachment through mechanical tethering, but did activate integrin-mediated attachment. In endothelial cells, attachment to RGD peptides or fibrinogen was mediated through $\alpha_v\beta_3$ integrin. EphB1 transfection conferred ephrin-B1-responsive activation of $\alpha_5\beta_1$ integrin-mediated cell attachment in human embryonic kidney cells. Activation-competent but signaling-defective EphB1 point mutants failed to stimulate ephrin-B1 dependent attachment. These findings lead us to propose that EphB1 functions as a 'ligand density sensor' to signal integrin-mediated cell–matrix attachment.

Keywords: EphB1/integrins/ligand density discrimination

Introduction

Eph receptors and their ligands have been implicated in developmental patterning events, including assembly of the vasculature (Wang *et al.*, 1998), retinotectal axonal targeting (Cheng *et al.*, 1995), and developmental segmentation of embryonic tissues into boundary zones defined by reciprocal spatial gradients of ligands and their receptors (Gale *et al.*, 1996). The EphB class receptors engage their type I transmembrane ephrin-B class ligands (including ephrins B1–3) through juxtacrine cell–cell contact (Bohme *et al.*, 1996) to mediate changes in cell migration (Drescher *et al.*, 1995; Wang and Anderson, 1997) and attachment (Stein *et al.*, 1998b). EphB–ephrin interactions are critical for vascular development, as ephrin-B2 gene deletion causes developmental vascular malformations and embryonic lethality (Wang *et al.*, 1998).

Ephrins, including ephrin-B subclass ligands, are displayed in developmental gradients along two different axes on cell surfaces of the tectum, where discrimination of ligand density guides migrating retinal axons to their correct destinations. Focal overexpression of ephrin-A2 within the tectal migratory field miscues retinal axons to inappropriate destinations (Nakamoto *et al.*, 1996), presumably as a receptor-mediated response to discontinuity in the 'addresses' provided by spatial ligand gradients, as suggested by Sperry (1963). If ephrin density is a primary signal to direct cell–cell recognition, or to modulate attractive and repulsive responses in migrating axons, receptor responses within a migrating process should be capable of discriminating the ligand density on membranes they encounter. To date, a receptor coupling mechanism capable of transmitting such discriminatory signals through surface receptors has not been defined, either biochemically or functionally. Explicitly, how does a specific complement of receptors expressed on a migrating cell first distinguish, and secondly alter, downstream responses based on the density of ligand displayed in a spatial context?

Recent evidence also suggests that ephrin-B oligomerization may be actively regulated. Ephrin-Bs share conserved cytoplasmic domain sequences that include protein–protein interactive domains. These include C-terminal PDZ recognition motifs (Songyang *et al.*, 1997) and conserved tyrosine residues within phosphorylated domains (Holland *et al.*, 1996; Bruckner *et al.*, 1997). EphB1 signaling complexes recruit different component proteins and direct distinct cell attachment and assembly responses depending upon the oligomeric state of the ephrin-B1 they engage (Stein *et al.*, 1998b). In aggregate, these findings indicate that Eph-mediated cell responses may be critically determined by the spatial array or 'density' of ephrins displayed on cell surfaces engaged by contact.

To address these questions, we plated endothelial and transfected HEK293 cells on coated surfaces that were reconstituted to display defined densities of ephrin-B1. Marked differences in cell attachment were observed, depending upon the ephrin-B1 density and the presence of defined extracellular matrix (ECM) components that were shown to interact with specific cellular integrins. The findings support a model in which EphB1 discriminates ephrin-B1 surface density to direct integrin-mediated cell attachment. Moreover, the assay provides new opportunities to define components of juxtacrine receptor activation and response.

Results

Defined surface densities of ephrin-B1 activate cell attachment through EphB1

To reconstitute surfaces displaying biologically active ephrin-B1, we coated plastic culture dishes with nitrocellulose, then adsorbed the ephrin-B1 ectodomain fusion protein, ephrin-B1/Fc (Beckmann *et al.*, 1994), in the

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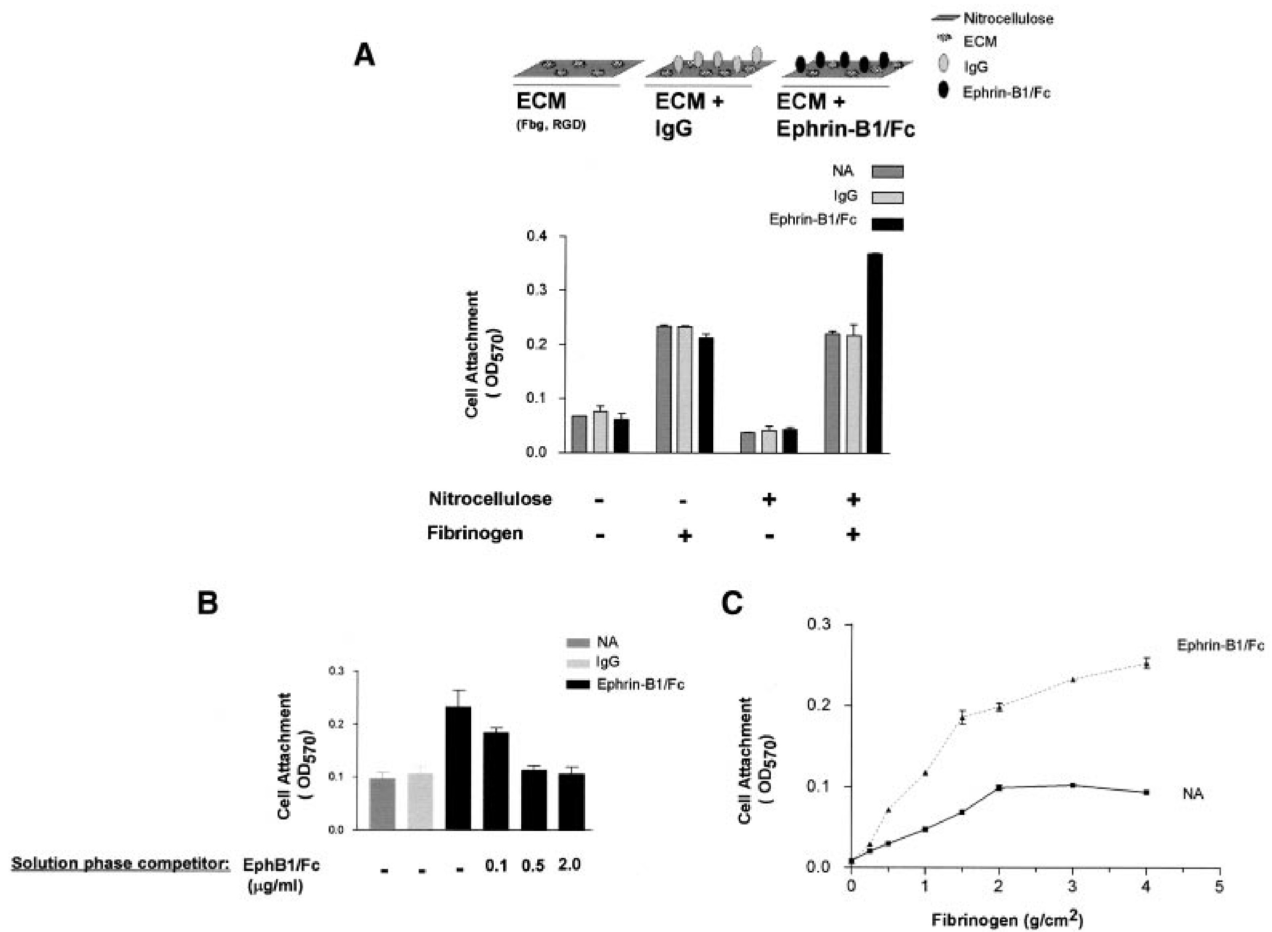


Fig. 1. Solid-phase presentation of ephrin-B1/Fc. (**A**) Nitrocellulose-bound ephrin-B1/Fc promotes microvascular endothelial cell attachment to fibrinogen. HRMEC cultured as described in Materials and methods were serum-deprived in Optimem. Forty-eight-well plates were untreated or precoated with nitrocellulose, in the presence or absence of fibrinogen (2×10^{-6} g/cm^2); designated amounts of no addition (NA), an Fc fusion control (IgG, 3×10^{-7} g/cm^2), or ephrin-B1/Fc (3×10^{-7} g/cm^2) were adsorbed overnight at 4°C. Wells were washed, blocked with medium containing 1% bovine serum albumin (BSA) and 1×10^5 HRMEC were plated for 1 h, then attached cells were determined as described. (**B**) EphB1/Fc blocks endothelial cell response to solid phase-displayed ephrin-B1/Fc. HRMEC attachment to surfaces coated with fibrinogen (2×10^{-6} g/cm^2) and ephrin-B1/Fc (3×10^{-7} g/cm^2) was assessed in the absence or presence of increasing concentrations of a competitive EphB1 ectodomain antagonist, EphB1/Fc. The IC_{50} of EphB1/Fc is 0.25 μg/ml. (**C**) Fibrinogen density effects on endothelial cell attachment. Indicated amounts of fibrinogen were adsorbed to surfaces coated with no addition (NA) or ephrin-B1/Fc (3×10^{-7} g/cm^2) and endothelial cell attachment was assayed after 60 min.

absence or presence of defined ECM proteins. Human renal microvascular endothelial cells (HRMEC) expressing endogenous EphB1 (Stein *et al.*, 1998b) adhered poorly to plastic or nitrocellulose-coated plastic in serum-free medium, but attached effectively to fibrinogen-coated surfaces (Figure 1A). Adsorbed ephrin-B1/Fc promoted endothelial attachment, but only when bound to nitrocellulose in the presence of provisional ECM components, such as fibrinogen or vitronectin (not shown). Ephrin-B1-stimulated differences were not observed on surfaces supplemented with either collagen I or laminin (not shown). A human IgG1, class-matched for the Fc domain used in the ephrin-B1/Fc fusion protein, had no effect on cell attachment at coating densities between 1×10^{-7} and 1×10^{-6} g/cm^2 (data are shown for 3×10^{-7} g/cm^2 only). Consistent with responses mediated through endothelial EphB receptors, addition of soluble EphB1/Fc coincident with plating abrogated the cell attachment stimulated by ephrin-B1/Fc, with an IC_{50} of ~2 nM (0.25 μg/ml) (Figure 1B).

The requirement for defined ECM proteins suggested that integrins participate in the ephrin-B1-induced cell attachment. In light of evidence that ECM surface density is a critical determinant of cell migration (Palecek *et al.*, 1997), we compared cell attachment to surfaces coated with different densities of fibrinogen, in the absence or presence of ephrin-B1. Cell attachment was maximal at fibrinogen surface densities of 2×10^{-6} g/cm^2 and higher, when surfaces were coated with fibrinogen alone (Figure 1C, solid line). Coincident coating of surfaces with ephrin-B1/Fc, at a constant density (3×10^{-7} g/cm^2), enhanced attachment across fibrinogen surface densities approaching saturation at 3×10^{-6} g/cm^2.

Motivated by the spatial gradients of ephrins displayed in developing tissues (Cheng *et al.*, 1995), we explored whether the surface density of ephrin-B1 affected cell attachment. A biphasic cell attachment response was identified as increasing surface densities of ephrin-B1/Fc were displayed (Figure 2A). Maximal ephrin-B1-induced cell attachment was promoted at ~3×10^{-7} g/cm^2 ephrin-B1/Fc, corresponding to 1.2×10^4 molecules/μm^2. A prominent decline in cell attachment was evident at higher densities, despite evidence that EphB1 was similarly activated (tyrosine phosphorylated) by engaging ephrin-B1 at the

higher surface densities (Figure 2A, lower panel, left). This biphasic response suggested that adsorbed ephrin-B1/Fc does not simply promote cell attachment through a receptor tethering mechanism, but rather that receptors may signal different responses based on their discrimination of ephrin-B1 surface densities. A similar biphasic effect of increasing ephrin-B1/Fc densities upon cell attachment was observed in murine teratocarcinoma-derived P19 cells, a neuronal progenitor line that expresses endogenous EphB1 receptors (Figure 2B, right panel) (Stein *et al.*, 1998b).

Recent data from our laboratory showed that different EphB1 signaling complexes assemble in response to soluble forms of ephrin-B1/Fc presented as a dimeric or a tetrameric ligand (Stein *et al.*, 1998b). This finding suggested that pre-clustering ephrin-B1/Fc could shift the surface density at which attachment was promoted. As shown in Figure 2B, pre-clustered ephrin-B1/Fc reduced the density of ephrin-B1/Fc required to stimulate attachment and reduced the density at which maximal attachment was seen. This finding, seen in both endothelial and P19 cells, suggests that spatial constraints are placed on receptor oligomerization by the intermolecular distance between surface tethered ephrin-B1 molecules, and thereby direct alternative downstream responses.

Ephrin-B1-signaled attachment is mediated through $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins

To determine whether this ephrin-B1 surface density effect was mediated through integrins, a defined peptide, GRGDSPK, was substituted for fibrinogen and applied to surfaces in the absence and presence of ephrin-B1/Fc (Figure 2C). This peptide was sufficient to support the ephrin-B1-induced increase in endothelial cell attachment, and this attachment was sensitive to competition by solution-phase competitors, including an RGD peptide or an antibody that blocks RGD engagement by $\alpha_v\beta_3$ integrin (LM609), but not $\alpha_v\beta_5$ or $\alpha_5\beta_1$ integrins. The $\alpha_v\beta_3$ integrin-mediated attachment occurs without any ephrin-B1-induced changes in the surface expression of $\alpha_v\beta_3$ integrin, as determined by cell surface protein biotinylation and immunoprecipitation with LM609 (Figure 2C, upper panel). The ephrin-B1/Fc-stimulated attachment of P19 cells was similarly sensitive to RGD peptide (data not shown), but the specific integrin mediating this P19 attachment response was not yet defined, as specific blocking antibodies against murine integrins are not available.

The functional link between integrin-mediated cell attachment and cell migration led us to evaluate whether the ephrin-B1/Fc oligomeric state, in a soluble complex, impacted endothelial cell migratory responses. It is important to note that this is a contrived situation using soluble forms of the normally membrane-bound ephrin-B1. We were particularly interested in whether ephrin-B1/Fc oligomeric forms exceeding an optimum would impose a biphasic effect on cell migration. Figure 3 shows the rates at which endothelial migration closed circular 'wounds' of ~400 μm diameter. The state of ephrin-B1/Fc oligomerization in the soluble complexes was varied by increasing the concentrations of anti-Fc used to pre-cluster the complexes. Consistent with the effect of ligand density to alter signaling responses mediated through EphB1 receptors, a biphasic migration response was identified. The maximal ephrin-B1-induced migration rate was attained when a defined oligomeric form of ephrin-B1 (which we independently confirmed to be a tetramer) was achieved; higher order ephrin-B1 oligomers did not promote either endothelial migration (shown here) or attachment (Stein *et al.*, 1998b). Efforts to assign this ephrin-B1-stimulated migration response to $\alpha_v\beta_3$ integrin were confounded by effects of the blocking antibody in reducing basal (unstimulated) endothelial migration.

To address whether EphB1 signaling was required for the attachment responses evoked by ephrin-B1, we identified an EphB1 null cell line. Transfection of HEK293 cells with EphB1 conferred increased attachment responses to surfaces coated with ephrin-B1 (Figure 4A). The increased attachment was evident within 30 min after plating and persisted for up to 6 h. Given transfection efficiency approaching 50%, the findings suggest that virtually all the transfected cells acquire increased avidity for the fibrinogen-coated surfaces based on EphB1 expression. As observed with the endothelial cells, the ephrin-B1-induced attachment was biphasic with optimal attachment at ephrin-B1 surface densities of $3–6\times10^{-7}$ g/cm^2 (Figure 4B). In contrast to the endothelial cell findings, competition experiments showed that this EphB1-mediated attachment was mediated by $\alpha_5\beta_1$ integrin, a dominant integrin expressed in HEK293 cells (Figure 4C). Integrin $\alpha_5\beta_1$, a prominent fibronectin receptor, has been shown recently to mediate fibrinogen binding as well (Suehiro *et al.*, 1997). As in the endothelial cell experiments, ephrin-B1 engagement did not alter surface expression of $\alpha_5\beta_1$ (Figure 4C, upper panel left).

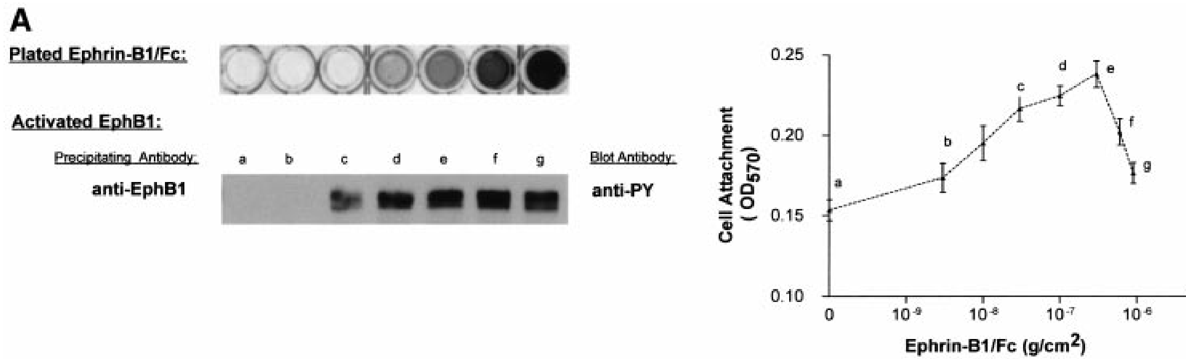
Signaling-competent EphB1 is required for coupled attachment responses

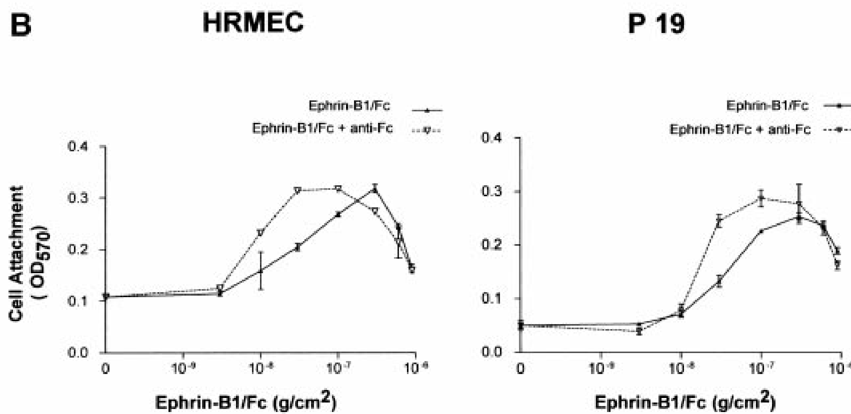
In recent reports, we demonstrated that activated EphB1 recruits Nck (Lehmann *et al.*, 1990) and the low mol. wt protein tyrosine phosphatase LMW-PTP (Zhang *et al.*, 1995) into precipitable receptor complexes through involvement of specific tyrosine residues, Y594 and Y929, respectively (Stein *et al.*, 1998a,b). In those experiments, point mutation of either tyrosine residue to phenylalanine abrogated cell attachment to fibronectin-coated surfaces, suggesting collaborative roles for receptor interactions with Nck and LMW-PTP. Here, transfection of kinase inactive (K652R), or point mutant (Y594F or Y929F) EphB1 failed to confer ephrin-B1-responsive increases in cell attachment (Figure 5). Each mutant EphB1 protein, including Y594F and Y929F point mutants, was expressed at levels similar to wild-type receptors, and each was activated similarly upon engagement of the surface-bound ephrin-B1, yet each mutant failed to alter $\alpha_5\beta_1$ integrin-mediated attachment. Thus, signaling-competent EphB1 is required to mediate the ephrin-B1-induced attachment responses. Furthermore, the capacity for EphB1 to recruit both Nck and LMW-PTP appears to be necessary. A change in integrin function other than cell surface abundance is required to mediate the response. The ephrin-B1-induced increase in cell attachment suggests 'inside-out' activation of $\alpha_5\beta_1$ integrin in HEK293 and $\alpha_v\beta_3$ integrin in endothelial cells.

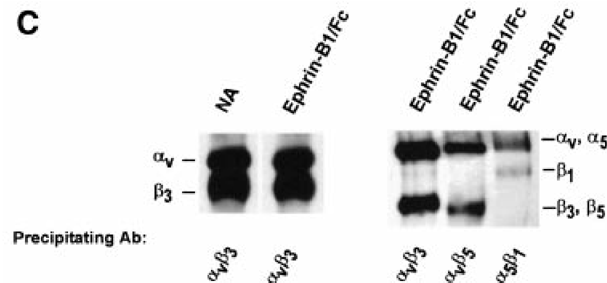
Discussion

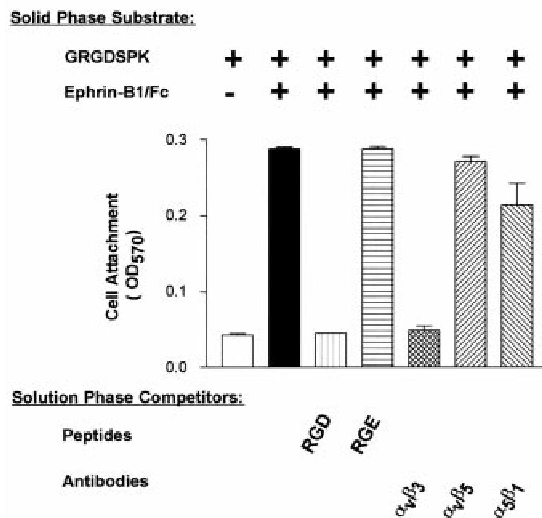
These findings demonstrate that EphB1 receptors discriminate the surface density of displayed ephrin-B1 to signal

changes in cell–matrix attachment. The integrins that participate in this response differ in the two cell systems tested. In endothelial cells, $\alpha_v\beta_3$ mediates the increased attachment, while $\alpha_5\beta_1$ responses predominate in transfected HEK293 cells. This EphB1-coupled cell attachment is remarkably restricted by the surface density at which ephrin-B1 is displayed in this reconstituted system. In particular, the coupled response does not correlate with









the capacity of ephrin-B1 engagement to stimulate receptor tyrosine phosphorylation, as an index of activation (Figure 2A). At ligand densities exceeding 3×10^{-7} g/cm^2, corresponding to ~1.2×10^4 molecules/μm^2, EphB1 was tyrosine phosphorylated, yet integrin-mediated attachment declined progressively toward the unstimulated baseline. This finding demonstrates the capacity of an Eph class receptor to signal alternative cell attachment responses based on its discrimination of the density of surface-displayed ephrin. Moreover, the results suggest a model that may describe aspects of cell targeting.

In this Eph/ephrin receptor/ligand system, spatial gradients provide positional information that directs neural targeting, in settings such as retino-tectal development (Cheng *et al.*, 1995; Nakamoto *et al.*, 1996). Based on our findings, we propose that the oligomerization of EphB1 is determined by the defined surface density of ephrin-B1, here spatially constrained by adsorption to nitrocellulose. Previous data have shown that the stoichiometry of Eph–ephrin interactions is 1:1 and that binding of solution phase ligand is high affinity (nM) (Beckmann *et al.*, 1994; Labrador *et al.*, 1997). Recent evidence shows that EphB1 signaling-complex composition is importantly determined by the oligomeric composition of solution phase ephrin-B1 (Stein *et al.*, 1998b). Here, the downstream coupled response, namely to activate integrin-mediated attachment, is critically dependent upon the surface density of ephrin-B1. If a strict correlation exists between the density of ephrin-B1 displayed and the oligomerization of EphB1 engaged, alternative signals may be importantly defined by the composition of the signaling complex recruited to EphB1.

Among EphB1 signaling complex constituents that we and others have identified, Nck and LMW-PTP appear critical for coupling receptor activation to cell attachment responses. Shown here, point mutants of EphB1 that are defective in recruiting either Nck or LMW-PTP are activated by engagement of surface-displayed ephrin-B1, but are not capable of mediating increased cell attachment responses (Figure 5). We previously showed that Nck is recruited to EphB1 following activation by either ephrin-B1 dimers or pre-clustered multimers (Stein *et al.*, 1998a) and Nck participation has been further implicated by its association with p62^{dok} and RasGAP (Holland *et al.*, 1997). Our findings suggest that Nck recruitment and signaling events are necessary, but insufficient, to link EphB1 activation with the coupled integrin response defined here. Recent findings implicate Nck interactions with the serine/threonine kinase NIK in coupled responses (E.Becker and U.Huynh-Do, unpublished results).

In contrast, LMW-PTP recruitment into EphB1 complexes is critically dependent upon EphB1 oligomerization promoted by binding to tetrameric ephrin-B1 (Stein *et al.*, 1998b). The EphB1–LMW-PTP interaction involves the C-terminal domain of EphB1, comprising a sterile alpha motif (SAM) domain (Ponting, 1995). The structure for an isolated recombinant EphB2 SAM domain has recently been solved, demonstrating tandem head-to-head/tail-to-tail repeats that impose ordered oligomeric structure on this domain (Thanos *et al.*, 1999). Based on the effect of the Y929F substitution of uncoupling the EphB1 activation of integrin-mediated signaling, we suggest that stoichiometric engagement of specific densities of displayed ephrin-B1 may promote the assembly of specific oligomers within the EphB1 SAM domain to define the signaling intermediaries recruited. At present, data favor tetrameric

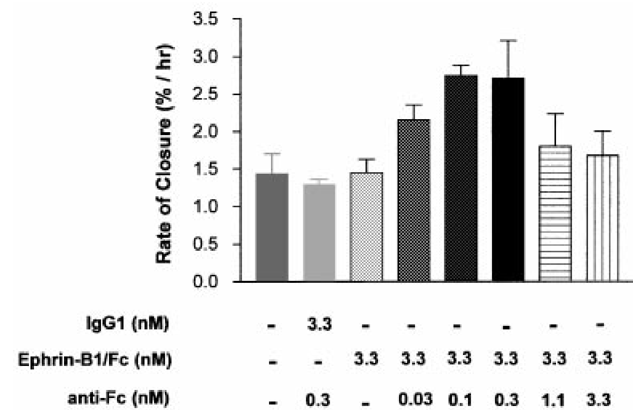


Fig. 3. Oligomeric state of soluble ephrin-B1 complex defines endothelial cell migration responses. Confluent HRMEC were serum depleted prior to mechanical 'wounding' to create circular defects of 400–500 μm (Materials and methods). Soluble ephrin-B1 complexes were generated by pre-incubation of a fixed amount of ephrin-B1/Fc with variable amounts of anti-Fc (133-fold final concentration) on ice for 60 min. Medium was then supplemented with no addition (NA), pre-clustered IgG control or pre-clustered ephrin-B1/Fc as indicated. Closure rates were calculated by linear regression of residual wound areas plotted over time. The maximal ephrin-B1-induced migration rate was attained with complexes formed at molar ratios of 30:1–10:1.

Fig. 2. Defined densities of surface-adsorbed ephrin-B1/Fc stimulate $\alpha_v\beta_3$ integrin-mediated endothelial cell attachment. (**A**) Ephrin-B1/Fc surface densities determine cell attachment within the range evoking receptor activation. Adsorption of ephrin-B1/Fc to coated surfaces was linear and non-saturating below densities of 1×10^{-6} g/cm^2, as determined by extraction and immunoblot analysis (not shown) and by a colorimetric *in situ* assay using biotin-conjugated ephrin-B1/Fc and streptavidin–horseradish peroxidase (HRP) (upper panel, left). In the lower panel (left), HRMEC plated on surfaces coated with ephrin-B1/Fc (at the indicated densities) were immunoprecipitated using EphB1 polyclonal antibodies and receptor activation was assayed by phosphotyrosine immunoblot. In the lower panel, cell attachment to the surfaces displaying the indicated densities of adsorbed ephrin-B1/Fc was assayed. On surfaces displaying greater than 3×10^{-7} g/cm^2 of ephrin-B1 cell attachment declined progressively toward the unstimulated baseline. (**B**) Pre-clustered ephrin-B1/Fc promotes receptor-mediated attachment at reduced surface densities. Pre-incubation of ephrin-B1/Fc with anti-Fc at defined molar ratios generates tetrameric complexes (Stein *et al.*, 1998b). Indicated densities of ephrin-B1/Fc dimers (ephrin-B1/Fc) or tetramers (ephrin-B1/Fc + anti-Fc) were adsorbed to fibrinogen-coated surfaces and endothelial cell attachment assayed (left panel). Similar results were obtained with the murine P19 cell line (right panel). (**C**) Ephrin-B1/Fc-promoted endothelial cell attachment is mediated through $\alpha_v\beta_3$ integrin. Upper panel: HRMEC were plated for 60 min at 37°C onto nitrocellulose-coated 60 mm dishes pre-adsorbed with fibrinogen (2×10^{-6} g/cm^2) in the absence or presence of ephrin-B1/Fc (3×10^{-7} g/cm^2), then cell surface proteins were biotinylated for 30 min at 4°C using 0.5 mg/ml Sulfo-NHS-LC-Biotin. Cells were harvested and integrins immunoprecipitated using the indicated antibodies. Labeled integrins were detected by streptavidin–HRP and enhanced chemiluminescence. Endothelial surface $\alpha_v\beta_3$ integrin abundance was unaltered by cell attachment to ephrin-B1/Fc-coated surfaces (left panel), and $\alpha_v\beta_3$ integrin was the dominant integrin in HRMEC. Lower panel: an integrin-interactive peptide (GRGDSPK, 1×10^{-5} g/cm^2) was adsorbed to nitrocellulose-coated plates in the absence or presence of ephrin-B1/Fc (3×10^{-7} g/cm^2), as indicated. Endothelial cells were plated in the absence or presence of the indicated peptide (100 μM) or antibody (5 μg/ml) competitors, including GRGDTP (RGD), GRGESP (RGE), anti-$\alpha_v\beta_3$ (LM609), anti-$\alpha_v\beta_5$ (P1F6) and anti-$\alpha_5\beta_1$ (JBS5). Similar results were obtained using fibrinogen (not shown).

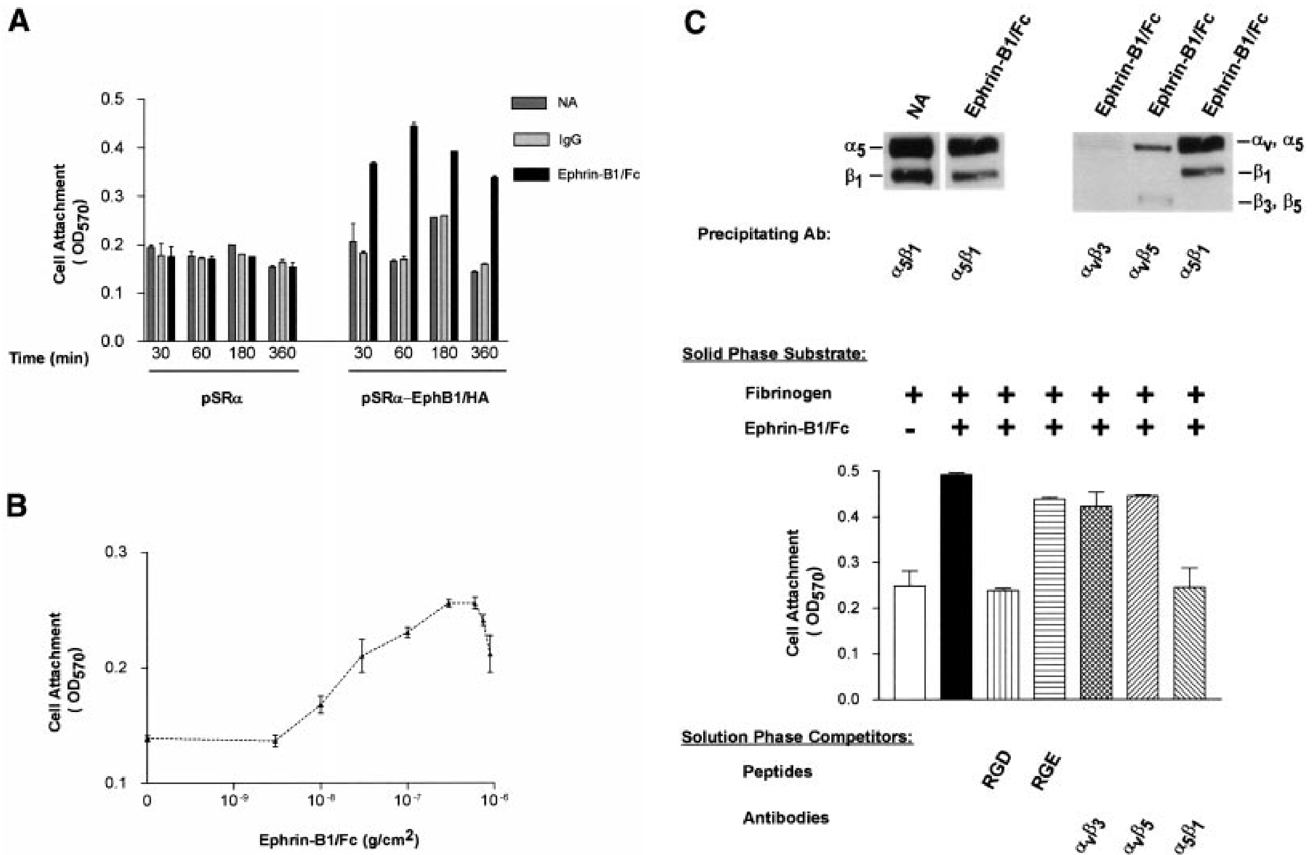


Fig. 4. EphB1 confers an ephrin-B1-dependent increase in HEK293 cell adhesion to fibrinogen. (**A**) Time course of HEK293 cell attachment. HEK293 were transiently transfected with vector alone (pSRα) or pSRα-EphB1/HA using LipofectAMINE Plus. After 48 h, cells were replated on surfaces coated with fibrinogen (2×10^{-6} g/cm^2) supplemented with no addition (NA), an Fc fusion control (IgG) or ephrin-B1/Fc (both at 3×10^{-7} g/cm^2). Duplicate cell attachment assays were conducted at the times indicated. (**B**) Specific densities of adsorbed ephrin-B1/Fc promotes HEK293 attachment. Indicated amounts of ephrin-B1/Fc were adsorbed to surfaces coated with fibrinogen (2×10^{-6} g/cm^2) and attachment of HEK293 transfected with pSRα-EphB1 was assayed as described. (**C**) Ephrin-B1/Fc-promoted HEK293 cell attachment is mediated through $\alpha_5\beta_1$ integrin. Upper panel: cell surface integrins of transfected HEK293 exposed to NA or ephrin-B1/Fc (3×10^{-7} g/cm^2) were biotinylated as described for Figure 2, and surface integrins were quantified by immunoprecipitation with the indicated antibodies, and detection using streptavidin-conjugated HRP. HEK293 surface $\alpha_5\beta_1$ integrin abundance is unaltered by cell attachment to ephrin-B1/Fc-coated surfaces (left panel), and $\alpha_5\beta_1$ integrin is the predominant integrin. Lower panel: fibrinogen (2×10^{-6} g/cm^2) was adsorbed to nitrocellulose-coated plates in the absence or presence of ephrin-B1/Fc (3×10^{-7} g/cm^2), as indicated. HEK293 cells were plated in the absence or presence of the indicated peptide (100 µM) or antibody (5 µg/ml) competitors, including GRGDTP (RGD), GRGESP (RGE), anti-$\alpha_v\beta_3$ (LM609), anti-$\alpha_v\beta_5$ (P1F6) and anti-$\alpha_5\beta_1$ (JBS5).

receptor cytoplasmic domains as mediators of responses we have identified here (Stein *et al.*, 1998b).

Recent evidence supports an important role for the surface density of integrin ligands, such as fibronectin, in determining cell migration and spreading (Palecek *et al.*, 1997). In our studies, the surface density of fibrinogen displayed did not greatly impact on cell attachment, either in the absence or presence of EphB1 signaling (Figure 1C), yet an intriguing parallel exists between our findings and the alternative responses ligands effect through integrins themselves. Integrins form distinct signaling complexes in response to receptor aggregation alone, ligand occupancy alone or a combination of aggregation and occupancy (Miyamoto *et al.*, 1995). In the case of EphB1, it appears that ligand density alone is capable of altering the signals communicated to regulate integrin-mediated attachment. The responses to EphB1 point mutants suggest that multiple pathways are required. For the integrin-mediated attachment responses downstream of EphB1, Nck and LMW-PTP seem to play a crucial role. However, since ligand stimulation of EphB1 and EphB2 results in different signaling complexes (Holland *et al.*, 1997; Stein *et al.*, 1998a), one may expect other intermediary molecules linking EphB signaling to the regulation of integrin functions. Future evaluation of individual cell behavior in response to stamped gradients of ephrins displayed on alternative surfaces in conjunction with membrane localization of EphB1 should be informative (Singhvi *et al.*, 1994).

The affinity state of $\alpha_5\beta_1$ integrin is importantly regulated by cytoplasmic domain interactions through inside-out signaling (Hughes *et al.*, 1995; Pfaff *et al.*, 1998). It is likely that EphB1 is signaling such a process in transfected HEK293 cells. Although definition is currently less comprehensive, it appears that $\alpha_v\beta_3$ integrins may also acquire a high affinity state through 'inside-out' signaling. Comparison of the affinity states of $\alpha_v\beta_3$ integrins in intact cells with those of cytoplasmic domain truncations suggest that cytoplasmic domain interactions impose tonic suppression on the affinity of ectodomain binding to integrin ligands (Mehta *et al.*, 1998). ADP-activated platelet adhesion to osteopontin is mediated through $\alpha_v\beta_3$ integrins, and appears to reflect inside-out activation (Bennett *et al.*, 1997). Finally, recent evidence has demonstrated association of $\alpha_v\beta_3$ integrin with platelet-

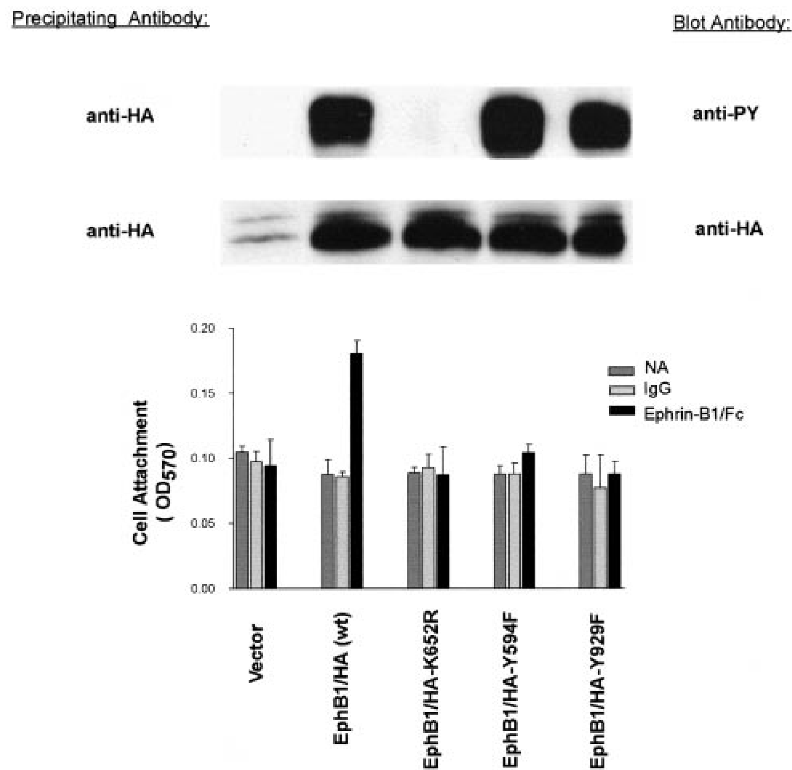


Fig. 5. EphB1 mutant analysis defines specific interactive partners necessary to mediate cell attachment. HEK293 were transfected with plasmids pSRα (vector), pSRα-EphB1/HA, pSRα-EphB1/HA-K652R, pSRα-EphB1/HA-Y594F, pSRα-EphB1/HA-Y929F (Stein *et al.*, 1998a,b), to drive expression of the following EphB1/HA proteins: wild type [EphB1/HA (wt)], kinase inactive (EphB1/HA-K652R), Nck recruitment defective (EphB1/HA-Y594F) or LMW-PTP recruitment defective (EphB1/HA-Y929F). Forty-eight hours after transfection, solid-phase attachment assays were conducted on fibrinogen-coated 48-well plates displaying ephrin-B1/Fc (3×10^{-7} g/cm^2). To confirm that quantitatively similar levels of EphB1 receptor expression (middle panel) and activation (upper panel) were achieved, EphB1 was immunoprecipitated with HA antibody (12CA5), samples were divided and separated on two different gels, transferred and immunoblotted using anti-HA and 4G10-HRP (anti-PY), respectively.

derived growth factor β (PDGF-β) receptors, both in conjunction with integrin-dependent migration (Woodard *et al.*, 1998), and in promoting PDGF-stimulated proliferation on $\alpha_v\beta_3$ integrin binding substrata (Schneller *et al.*, 1997). Although our findings demonstrate functional coupling of EphB1 to two different integrins, EphB1 does not co-precipitate with $\alpha_v\beta_3$ or $\alpha_5\beta_1$ integrin (data not shown).

The relevance of EphB family signaling to vascular development has been demonstrated with the embryonic lethal vascular dysgenesis phenotype of ephrin-B2 gene deletion mice (Wang *et al.*, 1998). Our findings provide a direct functional link between EphB–ephrinB signaling and integrins known to participate in angiogenesis. $\alpha_v\beta_3$ integrin is implicated in tumor angiogenesis and in ocular neovascularization, settings where its expression is induced in endothelial cells and where it binds preferentially to provisional ECM components such as vitronectin and fibrinogen (Brooks *et al.*, 1994). Although the levels of expression of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are quite similar in HRMEC, ephrin-B1-mediated attachment responses are dominantly dependent on $\alpha_v\beta_3$ integrin. This finding may be relevant in the context of the dependence of two alternative angiogenic pathways upon either $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins, evoked by bFGF or VEGF, respectively (Friedlander *et al.*, 1995).

Bader *et al.* (1998) recently showed that vascular development proceeds normally in mice lacking all five α_V integrins. The authors suggested that integrin blocking experiments may overestimate contributions of α_V integrins. Conversely, the genetic experiments could underestimate their contribution as a consequence of overlapping functions, or compensation by other adhesion receptors. In view of these seemingly contradictory results, it is not possible at present to draw definitive conclusions as to the relative importance of specific integrins in different angiogenic processes. Since the phenotype of α_V knock-out mice is considerably less severe than that of ephrin-B2 mutants (Wang *et al.*, 1998), any link between EphB receptors and integrin regulation can only partially explain the vascular dysgenesis phenotype following ephrin-B2 gene deletion.

At present, it appears clear that juxtacrine EphB–ephrinB engagement is a critical determinant of integrin-mediated responses, and that these are important intermediaries of cell attachment, migration and cell–cell recognition in the vasculature.

Materials and methods

Antibodies, peptides and matrix proteins

Polyclonal anti-EphB1 was generated by immunizing sheep with the full-length cytoplasmic domain of human EphB1 fused to glutathione *S*-transferase (GST) and expressed in Sf9 cells. The antibody recognizes endogenous human and murine EphB1, as well as the HA-tagged form of human EphB1. Ephrin-B1/Fc was provided by Immunex (Seattle, WA), monoclonal anti-human IgG1 (anti-Fc) was from the Binding Site (Birmingham, UK) and monoclonal anti-HA (12CA5) from Boehringer Mannheim (Indianapolis, IN). The HRP-conjugated antibody 4G10-HRP was from Upstate Biotechnology (Lake Placid, NY) and the streptavidin–HRP from Jackson (Westgrove, PA). Human IgG1 and plasma fibrinogen were from Sigma (St Louis, MO). The GRGDTP and GRGESP peptides

were from Calbiochem (La Jolla, CA), the GRGDSPK peptide from American Peptide Company (Sunnyvale, CA). The following anti-integrin blocking mAbs from Chemicon (Temecula, CA) were used: LM609 ($\alpha_v\beta_3$), P1F6 ($\alpha_v\beta_5$) and JBS5 ($\alpha_5\beta_1$).

Cell culture and transfection

HEK293 cells were passaged in minimum essential medium (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT). P19 cells were passaged in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. HRMEC were cultured as described (Martin *et al.*, 1997). HEK293 cells were transfected with LipofectAMINE Plus (Life Technologies) as described by the manufacturer. The expression constructs pSRα-hEphB1/HA, pSRα-hEphB1/HA-K652R, pSRα-hEphB1/HA-Y594F and pSRα-hEphB1/HA-Y929F have been described previously (Stein *et al.*, 1996). Cell attachment assays were routinely performed 48 h after transfection. Receptor recovery and phosphorylation of transfected EphB1 were assessed by immunoprecipitation with anti-HA or polyclonal sheep anti-EphB1 followed by anti-HA or anti-phosphotyrosine (4G10-HRP) immunoblots respectively.

Solid phase cell attachment assays

Forty-eight-well plates (Falcon) were coated with a layer of nitrocellulose (Schleicher & Schuell) as described previously (Wang and Anderson, 1997). They were incubated overnight at 4°C with phosphate-buffered saline (PBS) containing matrix proteins at the indicated concentrations alone (no addition, NA) or in combination with dimeric ephrin-B1/Fc. Two hours prior to assay, wells were washed twice and then blocked at 37°C with 1% BSA. Cells were starved for 24–40 h in Optimem (Life Technologies), detached using low concentrations of trypsin at room temperature, washed twice in medium containing 1% BSA, then plated at a density of $0.7–1\times10^5$ cells per well. After incubation at 37°C for 1 h (or as specified), unattached cells were dislodged by five brisk slaps of the plate on a horizontal surface. Wells were washed with PBS, monitoring albumin-coated wells as a control. Adherent cells were fixed with 2% glutaraldehyde, stained with 0.5% crystal violet and quantified by OD reading at 570 nm. In the competition experiment, EphB1/Fc was added at the indicated concentrations at the time of plating (Figure 1B). In experiments using competitive peptides or blocking antibodies, cells were pre-incubated with the indicated peptides (100 μM) or antibodies (5 μg/ml) for 15 min at 22°C before plating. Results representative of three or more independent experiments are given as OD values and represent the mean of triplicate wells ± SEM, unless otherwise stated.

Surface biotinylation and integrin immunoprecipitation

To confirm that surface integrin expression was not altered by transfection and/or exposure to ephrin-B1/Fc, cells were plated on 60 mm dishes coated with nitrocellulose, fibrinogen and ephrin-B1/Fc for 60 min at 37°C, then biotinylated for 30 min at 4°C with 0.5 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). Cells were then harvested in WG buffer (Stein *et al.*, 1996) and immunoprecipitation was performed with 2 μg of the same antibodies as used in the competition experiments. After SDS–PAGE (non-reducing conditions) and immunoblotting, biotinylated proteins were detected by enhanced chemiluminescence using ECL Western blotting detection (Amersham).

Wound closure assay

Replicate (triplicate) circular 'wounds' or defects (400–500 μm diameter) were generated in confluent HRMEC monolayers using a silicon-tipped drill press. Serum-free medium was supplemented with the indicated agonists at the time of wounding. Residual 'wound' areas were quantified at 6, 9 and 12 h, by analysis of digitized images using a Bioquant (Nashville, TN) software package calibrated to a Nikon Diaphot microscope. Rates were calculated by linear regression of residual wound areas plotted over time. r^2 values exceeded 0.980 for each regression.

Acknowledgements

The authors express thanks to Brigid Hogan, Peter Dempsey, Ray Harris and Mark Koury, for input to the manuscript, and to Roger Chalkley, Stanley Cohen, David Cheresh and Mark Ginsberg for helpful discussions. This work was supported by PHS award RO1-DK47078 (T.O.D.), NCI Center Grant CA68485, the T.J.Martell Foundation and the Swiss Science Foundation (U.H.-D.).

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Received December 29, 1998; revised and accepted February 23, 1999