Juxtmembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells

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Eph-related receptor tyrosine kinases have been implicated in the control of axonal navigation and fasciculation. To investigate the biochemical mechanisms underlying such functions, we have expressed the EphB2 receptor (formerly Nuk/Cek5/Sek3) in neuronal NG108-15 cells, and have observed the tyrosine phosphorylation of multiple cellular proteins upon activation of EphB2 by its ligand, ephrin-B1 (formerly Elk-L/Lerk2). The activated EphB2 receptor induced the tyrosine phosphorylation of a 62–64 kDa protein (p62 dok), which in turn formed a complex with the Ras GTpase-activating protein (RasGAP) and SH2/SH3 domain adaptor protein Nck. RasGAP also bound through its SH2 domains to tyrosine-phosphorylated EphB2 in vitro, and complexed with activated EphB2 in vivo. We have localized an in vitro RasGAP-binding site to conserved tyrosine residues Y604 and Y610 in the juxtmembrane region of EphB2, and demonstrated that substitution of these amino acids abolishes ephrin-B1-induced signalling events in EphB2-expressing NG108-15 cells. These tyrosine residues are followed by proline at the +3 position, consistent with the binding specificity of RasGAP SH2 domains determined using a degenerate phosphopeptide library. These results identify an EphB2-activated signalling cascade involving proteins that potentially play a role in axonal guidance and control of cytoskeletal architecture.

Keywords: Eph family receptor/ephrin/neuronal signalling/Ras GTpase-activating protein/tyrosine phosphorylation

Introduction

Receptor tyrosine kinases (RTKs) are known to play important roles in processes of cell–cell communication, exemplified by patterning of the developing embryo (reviewed in Pawson and Bernstein, 1990). EphB2 (previously termed Nuk/Cek5/Sek3) belongs to the largest subgroup of RTKs, the Eph-related receptors (Tuizi and Gucluck, 1993), which have been implicated in regulating pattern formation in the nervous system. High levels of EphB2 protein are detected in specific regions of the developing central nervous system (CNS) and in pioneering axon tracts of the peripheral nervous system (PNS; Henkemeyer et al., 1994). Consistent with these expression patterns, mice bearing a homozygous null mutation in the EphB2 gene exhibit a specific defect in the pathfinding of anterior commissure axons in the forebrain (Henkemeyer et al., 1996). Additional defects in axonal pathfinding and fasciculation, and in formation of the secondary palate, have been observed in mice lacking both EphB2 and a related Eph family RTK, EphB3 (previously termed Sek4/Hek2/Cek10; Orioli et al., 1996). [In this manuscript we refer to the Eph family receptors and their ligands using a new nomenclature that has been agreed to by the community and is being submitted for publication to Cell (M.Tessier-Lavigne, J.Flanagan, N.Gale, T.Hunter and E.Pasquale, personal communication).]

A family of eight related Eph receptor ligands (ephrins) have recently been identified (Pandey et al., 1995a). These ligands are cell surface-anchored glycoproteins which fall into two subgroups based on their method of membrane attachment. Ephrin-B1 (Beckmann et al., 1994; Davis et al., 1994; Shao et al., 1994), ephrin-B2 (Bennett et al., 1995; Bergemann et al., 1995; Cerretti et al., 1995) and ephrin-B3 (Gale et al., 1996b; Nicola et al., 1996) are transmembrane (TM) proteins which contain highly conserved cytoplasmic domains. These TM ligands bind to and activate a subgroup of Eph receptors which includes EphB1, EphB2, EphB3 and EphB4 (the EphB class; Brambilla et al., 1995; Gale et al., 1996a). The second group of ligands, ephrins-A1, -A2, -A3, -A4 and -A5, are bound to the cell surface by a glycosylphosphatidylinositol (GPI) linkage (Pandey et al., 1995a). Ligand membrane attachment appears crucial for efficient activation of Eph family receptors: in general, soluble ligand extracellular domains are only able to stimulate receptor autophosphorylation when artificially clustered, suggesting that surface attachment is required to facilitate ligand aggregation (Davis et al., 1994). Consistent with an in vivo function for Eph receptors in axonal guidance, cells expressing specific GPI-linked ephrins induce axonal repulsion, growth cone collapse and fasciculation of primary neurons in cell culture assays (Drescher et al., 1995; Winslow et al., 1995; Nakamoto et al., 1996). Furthermore,
TM ligands themselves become tyrosine phosphorylated in their cytoplasmic domains upon interaction with the EphB2 extracellular domain (Holland et al., 1996), and may therefore perform an intrinsic signalling function, as suggested by a genetic study of EphB2 (Henkemeyer et al., 1996).

Several groups have demonstrated tyrosine phosphorylation of Eph family receptors in response to binding of their cognate ligands (Bartley et al., 1994; Davis et al., 1994; Shao et al., 1994, 1995; Bennett et al., 1995; Gale et al., 1996a). However, phosphorylation of intracellular signalling proteins on tyrosine or their association with the activated receptors has not been readily detected in the cell lines studied. A highly conserved motif, corresponding to the sequence Y604DPTY610EDP in EphB2, is found in the internal juxtamembrane region of all Eph family receptors. The corresponding tyrosine residues are major in vitro autophosphorylation sites for the EphA4 RTK (previously Sek/Sek1/Heck/Cek8), and it is likely that they play an important role in downstream signal transduction (Lhotak and Pawson, 1993; Ellis et al., 1996). Associations have been demonstrated between the autophosphorylated Eph family RTKs EphA4 or EphA2 and the SH2 domain-containing proteins Fyn, the phosphatidylinositol (PI) 3′-kinase 85 kDa subunit and a novel adaptor protein, SLAP (Pandey et al., 1994, 1995b; Ellis et al., 1996), and recently between EphB1 and adaptor proteins Grb10 and Grb2 (Stein et al., 1996). However, downstream signalling events resulting from engagement of these effectors have not been investigated.

Unlike growth factor receptors, Eph family kinases are inefficient in stimulating cell proliferation in fibroblasts or epithelial cells (Lhotak and Pawson, 1993; Davis et al., 1994; Brambilla et al., 1995; Pandey et al., 1995c). Rather, Eph-related RTKs have been implicated in the control of axonal pathfinding and cell migration, processes which might be expected to involve regulation of the cell cytoskeleton (Drescher et al., 1995; Pandey et al., 1995c; Tessier-Lavigne, 1995; Winslow et al., 1995; Henkemeyer et al., 1996; Orioli et al., 1996). Signalling processes within the axonal growth cone that mediate its response to environmental cues are largely unknown. However, several cytoplasmic proteins including Rho family small GTPases have been implicated in axonal pathfinding in vertebrates (Jalink et al., 1994; reviewed in Mackay et al., 1995). In Drosophila, the Drac GTPase and dock, a homologue of the mammalian SH2 and SH3 domain-containing adaptor protein Nck, have been demonstrated to play important roles in axonal navigation (Luo et al., 1994; Garrity et al., 1996). The proposed involvement of Rho and Rac in growth cone responses is consistent with data showing that the Rho/Rac/Cdc42 family of GTPases control formation of actin-based cytoskeletal structures and cell matrix adhesions (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; reviewed in Chant and Stowers, 1995). In addition, the N-terminal region of the Ras GTPase-activating protein (RasGAP) is also able to influence cytoskeletal architecture (McGlade et al., 1993), possibly as a consequence of its interaction with p190, a GTPase-activating protein for Rho (Settleman et al., 1992).

In this study, we have expressed EphB2 in a neuronal cell line. We find that following stimulation by ephrin-

B1, autophosphorylated EphB2 associates with multiple proteins, including RasGAP. As a consequence of EphB2 activation, the RasGAP-associated protein, p62<sub>SLAP</sub>, becomes tyrosine phosphorylated and appears to mediate formation of a ternary complex between RasGAP and Nck. A degenerate peptide library screen performed with the SH2 domains of RasGAP revealed consensus binding sites in good agreement with the juxtamembrane sequence of EphB2. Substitution of Y604 and Y610 within this sequence by phenylalanine obviated both binding of RasGAP to EphB2 and downstream ephrin-B1-induced signalling events, demonstrating the importance of these conserved tyrosine residues in EphB2-mediated signalling.

**Results**

**Expression of the EphB2 cDNA in a neuronal cell line**

In the developing mouse embryo, EphB2 expression is most pronounced in neuronal structures (Henkemeyer et al., 1994). In several studies, prominent signalling responses were not observed after activation of Eph family receptors in Cos-1, endothelial and fibroblast cell lines (Pandey et al., 1994, 1995c; Bennett et al., 1995). In order to study the signalling properties of EphB2, we therefore ectopically expressed the EphB2 RTK in the neuronal cell line NG108-15 (NG108), which elaborates neurites in response to prolonged treatment with dibutyryl cAMP (Daniels and Hamprecht, 1974; Nelson et al., 1976). NG108 cells, which do not contain endogenous EphB2 protein (Figure 1A), were transfected with the mammalian expression vector pcDNA3 containing the full-length EphB2 cDNA, and single G418-resistant clones were isolated (NG-EphB2 cells). Western blot analysis of total cell lysates revealed high level production of the 130 kDa EphB2 protein in NG-EphB2 cells (Figure 1A), whereas no EphB2 protein could be detected in parental NG108 cells. EphB2 immunoprecipitated from NG-EphB2 cells was autophosphorylated in an in vitro kinase reaction (Figure 1B). No endogenous kinases were immunoprecipitated by the EphB2 antisera in NG108 cells, confirming the specificity of the EphB2 antibody (Henkemeyer et al., 1994).
EphB2-mediated signalling

Fig. 2. Stimulation of EphB2-expressing NG108 cells with ephrin-B1 induces tyrosine phosphorylation of cellular proteins. Parental and EphB2-expressing NG108 cells were challenged with 2 μg/ml clustered ephrin-B1Fc or Fc alone (c). (A) Anti-phosphotyrosine immunoblot of total cellular proteins. Proteins exhibiting ephrin-B1-induced tyrosine phosphorylation are indicated by — and . Ephrin-B1 treatment was for the indicated times. 60* = long exposure of the 60 min lane. (B) Anti-phosphotyrosine immunoblot of anti-EphB2 immunoprecipitates from ephrin-B1Fc (+) and Fc- (c) stimulated cells (upper panel). Anti-EphB2 reprobe (lower panel). WCL = whole cell lysate.

Tyrosine phosphorylation of cellular proteins is induced upon stimulation of EphB2-expressing cells with ephrin-B1

Several groups have demonstrated induced tyrosine phosphorylation of specific Eph receptor family members after stimulation with their cognate ligand(s) (Bartley et al., 1994; Davis et al., 1994; Shao et al., 1994, 1995; Bennett et al., 1995; Gale et al., 1996a). EphB2 becomes highly phosphorylated on tyrosine in response to treatment with ephrin-B1, ephrin-B2 and ephrin-B3 but not the GPI-linked subgroup of ephrins (Brambilla et al., 1995; Gale et al., 1996a). We wished to identify proteins other than EphB2 itself which become tyrosine phosphorylated after ephrin-B1 stimulation, and may therefore be involved in signalling downstream of this receptor. Parental and EphB2-expressing NG108 cells were stimulated for varying times with ephrin-B1 extracellular domain–IgG Fc region fusion protein (ephrin-B1Fc) which had been clustered using anti-human Fc. Total cell lysates were then analysed by immunoblotting with anti-phosphotyrosine antibodies. A marked increase in the tyrosine phosphorylation of a number of cellular proteins was observed in response to stimulation of NG-EphB2 cells with ephrin-B1 (Figure 2A). As previously noted (Davis et al., 1994), the onset of tyrosine phosphorylation in response to ephrin-B1 was slow and still developing at 30 and 60 min. The two most prominently phosphorylated polypeptides were EphB2 itself, and a protein of 62–64 kDa (indicated by the arrow in Figure 2A). Upon longer exposure, at least nine tyrosine-phosphorylated proteins of sizes ranging from ~48 to ~145 kDa could be detected in ephrin-B1-stimulated cells. No increase in tyrosine phosphorylation was observed when NG-EphB2 cells were exposed to aggregated Fc alone, or when the parental NG108 cells were exposed to ephrin-B1, indicating that the parental cells lack related EphB class receptors which can be activated by transmembrane ephrins (Figure 2A). The increase in phosphotyrosine content of proteins in NG-EphB2 cells observed upon ephrin-B1 stimulation can, therefore, be attributed to signalling in an EphB2-dependent pathway. Faint tyrosine phosphorylation of a 62–64 kDa polypeptide was also observed in response to ephrin-B1 treatment of Cos-1 cells, which endogenously express EphB2 (data not shown).

In order to determine potential signalling pathways initiated by EphB2 autoprophosphorylation, proteins associating with activated EphB2 in NG108 cells were investigated. As shown in Figure 2B, a number of tyrosine-phosphorylated proteins co-prefiected with EphB2 after ephrin-B1 stimulation. A duplicate EphB2 immunoprecipitation was electrophoresed on a long gel in order to determine the molecular sizes of these proteins more accurately (data not shown). The most prominent EphB2-binding protein identified by anti-phosphotyrosine antibodies had a mol. wt of ~104 kDa, and six other proteins of molecular sizes ~120, 88, 84, 76, 65 and 43 kDa were readily detected. None of these proteins cross-reacted with EphB2 antisera upon stripping and reprobing the blot, indicating that they are not likely to be degradation products of the phosphorylated receptor.

RasGAP and Nck SH2 domains interact with phosphorylated EphB2 in vitro

Phosphorylated tyrosine residues on activated receptors often provide docking sites for the SH2 domains of cytoplasmic signalling proteins. Initial experiments performed with a panel of GST–SH2 domain fusion proteins suggested that the SH2 domains of RasGAP, phospholipase Cγ (PLCγ; C-terminal SH2) and Src bound strongly to autophosphorylated EphB2 in vitro, whilst those of the P13’-kinase 85 kDa subunit and Nck associated more weakly, and the Grb2 SH2 domain appeared not to interact (data not shown). In Figure 3A, immobilized GST–RasGAP and GST–Nck SH2 domain fusion proteins were
Fig. 3. EphB2 associates with RasGAP in vitro and in vivo in intact cells. (A) Parental and EphB2-expressing NG108 cells were serum starved and stimulated with 2 μg/ml clustered ephrin-B1Fc (+) or Fc (c) for (A) 15 or (B) 30 min. (A) Lysates were incubated with immobilized GST fusion proteins and subjected to an in vitro kinase reaction in the presence of [γ-32P]ATP. Dissociated complexes were diluted, re-immunoprecipitated with anti-EphB2 serum and detected by autoradiography. (B) Anti-RasGAP immunoblot of anti-EphB2 immunoprecipitates (upper panel). Anti-EphB2 reprobe (lower panel).

mixed with lysates of control and ephrin-B1-stimulated NG-EphB2 cells and the washed beads were subjected to an in vitro kinase reaction. Labelled proteins were boiled in order to dissociate protein complexes and re-immunoprecipitated with EphB2 antiserum to specifically identify the receptor. EphB2 was precipitated specifically by the SH2 domain fusion proteins from lysates of ephrin-B1-stimulated cells. Phosphorylated EphB2 bound strongly to the RasGAP N-terminal SH2 domain (GAP SH2N), and with lower affinity to RasGAP C-terminal (GAP SH2C) and Nck SH2 domain fusion proteins. EphB2 was not detected in mixes with GST alone.

RasGAP associates with tyrosine-phosphorylated EphB2 in intact cells

In order to investigate the biological relevance of the RasGAP SH2 domain–EphB2 interaction detected in vitro, we tested whether RasGAP associates with EphB2 in vivo in intact cells. Parental and NG-EphB2 cells were stimulated with clustered ephrin-B1. Cell lysates were immunoprecipitated with EphB2 antiserum and immunoblotted with anti-RasGAP antibodies. RasGAP was detected in EphB2 immunoprecipitates from NG-EphB2 cells, but not parental cells (Figure 3B). The interaction was dependent on stimulation of the cells with ephrin-B1 and thus correlates with autophosphorylation of the receptor. Identification of the RasGAP–EphB2 complex required the use of a lysis buffer containing lowered (0.25%) Triton X-100, suggesting that this interaction may be of relatively low affinity, or transient in nature. In contrast, co-immunoprecipitation of Nck with phosphorylated EphB2 could not be detected (data not shown).

RasGAP-associated p62 becomes tyrosine phosphorylated upon ephrin-B1 stimulation of EphB2-expressing cells

The inducible association of RasGAP with EphB2 suggests that RasGAP may be involved in ephrin-B1-stimulated signal transduction. Studies using cells stimulated with growth factors or oncogenic tyrosine kinases have shown RasGAP to interact with several proteins including a Rho family GTPase-activating protein (p190 RhoGAP) and the p62 phosphoprotein (Ellis et al., 1990). We therefore investigated whether these two proteins bound to RasGAP or became tyrosine phosphorylated after stimulation of NG-EphB2 cells with ephrin-B1. Lysates of control and ephrin-B1-stimulated NG-EphB2 cells were immunoprecipitated with RasGAP antiserum and immunoblotted with anti-phosphotyrosine antibodies (Figure 4A). A phosphoprotein of 62–64 kDa was clearly seen in association with RasGAP upon ephrin-B1 stimulation, demonstrating that RasGAP not only binds to EphB2, but also interacts with other proteins in ephrin-B1-stimulated cells. A tyrosine-phosphorylated protein which co-migrated with the 62–64 kDa phosphoprotein identified in the RasGAP immune complex was also immunoprecipitated using a monoclonal
EphB2-mediated signalling

Fig. 5. RasGAP and Nck associate after ephrin-B1 stimulation of EphB2-expressing cells. Parental and EphB2-expressing NG108 cells were serum starved and stimulated with 2 μg/ml clustered ephrin-B1Fc (+) or Fc (−). (A) Anti-p62 immunoprecipitation followed by anti-Nck blot. The blot was stripped and reprobed with anti-phosphotyrosine antibodies. (B) Anti-RasGAP immunoprecipitation followed by anti-Nck blot. The blot was stripped and reprobed with RasGAP antiserum. (C) Anti-Nck immunoprecipitation followed by anti-RasGAP blot. The blot was stripped and reprobed with Nck antiserum.

antibody, mAb 2C4 (Hosomi et al., 1994), directed against the previously described RasGAP-associated p62 (Figure 4A). No p62 phosphorylation was observed in parental NG108 cells (data not shown). A modest increase in p62 phosphorylation was also observed in ephrin-B1-stimulated Cos-1 cells (data not shown). RasGAP is constitutively associated with a tyrosine-phosphorylated protein of 190 kDa in NG108 and NG-EphB2 cells (Figure 4A and data not shown). This protein was identified as p190 RhoGAP by immunoblotting with anti-p190 antibodies (data not shown). Neither the extent of association of RasGAP and p190 RhoGAP nor tyrosine phosphorylation of p190 were obviously affected by ephrin-B1 stimulation. Whilst the 120 kDa RasGAP protein itself did not become detectably tyrosine phosphorylated (Figure 4A), the protein did undergo a mobility shift after ephrin-B1 stimulation (data not shown), suggesting that it may become covalently modified, possibly by serine phosphorylation, as has been described following growth factor stimulation (Liu and Pawson, 1991).

Intriguingly, RasGAP-associated p62 co-migrated with the prominently tyrosine-phosphorylated 62–64 kDa protein observed in lysates of NG-EphB2 cells stimulated with ephrin-B1 (Figure 4A). In order to test the identity of this protein, cleared lysates from ephrin-B1-stimulated NG-EphB2 cells were depleted of RasGAP-binding proteins using the GST–GAP SH2N fusion protein. Relative to a protein of ~190 kDa, repeated incubation with GST–GAP SH2N depleted several phosphotyrosine-containing proteins from the lysate, including the 62–64 kDa phosphoprotein and EphB2. The 62–64 kDa polypeptide was the major phosphoprotein removed in the first round of incubation with GST–GAP SH2N (Figure 4B), suggesting that it corresponds to RasGAP-associated p62 or a related RasGAP-binding protein. Incubation with GST alone did not alter the abundance of these proteins. These data suggest that RasGAP-associated p62 may be the most abundant tyrosine-phosphorylated protein in the ephrin-B1-stimulated NG-EphB2 cell lysate after EphB2 itself, and therefore may have an important function in EphB2-dependent signalling.

Nck forms a complex with p62 and RasGAP upon ephrin-B1 stimulation of EphB2-expressing cells

As Nck is known to be required for axonal pathfinding in Drosophila (Garrity et al., 1996), we investigated further whether Nck might play a role in EphB2-mediated signalling. Nck immunoprecipitated from ephrin-B1-stimulated NG-EphB2 cells was not detectably tyrosine phosphorylated; however, a phosphotyrosine-containing protein of ~62–64 kDa was present in Nck immune complexes from stimulated lysates (Figure 4A). The Nck-associated 62–64 kDa polypeptide co-migrated with the RasGAP-associated p62 phosphoprotein and with the polypeptide immunoprecipitated directly using the anti-p62 monoclonal antibody. Furthermore, the Nck-associated protein
Fig. 6. Phosphopeptide specificity of the N- and C-terminal RasGAP SH2 domains. Results are from the pY+1 (A and D), pY+2 (B and E) and pY+3 (C and F) cycles of peptide sequencing. The value represents the ratio of the amount of each amino acid eluted from GST–SH2N (A–C) or GST–SH2C (D–F) bead columns divided by that of the control GST bead column at the same cycle. Amino acids are presented in single letter code. A strong selection for Pro is observed at the pY+3 position using GST–SH2C.

exhibited a similar diffuse pattern of migration to RasGAP-associated p62 (Figure 4A). After boiling Nck immune complexes to dissociate interacting proteins, the Nck-associated 62–64 kDa protein could be re-precipitated by incubation with the immobilized GST–GAP SH2N fusion protein (data not shown), further suggesting that p62 associates with Nck as well as RasGAP. In order to confirm this, proteins were immunoprecipitated from control and ephrin-B1-stimulated cells using the monoclonal anti-p62 antibody and immunoblotted with anti-Nck serum (Figure 5A). This experiment provided direct evidence that Nck binds inducibly to p62 following ephrin-B1 stimulation of EphB2-expressing NG108 cells. This interaction is likely to be mediated by the Nck SH2 domain, as we have observed a 62–64 kDa tyrosine phosphorylated protein in GST–Nck SH2 precipitates from ephrin-B1-treated NG-EphB2 cells (data not shown). As RasGAP also binds p62 via its SH2 domains (Moran et al., 1990), one can envisage
that tyrosine-phosphorylated p62 may act as a docking protein by binding to SH2 domain-containing proteins and mediating multiprotein complex formation. We therefore tested whether ephrin-B1 induced an association between RasGAP and Nck in NG-EphB2 cells. Following stimulation with clustered ephrin-B1, cells were lysed and immunoprecipitated with Nck or RasGAP antibodies. Immunoblots were then performed using RasGAP or Nck antisera respectively. The presence of Nck was clearly detected in RasGAP immunoprecipitates and, conversely, RasGAP was observed in Nck immune complexes (Figure 5B and C). The association between RasGAP and Nck was observed in NG-EphB2 but not parental cells, and was dependent on stimulation by ephrin-B1. Taken together, these data imply that following EphB2 activation, a complex is formed between RasGAP and Nck, which is mediated via the binding of the SH2 domains of both proteins to tyrosine-phosphorylated p62. It is possible that the weak association between EphB2 and the Nck SH2 domain observed in Figure 3A occurs indirectly through formation of this complex.

RasGAP binds to the internal juxtamembrane region of EphB2 in vitro

A degenerate peptide library randomized for all amino acids other than Cys or Trp at the three positions immediately C-terminal to the phosphotyrosine was used to examine the binding specificity of RasGAP SH2 domains. The isolated N-terminal SH2 domain of RasGAP bound preferentially to peptides with the motif pY-I/L/V-X-hydrophobic (including Pro) (Figure 6A–C), whereas the C-terminal SH2 domain strongly selected a Pro residue at position pY+3 and could accommodate Asp at position pY+2, with the consensus motif being pY-X-X-P (Figure 6D–F). Pro was also selected exclusively at pY+3 by fusion proteins containing both the N- and C-terminal SH2 domains of RasGAP (data not shown).

The internal juxtamembrane region of EphB2 contains the sequence Y<sub>604</sub>DPFTY<sub>610</sub>EDP, corresponding to a motif which is conserved in all Eph-related receptors (Lhotak and Pawson, 1993). In the EphA4 receptor, the two tyrosine residues in this motif are the major sites for in vitro autophosphorylation (Ellis et al., 1996). As these tyrosine residues are located in sequence elements which accord well with the consensus motifs selected by RasGAP SH2 domains, we sought to determine whether these sites are involved in the binding of RasGAP to EphB2. Immobilized GST–GAP SH2N was mixed with lysates of ephrin-B1-stimulated NG-EphB2 cells, followed by an in vitro kinase reaction, dissociation and re-immunoprecipitation by anti-EphB2 serum, as in Figure 3A. We then tested whether a phosphopeptide, which included the conserved Y<sub>604</sub>DPFTY<sub>610</sub>EDP sequence and in which both tyrosine residues were phosphorylated, could compete binding of the GST–GAP SH2N domain to EphB2 (Figure 7A). Association of EphB2 with GST–GAP SH2N was markedly inhibited by 5 μM, and almost totally abolished by 50 μM of the di-phospho-EphB2 peptide, whereas 50 μM unphosphorylated EphB2 peptide or phosphorylated insulin receptor peptide gave negligible competition. In further experiments using the Biacore, an IC<sub>50</sub> value of 32 μM was obtained for binding of the di-phospho-EphB2 peptide to a GST fusion protein containing both the N- and C-terminal SH2 domains of RasGAP (data not shown). In addition, a biotinylated form of the di-phospho-EphB2 peptide coupled to streptavidin beads was able to precipitate RasGAP from a lysate of NG108 cells, whereas the same peptide which had been dephosphorylated using potato acid phosphatase (PAP; Figure 7B) was not. These in vitro data strongly suggest that RasGAP associates with tyrosine-phosphorylated Y604 and/or Y610 of EphB2.

Substitution of Y604 and Y610 of EphB2 with phenylalanine abolishes ephrin-B1-induced RasGAP binding and downstream signalling events

In order to investigate the role of the EphB2 juxtamembrane tyrosine residues in interactions with potential targets in vivo in intact cells, the codons for Y604 and Y610 were substituted with those for phenylalanine within the EphB2 cDNA. NG108 cells were transfected with pcDNA3 containing the full-length mutated EphB2 cDNA (EphB2

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Fig. 7. RasGAP binds a di-phosphopeptide from the juxtamembrane region of EphB2. (A) Peptide competition. NG-EphB2 cells were serum starved and stimulated with 2 μg/ml clustered ephrin-B1Fc for 15 min. Lysates were mixed with GST fusion proteins, subjected to an in vitro kinase reaction and re-immunoprecipitated as in Figure 3A. Lane 1, mix with GST alone; lanes 2–7, mix with GST–GAP SH2N. Binding of EphB2 to GST–GAP SH2N was inhibited by addition of a di-phosphopeptide from the juxtamembrane region of EphB2 (EphB2pY<sub>604</sub>pY<sub>610</sub>) at concentrations of 0.5, 5 and 50 μM (lanes 3–5). The corresponding unphosphorylated EphB2 peptide (EphB2pY<sub>604</sub>A<sub>610</sub>; lane 6) or a phosphorylated peptide from the insulin receptor (lane 7) at concentrations of 50 μM gave negligible competition. (B) Anti-RasGAP immunoblot of proteins precipitated from NG108 lysate by immobilized di-phospho- (bio-EphB2 pep) or dephosphorylated (bio-EphB2 pep + PAP) EphB2 juxtamembrane peptide. PAP = potato acid phosphatase. NG108 whole cell lysate (WCL) was included to indicate the mobility of the RasGAP protein.
Fig. 8. Mutation of EphB2 juxtamembrane tyrosine residues abolishes ephrin-B1-induced RasGAP binding, p62 tyrosine phosphorylation and complex formation with Nck. Wild-type EphB2- and EphB2 Y604,610F-expressing NG108 cells were serum starved and stimulated with 2 μg/ml clustered ephrin-B1Fc (+)/Fc (−) or Fc (c). (A) Anti-phosphotyrosine immunoblot of anti-EphB2 immunoprecipitate (upper panel). The blot was stripped and reprobed with anti-EphB2 serum. (B) In vitro kinase reaction of anti-EphB2 immunoprecipitates using enolase as exogenous substrate. (C) Anti-EphB2 immunoprecipitation followed by anti-RasGAP blot. The blot was stripped and reprobed using anti-EphB2 serum. (D) Anti-phosphotyrosine immunoblot of anti-RasGAP (left panels) or anti-Nck (right panels) immunoprecipitates. Blots were stripped and reprobed with RasGAP or Nck antiserum. (E) Anti-RasGAP (left panels) or anti-Nck (right panels) immunoprecipitations followed by anti-Nck (left panel) or anti-RasGAP (right panel) immunoblots. Blots were stripped and reprobed with anti-RasGAP or anti-Nck serum.

Y604,610F) and single G418-resistant clones were isolated (NG-EphB2 Y604,610F cells). In contrast to the wild-type (WT) protein, EphB2 immunoprecipitated from lysates of ephrin-B1-stimulated NG-EphB2 Y604,610F cells contained almost no detectable phosphotyrosine (Figure 8A), although kinase activity towards enolase was not obviously affected (Figure 8B). Binding of RasGAP to EphB2 Y604,610F could not be detected following ephrin-B1 stimulation (Figure 8C), suggesting that Y604 and/or Y610 are required for binding of RasGAP to EphB2 in vivo, although it is formally possible that substitution of these residues structurally alters the EphB2 protein, leading to inhibition of RasGAP binding. Furthermore, tyrosine-phosphorylated p62 was not observed in RasGAP or Nck immunoprecipitates from lysates of ephrin-B1-induced NG-EphB2 Y604,610F cells (Figure 8D), nor was complex formation between Nck and RasGAP detected (Figure 8E). These data suggest that the association of signalling proteins such as RasGAP with the putative juxtamembrane tyrosine phosphorylation sites of EphB2 is required to mediate downstream ephrin-B1-stimulated signal transduction in NG-EphB2 cells.

Discussion

A neuronal system to study EphB2 signalling

In this report we have employed a neuronal cell line in order to study signalling by the EphB2 receptor tyrosine kinase. NG108 cells lack endogenous EphB2, and exhibit no increased tyrosine phosphorylation in response to treatment with ephrin-B1, indicating that they do not express functional EphB class receptors. We have, therefore, been able to analyse the activation of specific signalling pathways by WT or mutant EphB2 receptors ectopically expressed in this cell line.

Previous studies using Cos-1 cells, human endothelial cells, rat smooth muscle cells and NIH3T3 fibroblasts (Davis et al., 1994; Pandey et al., 1994, 1995c; Bennett et al., 1995) have failed to demonstrate association of tyrosine-phosphorylated cellular proteins with various activated Eph family kinases. In contrast, upon stimulation of EphB2-expressing NG108 cells with ephrin-B1, a marked increase in the phosphorytrosine content of several cellular proteins was detected. Moreover, a number of these tyrosine-phosphorylated proteins, in addition to the receptor itself, were detected in anti-EphB2 immunoprecipitates from ephrin-B1- and ephrin-B2-stimulated NG-EphB2 cells (Figure 2B and Holland et al., 1996). At present, it is not known whether each of these proteins binds directly to EphB2, or whether intermediate proteins are involved, and their identities remain to be determined. Nevertheless, these results suggest that autophosphorylated EphB2 physically associates with multiple potential target proteins, in a similar fashion to other receptor tyrosine kinases.
**Signalling downstream of EphB2**

The juxtamembrane region of EphB2 possesses a sequence motif which is highly conserved amongst Eph receptor family members, and contains two tyrosine residues (Y604 and Y610 of EphB2) which provide potential sites for autophosphorylation and interaction with downstream targets (Lhotak and Pawson, 1993). Indeed, the corresponding tyrosine residues in the EphA4 RTK have been identified recently as major in vitro autophosphorylation sites (Ellis et al., 1996). Consistent with these observations, a mutant EphB2 receptor in which both Y604 and Y610 were substituted with phenylalanine failed to become detectably tyrosine phosphorylated when expressed in NG108 cells and stimulated with ephrin-B1. The enzymatic activity of the mutant EphB2 protein appeared unimpaired, although we have not ruled out the possibility that a second kinase co-precipitates with the receptor. As residues Y604/Y610 appear to be the major sites of ephrin-B1-induced EphB2 tyrosine phosphorylation, it is possible that these tyrosine residues interact with several different signalling proteins, as is found in hematopoietic growth factor receptor-related RTKs (reviewed in Comoglio and Boccaccio, 1996).

Consistent with the notion that residues Y604/Y610 may act as docking sites for downstream targets, we have found that RasGAP co-precipitates with WT EphB2 from NG-EphB2 cells after ephrin-B1 stimulation, but fails to associate with the mutant EphB2 Y604,610F receptor. In vitro, RasGAP could be precipitated from NG108 cell lysates using a doubly phosphorylated biotinylated peptide encompassing residues Y604 and Y610. Furthermore, immobilized RasGAP SH2 domains, notably SH2N, bound to autophosphorylated EphB2 in lysates of stimulated NG-EphB2 cells, and this interaction was competed by the juxtamembrane di-phosphopeptide. The unphosphorylated form of the peptide had no effect in either assay. Taken together, these results support the conclusion that RasGAP physically associates through its SH2 domains with phosphorylated tyrosine residues Y604 and/or Y610 of EphB2 in vivo in intact cells. Both of these tyrosine sites have a proline residue at the +3 position, as found in a previously described physiological RasGAP-binding site in the β-receptor for platelet-derived growth factor (Fantl et al., 1992a; Kazlauskas et al., 1992); moreover, their flanking sequences are in excellent agreement with the consensus motifs selected by the RasGAP N- and C-terminal SH2 domains from a degenerate phosphopeptide library. In contrast, a previous report failed to detect an interaction between RasGAP and the Eph family member EphA4 in vitro (Ellis et al., 1996). It remains possible that the association of RasGAP with activated EphB2 is indirect or requires an additional polypeptide specifically found in neuronal cells.

Activation of EphB2 in NG-EphB2 and, to a lesser extent, Cos-1 cells resulted in tyrosine phosphorylation of the RasGAP-associated p62 polypeptide. Indeed, after EphB2 itself, a protein which co-migrated with p62 was the most prominent tyrosine-phosphorylated protein present in ephrin-B1-stimulated NG-EphB2 lysates. In contrast, p62 was not tyrosine phosphorylated upon stimulation of NG108 cells expressing the EphB2 Y604,610F mutant receptor, suggesting that these conserved juxtamembrane tyrosine residues are required for propagation of signals downstream of EphB2.

**EphB2-mediated signalling and axonal guidance**

Recent work on Eph family members and their ligands has suggested that these receptors can elicit responses such as growth cone collapse and axon fasciculation, and are important for correct axonal pathfinding in vivo (Dreesch et al., 1995; Winslow et al., 1995; Henkemeyer et al., 1996; Orioli et al., 1996). How might the potential EphB2 targets identified here regulate such biological processes? The axonal growth cone is a specialized structure (reviewed in Strittmatter and Fishman, 1991) which can respond to an array of external environmental cues (reviewed in Dodd and Schuchardt, 1995; Keynes and Cook, 1995) by local reinforcement or dissolution of its actin cytoskeleton, resulting in steering (reviewed in Tanaka and Sabry, 1995). A key mechanism for controlling the formation of actin stress fibres, lamellipodia, filopodia and the assembly of substrate adhesion complexes is the cycling of Rho/Rac/Cdc42 GTPases between inactive and active states (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; reviewed in Chant and Stowers, 1995). In NG-EphB2 cells, RasGAP is associated with a tyrosine-phosphorylated form of p190 RhoGAP, a negative regulator of Rho. Although this complex appears to be constitutive, it is possible that p190 RhoGAP function is modified in the ephrin-B1-stimulated cells through its association with RasGAP. Consistent with this, expression in fibroblasts of the N-terminal region of RasGAP, which is bound to p190 RhoGAP, leads to changes in the cytoskeleton, characterized by disruption of actin stress fibres and focal adhesion complexes (McGlade et al., 1993). Indeed, preliminary experiments suggest that modulation of the actin cytoskeleton follows ephrin-B1 treatment of NG-EphB2 cells bearing dibutyryl cAMP-induced neurites (S.J.Holland and T.Pawson, unpublished data).

During preparation of this manuscript, the cDNA for p62 (renamed p62<sup>2dk</sup>) was isolated and shown to encode a protein with an N-terminal PH domain, and six YXXP motifs that form potential sites of tyrosine phosphorylation and RasGAP binding (Carpino et al., 1997; Yamanashi and Baltimore, 1997). These p62<sup>2dk</sup> YXXP sites are closely related to those found in the EphB2 juxtamembrane region, and conform to the consensus motifs selected by the RasGAP SH2 domains from the phosophopeptide library. Furthermore, one of the p62<sup>2dk</sup> YXXP sites forms a predicted optimal binding motif for the Nck SH2 domain (pYDEP) (Songyang et al., 1993). One suggested function for p62<sup>2dk</sup> is to act as a tyrosine-phosphorylated scaffolding protein, allowing the assembly of multiprotein complexes at the membrane through the docking of SH2 domains (Neet and Hunter, 1995; Yamanashi and Baltimore, 1997). Consistent with this possibility, both RasGAP and Nck associated with p62<sup>2dk</sup> in ephrin-B1-stimulated NG-EphB2 cells. Indeed, RasGAP and Nck could be co-precipitated following ephrin-B1 stimulation, most likely through their mutual interaction with p62<sup>2dk</sup>. Such a complex may allow recruitment of RasGAP and Nck to sites where they might regulate the cytoskeleton.

Recent genetic data has directly implicated dreadlocks (dock), the *Drosophila* homologue of the SH2/SH3 adaptor protein Nck, in the control of axon guidance in the fly visual system (Garrity et al., 1996). In dock mutant flies, axons originating from the photoreceptor cells pathfind aberrantly and show defects in fasciculation. The wild-
type dock protein is localized in the growth cones of these axons. Our results suggest a possible role for the mammalian Nck protein in a signalling cascade initiated by activation of EphB2, which is crucial for axonal navigation in the mouse CNS (Orioli et al., 1996). Nck SH3 domains interact with two Cdc42/Rac-binding proteins; the serine/threonine kinase mPAK-3 (Bagrodia et al., 1995) and the multimodular protein WASP (Rivero-Lezano et al., 1995). WASP localizes to clusters that are rich in polymerized actin, and is able to inhibit the ability of a constitutively active Cdc42 to induce lamellipodia and stress fibre formation, thus linking Nck to the regulation of actin polymerization (Aspenström et al., 1996; Symons et al., 1996). It will be of interest to know whether the related neuronal protein, N-WASP, which possesses actin depolymerizing activity (Miki et al., 1996), is regulated upon EphB2 activation.

We have demonstrated here that autophosphorylated EphB2 associates with a number of potential downstream targets in neuronal cells. We have identified several proteins, including RasGAP, Nck and p62, that are connected in an ephrin-B1-induced signalling cascade which is coupled to activated EphB2 through conserved juxtamembrane tyrosine residues. Whilst additional EphB2-associated proteins and potential substrates detected in ephrin-B1-stimulated NG-EphB2 cells remain to be identified, these results pave the way for a more complete understanding of signalling by EphB2 and related Eph receptors.

Materials and methods

Expression constructs and mutagenesis
Full-length EphB2 cDNA (Henkemeyer et al., 1994) was cloned into the mammalian expression vector pcDNA3 (Invitrogen). Tyrosine residues Y604 and Y610 were mutated using a PCR-mediated protocol. The mutated PCR product was initially cloned into pcRII (Invitrogen) before a PstI fragment covering the mutated region was subcloned back into the full-length cDNA. The mutated region was sequenced on both strands using the ALF automated DNA sequencer (Pharmacia).

Antibodies, GST fusion constructs and peptides
Anti-EphB2 (Henkemeyer et al., 1994), anti-RasGAP (Ellis et al., 1990), affinity-purified polyclonal anti-phosphotyrosine antibodies (Letwin et al., 1995) and anti-p62 monoclonal antibody 2C4 (Hosomi et al., 1995) have been described previously. Anti-Nck antibodies (Garrity et al., 1996) were raised against the SH3 domains of Nck. For GST fusion constructs, cDNA sequences corresponding to the SH2 domains of human RasGAP (SH2N: residues 178–277; SH2C: 351–442; SH2N + C: 169–470) and Nck (residues 282–377) were cloned into pGEX2T. Phosphotyrosine-containing peptides were synthesized as outlined previously (Songyang et al., 1995). The EphB2 juxtamembrane peptide has the amino acid sequence G597MKYIDFTETEEDPNEAVRQ61K, in which residues Y604 and Y610 are either both phosphorylated (EphB2pY604pY610) or unphosphorylated (EphB2pY604Y610). The C-terminal lysine residue was added to facilitate biotinylation. The control insulin receptor peptide has the sequence GPLYASSNPePYSLSADSPV. Selection and analysis of peptides from a phosphotyrosine-containing degenerate peptide library by RasGAP SH2 domains was as described (Songyang et al., 1993).

Cell culture
NG108 cells (mouse neuroblastoma×rat glioma hybrid; Daniels and Hamprecht, 1974; Nelson et al., 1976) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL). Cos-1 cells were maintained in DMEM supplemented with 10% FBS. Transfection of cells was performed using Lipofectin reagent and Optimem medium (Gibco/BRL) according to the manufacturer’s instructions. To produce EphB2-expressing stable cell lines, NG108 cells were transfected with 10 μg of pcDNA3-EphB2 or -EphB2 Y604F, Y610F. Transfectants were selected in 400 μg/ml G418 (Gibco/BRL) and individual colonies picked and expanded. Experiments were carried out using two independent clones of each transfected cell line.

For stimulations, purified baculovirus-produced ephrin-B1F or Fc (Davis et al., 1994; Gale et al., 1996) was aggregated using human Fc (Jackson Immunoresearch) for 1–2 h at 4°C and diluted to 2 μg/ml in DMEM before applying to cells. Aggregation allows soluble ligand extracellular domain to stimulate the receptor efficiently (Davis et al., 1994). Stimulations were performed for 30 min at 37°C except where otherwise stated. Cells were incubated in medium containing 0.5% FBS for 6–8 h prior to stimulation where indicated.

Immunoprecipitation and Western blot analysis
Cells were rinsed once in ice-cold PBS-A and routinely lysed in PLC lysis buffer (Henkemeyer et al., 1996), is regulated upon EphB2 activation. For the GST fusion protein depletion experiment, lysates were incubated sequentially five times with 5 μg of GST fusion protein immobilized on glutathione-agarose for 1 h at 4°C before washing as above. For the GST fusion protein depletion experiment, lysates were incubated sequentially five times with 5 μg of GST fusion protein.

In vitro kinase reactions, re-immunoprecipitation and peptide competition
Proteins were collected by immunoprecipitation or incubation with GST fusion proteins as described. The beads were washed twice in kinase reaction buffer (KR; 20 mM HEPES, pH 7.5, 2.5 mM MgCl2, 4 mM MnCl2, and 0.1 mM Na3VO4) and incubated in 30 μl of KR with 5 μCi of [γ-32P]ATP (Amersham) for 30 min at room temperature. Unincorporated ATP was removed by washing 2–3 times in HNTG. Where indicated, labelled proteins were eluted from beads by boiling in 50 μl of 1% SDS, and diluted in PLC lysis buffer containing 0.25% Triton X-100 to a final concentration of 0.05% SDS before re-immunoprecipitation with anti-EphB2 serum. For the peptide competition, peptides were included in the incubation with GST–GAP SH2N at final concentrations of 0.5, 5 and 50 μM for the di-phospho-EphB2 peptide and 50 μM for control peptides. For the enolase assay, 5 μg of acid-denatured enolase was added to the kinase reaction, incubated for 20 min at room temperature, and the whole reaction was loaded on the gel.

Biotinylated peptide mix
Di-phospho-EphB2 peptide was biotinylated using NHS-LC-Biotin (Pierce) as outlined by the manufacturer, and purified by reverse-phase HPLC. Duplicate aliquots of ~40 nmol of peptide were bound to 50 μl of streptavidin beads for 1 h at 4°C, washed three times in dephosphorylation buffer (4 mM HEPES, pH 7.5, 0.6 mM MgCl2). Samples were incubated at 37°C for 2.5 h in 1 ml of dephosphorylation buffer, with or without 10 μl of PAP (Boehringer Mannheim) and washed three times with HNTG. Half of each aliquot was mixed with NG108 lysate for 1 h before washing three times with HNTG. The remaining peptide was immunoblotted with anti-phosphotyrosine antibodies to confirm that dephosphorylation had taken place.

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