

The *eps8* Family of Proteins Links Growth Factor Stimulation to Actin Reorganization Generating Functional Redundancy in the Ras/Rac Pathway

Nina Offenhäuser,^{*†} Alessandro Borgonovo,[†] Andrea Disanza,^{*†}
Pascale Romano,^{*} Isabella Ponzanelli,^{*†} Gioacchin Iannolo,[†]
Pier Paolo Di Fiore,^{*†‡} and Giorgio Scita^{*†§}

^{*}The FIRC Institute for Molecular Oncology, 20134 Milan, Italy; [†]University of Milan, Medical School, 20122 Milan, Italy; and [‡]Department of Experimental Oncology, Istituto Europeo di Oncologia, 20141 Milan, Italy

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Sos-1, a guanine nucleotide exchange factor (GEF), *eps8* and *Abi1*, two signaling proteins, and the lipid kinase phosphoinositide 3-kinase (PI3-K), assemble in a multimolecular complex required for Rac activation leading to actin cytoskeletal remodeling. Consistently, *eps8* ^{-/-} fibroblasts fail to form membrane ruffles in response to growth factor stimulation. Surprisingly, *eps8* null mice are healthy, fertile, and display no overt phenotype, suggesting the existence of functional redundancy within this pathway. Here, we describe the identification and characterization of a family of *eps8*-related proteins, comprising three novel gene products, named *eps8L1*, *eps8L2*, and *eps8L3*. *Eps8Ls* display collinear topology and 27–42% identity to *eps8*. Similarly to *eps8*, *eps8Ls* interact with *Abi1* and *Sos-1*; however, only *eps8L1* and *eps8L2* activate the Rac-GEF activity of *Sos-1*, and bind to actin *in vivo*. Consistently, *eps8L1* and *eps8L2*, but not *eps8L3*, localize to PDGF-induced, F-actin-rich ruffles and restore receptor tyrosine kinase (RTK)-mediated actin remodeling when expressed in *eps8* ^{-/-} fibroblasts. Thus, the *eps8Ls* define a novel family of proteins responsible for functional redundancy in the RTK-activated signaling pathway leading to actin remodeling. Finally, the patterns of expression of *eps8* and *eps8L2* in mice are remarkably overlapping, thus providing a likely explanation for the lack of overt phenotype in *eps8* null mice.

INTRODUCTION

Small GTPases act as binary switches that cycle between inactive GDP-bound and active GTP-bound states (Campbell *et al.*, 1998; Bar-Sagi and Hall, 2000). Among small GTPases, Rac, which belongs to the Rho-family of GTPases, is essential in the pathway originating from receptor tyrosine kinases (RTKs) and leading to the reorganization of the actin cytoskeleton (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998; Kaibuchi *et al.*, 1999).

The activation of small GTPases depends on guanine nucleotide exchange factor (GEFs; Cherfils and Chardin, 1999; Schmidt and Hall, 2002). The large number of GEFs, far exceeding that of GTPases, suggests that a single GTPase is under the control of multiple activators (Zohn *et al.*, 1998; Schmidt and Hall, 2002). GEFs are themselves regulated at multiple levels. The catalytic domain present in GEFs for RhoGTPases is the Dbl homology domain (DH), which is almost invariably preceded by a pleckstrin homology (PH) domain, suggesting an integrated function for the DH-PH tandem (Whitehead *et al.*, 1997). Accordingly, the DH-PH module frequently represents the minimal catalytic unit

(Crompton *et al.*, 2000; Hoffman and Cerione, 2002). However, in other cases, the DH-PH might function as a self-inhibitory device, as suggested by findings that isolated PH domains bind to and inhibit the activity of some DH domain (Nimnual *et al.*, 1998; Das *et al.*, 2000; Paduch *et al.*, 2001).

Additional regulatory complexity is implied by the observation that GEFs can assemble into multimolecular complexes (Cherfils and Chardin, 1999; Schmidt and Hall, 2002). An example is provided by the complex formed by *eps8*, *Abi1*, p85 (the regulatory subunit of phosphatidylinositol 3 kinase [PI3-K]), and the GEF *Sos-1* (Fazioli *et al.*, 1993; Biesova *et al.*, 1997; Scita *et al.*, 1999; Di Fiore and Scita, 2002; Innocenti *et al.*, 2002, 2003; Nimnual and Bar-Sagi, 2002). *Sos-1* belongs to a class of exchange factors endowed with dual catalytic ability, on Ras and Rac GTPases (Nimnual and Bar-Sagi, 2002; Schmidt and Hall, 2002). The catalytic specificity of *Sos-1* is dictated by its engagement into different protein complexes (Innocenti *et al.*, 2002). Thus, when coupled to the adaptor molecule Grb2, *Sos-1* is recruited to the plasma membrane by activated RTK and catalyzes nucleotide exchange on Ras. Conversely, *Sos-1*, when in complex with *eps8*, *Abi1*, and p85, displays Rac-GEF activity (Innocenti *et al.*, 2002).

Eps8 is critical for the activation of the *eps8*-*Abi1*-p85-*Sos-1* complex. Consistently, disruption of the complex, by genetic removal of *eps8* abrogates Rac activation and Rac-dependent actin remodeling induced by various stimuli (Scita *et al.*, 1999). Within the complex, *Abi1* acts as a scaffold on which *eps8*, p85, and *Sos-1* assemble (Scita *et al.*, 1999). This topological arrangement is thought to facilitate a low

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[§] Corresponding author. E-mail address: gscita@teo.it

Abbreviations used: RTK, receptor tyrosine kinase; dpc, days postconception; MEF, mouse embryo fibroblast; PI3K, phosphoinositides 3 kinase; GEF, guanine nucleotide exchange factor.

affinity interaction between eps8 and Sos-1, which is sufficient for the catalytic activation of Sos-1 (Scita *et al.*, 2001). Thus, two features are critical to the function of eps8: the association with Abi1 via its SH3 domain, and the activating interaction with Sos-1. Furthermore, eps8 binds directly to F-actin and directs *in vivo* the localization of Abi1 and Sos-1 to F-actin-rich structures (Scita *et al.*, 2001). Thus, the formation of an eps8-based macromolecular complex is essential for both Sos-1 catalytic activation and localization of the complex to sites where its activity is required.

Despite the essential role of eps8 in signaling, *eps8* null mice are healthy, fertile, and devoid of any obvious phenotypic alteration (Scita *et al.*, 1999). This is in contrast with the early embryonic lethal phenotype exhibited by null mice for other proteins involved in the same signaling pathway, such as Rac1, and Abi1 (Sugihara *et al.*, 1998, and A.M. Pendergast, personal communication). An obvious explanation for the lack of phenotype of the *eps8* $-/-$ mice is the existence of redundant functions *in vivo*. Here, we describe the identification and characterization of a family of eps8-related proteins (eps8Ls, standing for eps8-like) and provide biochemical and biological evidence that members of this family act redundantly in the pathway leading to Rac activation. Thus, the eps8Ls constitute a novel family of proteins, which links growth factor stimulation to actin dynamics and provide a safety device to ensure propagation of signals leading to the activation of Rac and Rac-dependent pathways.

MATERIALS AND METHODS

Cloning of EPS8-like Genes

A UNI-ZapXR Human Placenta library (CLONTECH, Palo Alto, CA), a pCEV-29-based library from M426 human fibroblasts (a kind gift of T. Miki), and a λ phage (λ NM1149) library derived from HeLa cells (a kind gift of C. Schneider) were used to obtain the entire open reading frames (ORFs) of human *EPS8L1*, *EPS8L2*, and *EPS8L3*, respectively. The ORFs of the *EPS8Ls* were individually cloned into pBSK+ vectors and subcloned into pCDNA3.1 flag-containing eucaryotic expression vectors. The mouse sequences were obtained by sequencing of EST clones obtained from the IMAGE consortium and by RT-PCR-based cloning from E16.5 dpc mouse head RNA. The human and mouse sequences were deposited in GenBank (AY074928, AY074931, AY074929, AY074930, AY074932).

In Situ Hybridization

Timed matings were set up between adult C57BL6 males and females. The day of plug was considered 0.5 d postconception (dpc). Embryos were dissected at the indicated stages, processed, and paraffin-embedded. *In situ* hybridization was carried out on 5- μ m sagittal sections as previously described (Rugarli *et al.*, 1993). Sense and antisense 35 S-labeled riboprobes were transcribed from 600-base pair mouse cDNA fragments, corresponding to positions 1480–2100 of *eps8L1*, 1560–2100 of *eps8L2*, and 1630–2250 of *eps8L3*, using T3 and T7 RNA polymerase (numbering is according to the GenBank-deposited sequences).

Northern Blot and Semiquantitative RT-PCR

Total RNA was extracted from adult mouse tissues using Triazol (GIBCO BRL, Rockville, MD) according to the manufacturer's instructions. Northern blotting was performed using 10 μ g of total RNA according to standard procedures. For RT-PCR, oligo dT-primed first-strand synthesis was performed using Superscript II (GIBCO BRL) on 1 μ g of total RNA. PCR was performed with specific primers located in the 5' coding region of the genes. Cycle number was determined for each gene individually. The sequences of the primers and the exact PCR conditions for each primer set are available on request.

Antibodies and Biochemical Procedures

Monoclonal antibodies to flag (M2, Sigma, St. Louis, MO), actin (Sigma), and eps8 (Transduction Laboratories, Lexington, KY) were obtained commercially. The polyclonal anti-Abi1 has been previously described (Biesova *et al.*, 1997). Anti-myc (9E10) was produced in synthetic medium. Standard procedures of protein analysis (Western blotting, immunoprecipitations, and co-immunoprecipitations) were performed as previously described (Fazioli *et al.*, 1993).

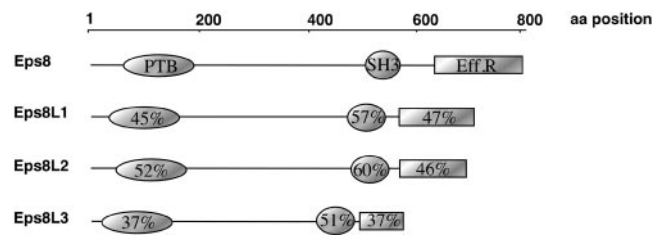


Figure 1. The eps8 family. Schematic representation of human eps8 and of the three related proteins eps8L1, eps8L2, and eps8L3. The PTB and SH3 domains were identified using the SMART module. The effector region (Eff.R., amino acids 649–822 in eps8) was previously functionally identified in eps8 (Scita *et al.*, 2001). The percentages of identity between eps8 and individual eps8Ls, in the three domains, are shown, as calculated by pairwise alignments using a BLOSUM62 matrix.

In vitro GEF activity toward Rac was performed as described (Scita *et al.*, 1999). Data are the mean \pm SE of at least three independent experiments performed in triplicates. Results are expressed as the percentage of [3 H]GDP released after 20 min relative to time 0, after subtracting the background counts released in control reactions (obtained in the presence of immunoprecipitates performed with control IgGs).

Actin cosedimentation assays were performed as described (Scita *et al.*, 2001). Briefly, monomeric rabbit G-actin (1 mg/ml) was induced to polymerize at 37°C in F-actin buffer (5 mM Tris/HCl, pH 7.8, 1 mM ATP, 0.5 mM DDT, 0.2 mM CaCl₂, 0.2 mM MgCl₂, and 100 mM KCl). Recombinant and purified GST-fusion proteins were subsequently incubated with 10 μ M F-actin for 45 min at 37°C, flowed by ultracentrifugation. Equal amount of starting materials, supernatants, and pellets were solubilized in loading buffer, boiled, and resolved on SDS-PAGE.

Transfection Procedures and Indirect Immunofluorescence

Cells seeded on gelatin were transfected with the indicated expression vectors using the LIPOfectamine reagent (GIBCO BRL), according to the manufacturer's instructions. Alternatively, nuclei of quiescent fibroblasts were injected with 100 ng/ml the appropriate expression vectors. Three to 6 h later, cells were processed for immunostaining. At least 100 microinjected cells were analyzed in each experiment. For indirect immunofluorescence cells were fixed in 4% paraformaldehyde for 10 min, permeabilized in 0.1% Triton X-100 and 2% BSA for 30 min, and then incubated with the primary antibody for 45 min, followed by the secondary antibody for 30 min. F-actin was detected by staining with rhodamine-conjugated phalloidin (Sigma), at a concentration of 6.7 U/ml (ca. 0.22 μ M). Where indicated, cells were treated with PDGF (10 ng/ml) for 10 min.

RESULTS

A Family of eps8-related Proteins Regulates the RacGEF Activity of Sos-1

An *in silico* screening of the expressed sequence tags (EST) database led to the identification of three human genes displaying high homology to the human *EPS8* gene. These genes were named *EPS8-Like1*, 2, and 3 (*EPS8L1*, 2, and 3), respectively (Mongiovi *et al.*, 1999; Tocchetti *et al.*, 2003). Full-length clones of *EPS8Ls* were obtained by screening of human cDNA libraries. The predicted proteins displayed collinear topology and an overall amino acid identity, among themselves, ranging from 27 to 42%. *Eps8Ls* share a common modular organization, consisting of three domains: an N-terminal region (37–52% identity with eps8), whose predicted fold resembles a phosphotyrosine binding domain (PTB; Tocchetti *et al.*, 2003; Calderwood, 2003); a central SH3 domain (51–60% identity with eps8); and a C-terminal region (37–47% identity with eps8) similar to the “effector region” of eps8 (Scita *et al.*, 2001) (Figure 1).

The conserved modular organization within the family suggested overlapping biochemical and functional properties among its members. *Eps8* is essential for the activation of the RacGEF activity of the eps8-Abi1-p85-Sos-1 complex.

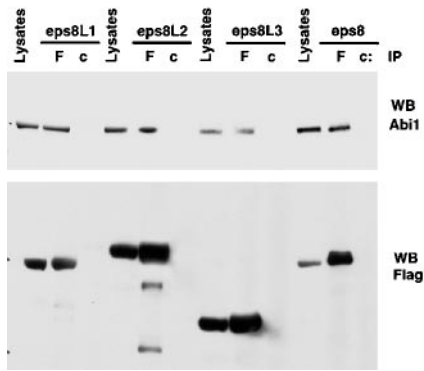


Figure 2. Eps8Ls coprecipitate with Abi1. 293T cells were transfected with flag-tagged eps8Ls (as indicated at the top, Tfx), followed by immunoprecipitation (IP) with an anti-flag (F) or control IgGs (c). Lysates and immunoprecipitates were immunoblotted (WB) with the indicated antibodies.

Thus, we initially tested the ability of eps8Ls to bind to Abi1 *in vivo*. As shown in Figure 2, all eps8 family members, expressed as flag-tagged proteins, coimmunoprecipitated with endogenous Abi1.

We next assessed whether RacGEF activity could be recovered in eps8Ls immunoprecipitates, under conditions in which eps8Ls-Abi1-Sos-1 complexes are formed. To do this, we engineered a model system in 293T cells, in which flag-tagged eps8 family members were individually transfected together with Sos-1 and either with or without an Abi1 expression vector (Figure 3, A and B). We have previously

demonstrated that an endogenous complex, containing eps8, Abi1, and Sos-1, can be recovered by immunoprecipitation with anti-eps8 antibodies from untransfected cells (Innocenti *et al.*, 2002). However, in order to perform enzymatic assays for Rac-GEF activity, recovery of sizable amounts of the complex can be obtained only under conditions of overexpression of the three components (Scita *et al.*, 1999). In addition, the low amounts of endogenous Abi1 (which scaffolds together eps8 and Sos-1) in 293T cells makes the detection of an eps8/Abi1/Sos-1 complex rather difficult, even in the presence of overexpressed eps8 and Sos-1 (Figure 3A, top left panel). However, this can be turned into an experimental advantage, because the simultaneous overexpression of Abi1 allows for the detection of the complex in an Abi1-dependent manner. Indeed, under conditions of triple transfection, all of the eps8 family members could be found in association with Abi1 and Sos-1 (Figure 3A). In addition, the formation of the complex was Abi1 dependent, as shown by lack of coimmunoprecipitation between eps8 family members and Sos-1, in the absence of overexpressed Abi1 (Figure 3A).

We then tested Rac-GEF activity in anti-flag immunoprecipitates from the triple transfectants (Figure 3B). Eps8-, eps8L1-, and eps8L2-, but not eps8L3-containing immunocomplexes displayed RacGEF activity (Figure 3B). Of note, the presence of the Rac-GEF activity, in the immunocomplexes, was dependent on the simultaneous transfection with Abi1, as predicted (Figure 3B).

The lack of Rac-GEF activity in eps8L3-containing complexes indicated that some additional molecular characteristics, shared by eps8, eps8L1, and eps8L2, are not present in eps8L3. We have previously demonstrated that the C-termi-

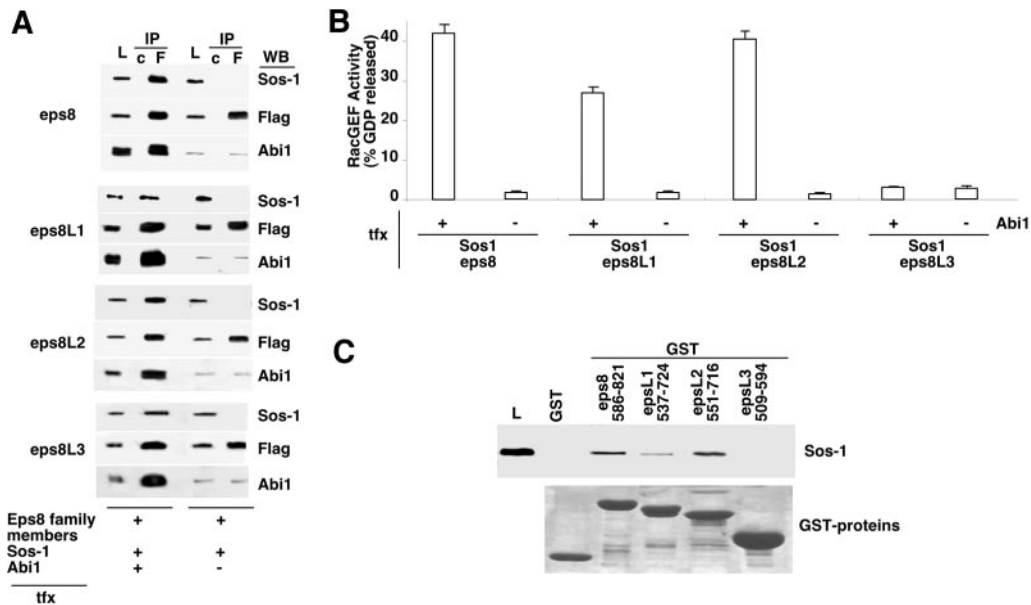


Figure 3. Rac-GEF activity of complexes containing various eps8 family members. (A) 293T cells were cotransfected with the constructs (Flag-tagged eps8 family members, Sos-1 and Abi1) indicated at the bottom. The individual eps8 family member transfected in each set is indicated on the left. Total cellular lysates were immunoprecipitated with an anti-Flag antibody (F) or control IgGs (c). Lysates (L) and immunoprecipitates (IP) were immunoblotted with the indicated antibodies (WB). (B) RacGEF activity was measured on aliquots of the IPs shown in A. For each set, the combination of transfected expression vectors (eps8 family members, Sos-1, with or without Abi1) is indicated at the bottom. Data are expressed as described in MATERIALS AND METHODS. (C) Immobilized, C-terminal fragments of various eps8 family members (the amino acid boundaries are indicated), or GST alone, were incubated with total cellular lysates from Sos-1-expressing 293T cells. Bound proteins were resolved by SDS-PAGE and immunoblotted with anti-Sos-1 (top panel). A Coomassie staining, to verify equal loading of GST fusions, is also shown (bottom panel, GST-proteins).

nal region of *eps8* (effector region) can contract a direct low-affinity interaction with Sos-1 (Scita *et al.*, 2001). We further showed that this interaction is necessary and sufficient to cause the catalytic activation of Sos-1 as a Rac-GEF, both in vitro and in vivo (Scita *et al.*, 2001). Using purified proteins, we showed in vitro that holo-*eps8* cannot interact with Sos-1; however, the interaction can be unmasked if the C-terminal fragment of *eps8* is used (Scita *et al.*, 2001). Although this probably reflects the need for some conformational change in vivo, which affects *eps8* in the *eps8*/Abi1/Sos-1 complex and which is mimicked by the isolation of the effector region from the context of the holoprotein, it provided an assay to investigate biochemical differences within the *eps8* family. Thus, we tested the ability of the C-termini of *eps8L1*, *eps8L2*, and *eps8L3* to associate to Sos-1 in vitro. Native Sos-1 could be recovered onto immobilized GST-C-terminal fragments of *eps8*, *eps8L2*, and *eps8L1* (in the latter case, at lower stoichiometry). No association could be detected with *eps8L3* (Figure 3C). Thus, *eps8L1* and *eps8L2*, but not *eps8L3* share critical biochemical properties with *eps8*.

Differential Interaction of *eps8* Family Members with Dynamic F-actin-rich Structures In Vivo

An important and biologically relevant feature of *eps8* is its ability to interact with actin. This interaction is essential for the intracellular localization of *eps8* and is thought to localize *eps8*-based complexes to sites where actin remodeling is required (Scita *et al.*, 2001). Similarly to *eps8*, *eps8L1* and *eps8L2*, but not *eps8L3* coprecipitated with actin (Figure 4A). The C-terminal region of *eps8*, encompassing the last 173 amino acids, mediates a direct interaction with F-actin (Scita *et al.*, 2001). Because this region is also highly conserved among the member of the *eps8*-related genes (Figure 1), we next tested the ability of purified C-terminal fragments of the various *Eps8Ls* to associate directly with polymerized actin in cosedimentation assays. Consistent with the immunoprecipitation results, *eps8*, *eps8L1*, and *L2*, but not *eps8L3* could directly associate to F-actin, albeit with different stoichiometry, pointing to the existence of a novel, conserved F-actin-binding surface present in the C-terminus of *eps8Ls*. This is further supported by the observation that *eps8L3*, whose C-terminal region is significantly shorter and the least conserved among the *eps8Ls*, failed to interact with F-actin. The ability of *eps8Ls* to associate to filamentous actin may contribute to their intracellular localization at site where F-actin is enriched, such as membrane ruffles. To this end, the cellular distribution of the *eps8Ls* was assessed. Flag-tagged *eps8Ls* were expressed in mouse fibroblasts. *Eps8L1* and *eps8L2*, similarly to *eps8*, were found enriched in PDGF-induced, F-actin-rich ruffles (Figure 4B). In contrast, *eps8L3* was inefficiently recruited to ruffles, in agreement with its lack of interaction with actin (Figure 4, A and B). It has to be pointed out that the direct binding to F-actin may not be sufficient to direct the localization of *eps8Ls* at membrane protrusions. Additional structural features, first and foremost the SH3 domains, may participate in dictating a proper cellular distribution. Consistent with this notion, all *eps8Ls* are capable of associating to Abi1, which was shown to localize specifically at the leading edge of protruding membranes (Stradal *et al.*, 2001), possibly contributing to the localization of *eps8Ls*.

Eps8L1 and *eps8L2*, but not *eps8L3*, Reconstitute the PDGF-induced Ruffling Response in *eps8* $-/-$ Fibroblasts

The ability to form an active complex with Abi1 and Sos-1 and to associate with F-actin-rich structures suggested that

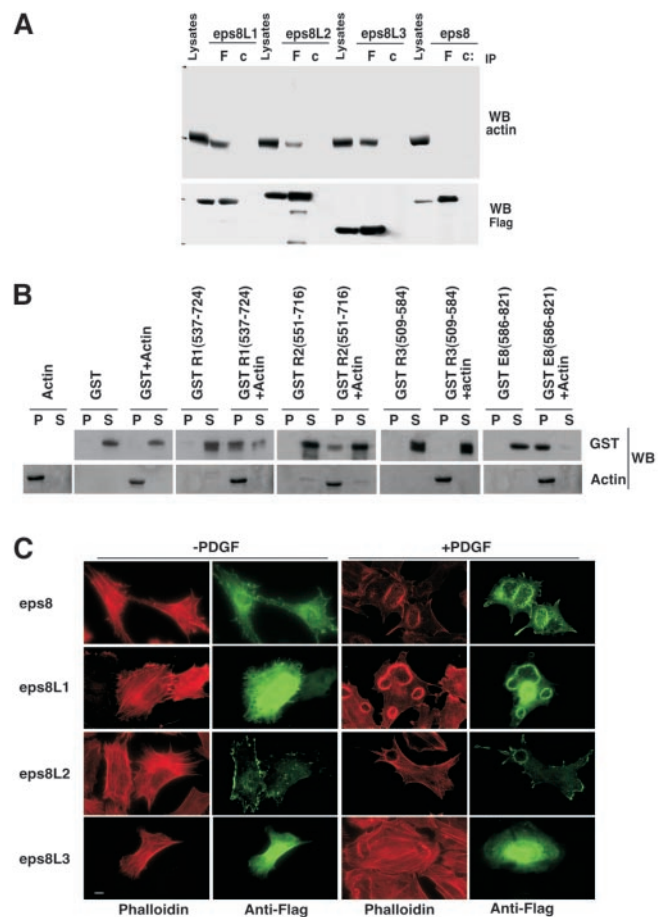


Figure 4. Interaction between actin and *eps8* family members. (A) 293T cells were transfected with flag-tagged *eps8* family members (as indicated at the top, Tfx), followed by immunoprecipitation (IP) with an anti-flag (F) or control IgGs (c). Lysates and immunoprecipitates were immunoblotted (WB) with the indicated antibodies. The flag (bottom) panel is the same as in Figure 2, bottom, because the two experiments were performed together. (B) Actin cosedimentation assay. The indicated C-terminal fragments of *eps8* and *eps8* related proteins (*eps8L1* [537–724]; *eps8L2* [551–716]; *eps8L3* [509–594]; *eps8* [586–821]) fused to GST (1 μ M) were mixed in the presence (+ actin) or absence of 10 μ M of purified F-actin, as described in MATERIALS AND METHODS, and subjected to ultracentrifugation. GST alone was used as a negative control. Each lane shows the supernatant (S) and the pellet (P) obtained after ultracentrifugation. The lanes (actin) on the left show the supernatant and the pellet of F-actin alone. Detection was by immunoblotting (WB) with the indicated abs. (C) MEFs, microinjected with flag-tagged *eps8* family members (indicated on the left), were serum-starved and stimulated with 10 ng/ml PDGF (+PDGF) or mock-treated (-PDGF). Cells were fixed and stained with an anti-flag antibody (Anti-Flag, green) or rhodaminated phalloidin to visualize F-actin (Phalloidin, red).

eps8L1 and *eps8L2* might compensate for loss of *eps8* function in vivo. To verify this prediction, we expressed the various *eps8Ls* in mouse embryo fibroblasts (MEFs) derived from *eps8* knockout mice and analyzed RTK-mediated ruffling. *Eps8* $-/-$ MEFs do not express detectable levels of mRNA for *eps8L* genes (Figure 5A) and are unable to form membrane ruffles in response to growth factor stimulation (Scita *et al.*, 1999). Ectopic expression of *eps8*, *eps8L1*, or *eps8L2*, but not of *eps8L3*, restored ruffling formation, in response to growth factor stimulation, in *eps8* null MEFs

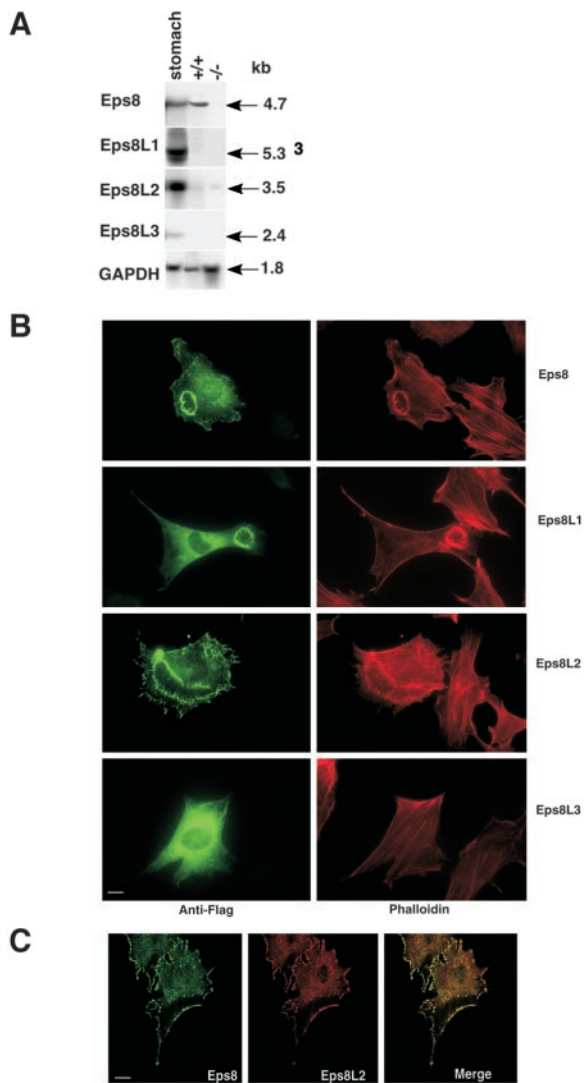


Figure 5. Eps8L1 and eps8L2, but not eps8L3 reconstitute ruffling in response to PDGF in *eps8*^{-/-} MEFs. (A) Northern blot analysis of total RNAs from wt (+/+) and *eps8* null (-/-) MEFs. An adult stomach RNA, in which the expression of all family members is visible, is also shown as a positive control. Probes specific for the various *eps8Ls* detected a single transcript of the indicated size (Kb). GAPDH was used to control for loading. (B) *eps8*^{-/-} MEFs were microinjected with flag-tagged *eps8* family members (indicated on the right), serum-starved, and stimulated with 10 ng/ml PDGF. Cells were fixed and stained with anti-flag (Anti-Flag, green) and rhodaminated phalloidin (Phalloidin, red). Quantitation (n = 3, at least 100 cells counted/experiment) was performed by determining the percentage of cells expressing *eps8* family members that underwent ruffling: *eps8*, 78 ± 3; *eps8L1*, 42 ± 2; *eps8L2*, 71 ± 4; *eps8L3*, 4 ± 1; and untransfected cells, 5 ± 1. (C) *eps8*^{-/-} MEFs, cotransfected with myc-*eps8* and flag-*eps8L2*, were fixed and stained with the appropriate antitag antibodies (*eps8*, antimyc in green; *eps8L2*, anti-flag in red). Colocalization of *eps8* and *eps8L2* is evidenced by the appearance of yellow color in the merge. Central confocal sections are shown. Bar, 10 μM

(Figure 5B). Notably, *eps8L2* was as efficient as *eps8* (see legend to Figure 5). This is consistent with the observation that *eps8* and *eps8L2* display the highest degree of amino acid identity, within the family, and quantitatively comparable biochemical properties (*eps8L2* and *eps8* bind to and

activate Sos-1 with similar efficiency; see Figure 3). Furthermore, they show a very similar intracellular distribution as witnessed by the large extent of colocalization between the two proteins by confocal analysis (Figure 5C). Thus, the members of the *eps8* family display overlapping functional properties, which can be ranked by their ability to elicit Rac-GEF activity and to substitute for *eps8* (*eps8* = *eps8L2* > *eps8L1* >> *eps8L3*), and generate functional redundancy in pathway connecting RTK to Rac.

Differential Patterns of Expression of *eps8Ls* in Developing and Adult Mice

The overlapping functions of *eps8L*-family members provide tentative hypothesis to explain the lack of phenotype in *eps8* null mice. To gain insight into this issue, we analyzed the pattern of expression of *eps8*-family members by in situ hybridization, in the developing mouse. The expression of *eps8*, but not the other *eps8Ls*, was detected as early as 10.5 d postconception (dpc; our unpublished results, and Avantagegiato *et al.*, 1995). However, at 16.5 dpc expression of all four genes could be observed. *Eps8* and *eps8L2* displayed a widespread and largely overlapping pattern of expression (Figure 6, A–D, and Table 1). *Eps8L1* and *eps8L3* in contrast showed restricted expression (Table 1 and Figure 6). *Eps8L1* was detectable almost exclusively in the naris externis (Figure 6, A and B). In this location, the expression of *eps8L1* overlapped with *eps8L2*, but not with *eps8*, whose expression, instead, was restricted to the cartilage that gives rise to the nasal bones (Figure 6B). Weak expression of *eps8L1* was also observed in the skin (Figure 6B). The expression of *eps8L3* was confined to the inner mucosal layer of the intestine, where it was first detectable at 14.5 dpc (Figure 6C), rising to relatively high levels by 16.5 dpc (Figure 6C). Expression of *eps8L3* was higher in the hindgut than in the mid and foregut (Figure 6A). In this location, *eps8L3* was coexpressed with *eps8* and *eps8L2*, whereas *eps8L1* was completely absent (Figure 6C).

Northern blot analysis and RT-PCR were used to assess the expression of *eps8Ls* in adult mice (Table 2). Consistently with data obtained in embryos, *eps8* and *eps8L2* showed a relative broad pattern of expression. In contrast, *eps8L1* and *eps8L3* displayed a restricted expression pattern also in adult tissues (Table 2). Of note, both in developing and adult mice, *eps8* was the sole family member that displayed detectable expression in the brain (Tables 1 and 2).

DISCUSSION

In this study, we describe a new family of proteins, the *eps8* family, whose founding member, *eps8*, was previously shown to be a key component of a multimolecular complex endowed with RacGEF activity (Scita *et al.*, 1999; Innocenti *et al.*, 2003). Our results show that members of this family link growth factor stimulation to actin organization, generating functional redundancy in the pathways that regulate actin cytoskeletal remodeling.

Members of the *eps8* family share a modular organization consisting of a putative PTB domain, a central SH3 domain and a C-terminal effector region. The SH3 domains of *eps8Ls* display unique binding preferences for peptides containing a proline-X-X-aspartate-tyrosine (pXXDY) consensus and constitute a phylogenetically distinct subfamily within the SH3 domain family (Mongiovi *et al.*, 1999). In keeping with this, all *eps8Ls* can associate in vivo, with comparable efficiencies, to Abi1, a known interactor of the SH3 of *eps8* that is required for the assembly of the multimeric Rac-GEF complex. However, in the case of *eps8L3*, this is not suffi-

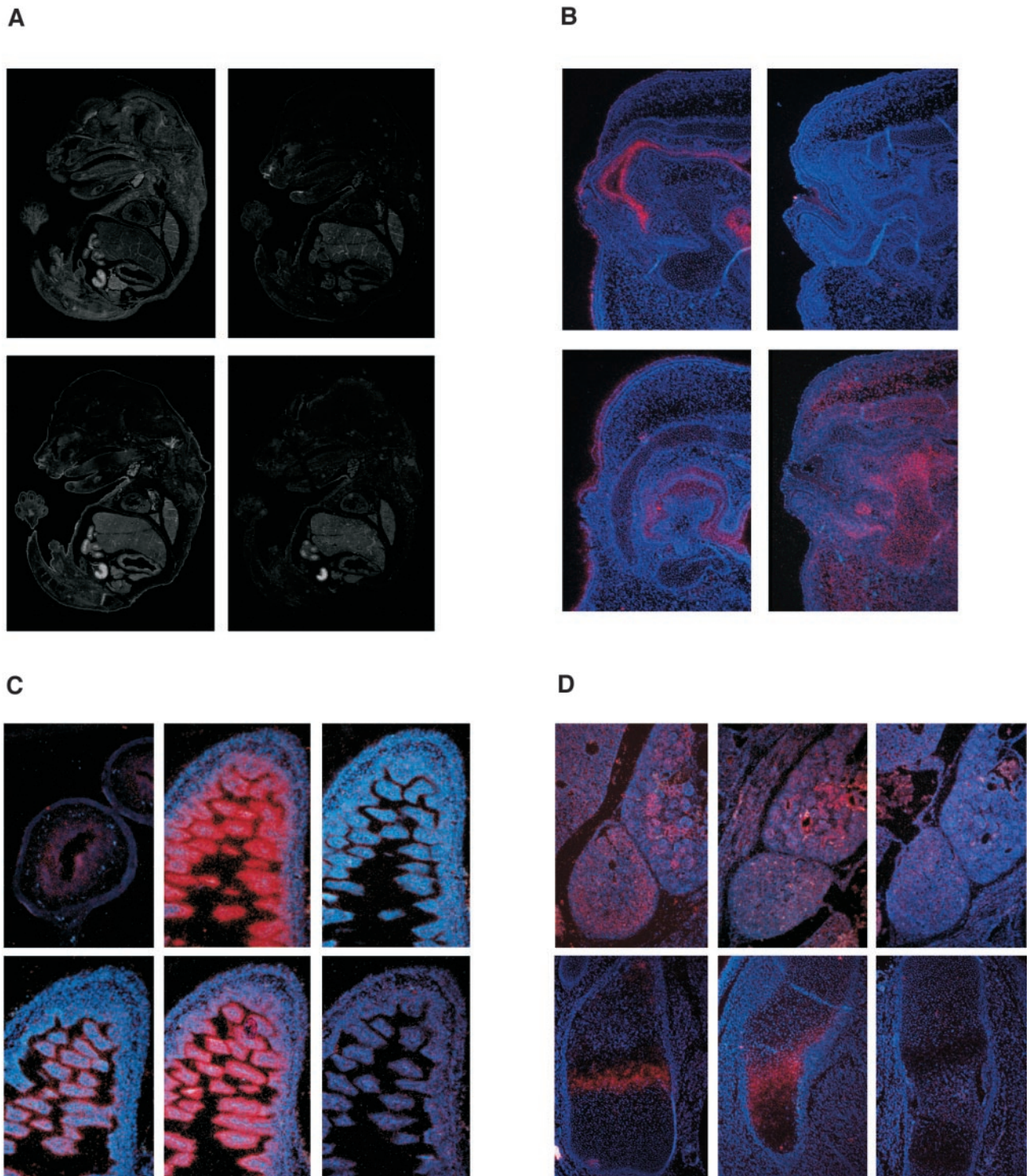


Figure 6. *Eps8Ls* displays a distinct expression pattern in the developing mouse embryo. (A) Expression of the *eps8Ls* in 16.5 dpc mouse embryos. Low-magnification photomicrographs demonstrate nearly ubiquitous expression of *eps8* and widespread expression of *eps8L2* in 16.5 dpc mouse embryos. *Eps8L1* and *eps8L3* in contrast show a very restricted expression, limited to the naris externis and the developing gut, respectively. (B) Expression of *eps8Ls* in the nasal anlage. The *eps8L1* antisense probe (a), but not the sense control (b) displays a specific signal on the naris externis. Expression of *eps8L2* was localized in the naris externis, in the surrounding nasal cartilage and in the skin (c). *Eps8* expression was limited to the nasal cartilage (d). (C) Expression of *eps8Ls* in the hindgut. An antisense probe for *eps8L3* detects expression in the embryonic gut starting from 14.5 dpc (a), increasing to very high levels at 16.5 dpc (b). No signal is obtained with the corresponding sense probe (c). *Eps8* (d) and *eps8L2* (e), but not *eps8L1* (f) were detected in the inner mucosal layer of the gut. (D) Examples of overlapping expression of *eps8* and *eps8L2*. An antisense probe for *eps8* detects expression in the kidney and adrenal gland (a), and in the cartilage primordium of the developing femur (d). *Eps8L2* displayed a similar expression pattern (b, kidney and adrenal gland; e, cartilage primordium of the scapula). No signal was detected with the sense probe for *eps8L2* (c and f). The red color represents the in situ hybridization signal and the blue color shows the nuclei stained with the Hoechst 33258 dye.

Table 1. Expression of *eps8* family members in the 16.5 dpc mouse embryo

	<i>eps8</i>	<i>eps8L1</i>	<i>eps8L2</i>	<i>eps8L3</i>
Brain	+	-	-	-
Dorsal root ganglia	-	-	-	-
External naris	-	+	+	-
Heart	-	-	-	-
Lung	±	-	±	-
Liver	-	-	±	-
Kidney	+	-	+	-
Adrenal gland	+	-	+	-
Salivary gland	+	-	+	-
Esophagus	±	-	±	-
Stomach	±	-	+	-
Gut	±	-	+	+
Bladder	±	-	±	-
Muscle	-	-	-	-
Cartilage	+	-	+	-
Skin	±	±	+	-

Expression levels were assessed by in situ hybridization and are expressed in a semiquantitative fashion: (-) negative, (±) weakly positive, (+) positive.

cient to form an active RacGEF or to substitute for *eps8* in *eps8* null MEFs, suggesting that additional structural or functional features are required. The C-terminal effector region fulfills this requirement. This region, in *eps8*, is essential for interaction with Sos-1 and its activation as a RacGEF. In addition, it mediates a direct interaction with F-actin, determining the intracellular localization of the protein at sites where actin is highly dynamic (Scita *et al.*, 2001). Consistently, *eps8L1* and *eps8L2*, which display the highest homology to the *eps8* in the C-terminal effector region, are capable of restoring ruffling response to RTK stimulation in *eps8* null fibroblasts. Moreover, they can associate to actin in vivo and accumulate in PDGF-induced ruffles, thus further

Table 2. Expression of *eps8* family members in mouse adult tissues

Tissue	Method ^a	<i>eps8</i>	<i>eps8L1</i>	<i>eps8L2</i>	<i>eps8L3</i>
Testis	N, P	+	±	±	++
Ovary	P	+	±	+	±
Placenta	P	+	++	++	++
Liver	N, P	-	-	±	-
Skin	P	+	+	++	±
Adrenal gland	P	+	±	+	+
Salivary gland	P	+	±	+	±
Mammary gland	P	+	+	±	-
Lymph node	P	±	±	±	-
Thymus	P	-	±	±	±
Bone marrow	P	+	+	-	-
Brain	N	+	-	-	-
Kidney	N	++	±	++	-
Heart	N	±	±	-	-
Intestine	N	++	-	++	++
Stomach	N	+	++	++	±

^a Expression levels were measured by either Northern blot analysis (N) or semiquantitative PCR (P). Results refer to intragene comparison (after normalization for control GAPDH) and are expressed in a semiquantitative fashion: (-) negative, (±) weakly positive, (+) positive, (++) strongly positive.

supporting the notion that relocalization of *eps8Ls*-based complexes is a prerequisite for their function in vivo. The observation that *eps8L3*, instead, does not contain a functional effector domain raises the question of its physiological role. We note that *eps8L3* displays a punctuate, cytoplasmic staining reminiscent of proteins typically associated with vesicular organelles. Thus, it is tempting to speculate that *eps8L3* may be involved in intracellular trafficking, a possibility currently under investigation.

Eps8L1 and *eps8L2*, similarly to *eps8*, mediate RTK-dependent actin remodeling, most likely by entering into a RacGEF complex with Abi1 and Sos-1. The existence of a family of proteins capable of forming multisubunit RacGEFs underscores additional complexity in the regulation of Rac. Multiple mechanisms have, indeed, been implicated in the activation of Rac by active plasma membrane receptors (Di Fiore and Scita, 2002). These rely mainly on the existence of a variety of Rac-specific GEFs (Schmidt and Hall, 2002), which are all comparably efficient in mediating Rac activation. Physiologically, however, the intracellular and the tissue distribution of the various RacGEFs is thought contribute to their specificity. The tissue distribution of the *eps8* family of proteins supports this hypothesis.

Finally, the lack of overt phenotypic alterations in mice lacking *eps8* can be rationalized in light of the observation that, at the mRNA level, the pattern of expression of *eps8* overlaps with that of *eps8L2*, which, in turn, exhibits complete functional redundancy with *eps8*, at the protein level. It is of note that in organisms where *eps8* gene duplication has not yet occurred or where only two members have evolved (*Caenorhabditis elegans* and in *Drosophila*, respectively) the genetic removal of *eps8* leads to a number of developmental defects (A. Croce and P.P. Di Fiore, unpublished observations, and K. Moffat, personal communication). In mammalian systems, altered phenotypes (impaired Rac activation and actin remodeling in response to growth factor stimulation), caused by genetic removal of *eps8*, could be unmasked only in mouse embryo fibroblasts, which express exclusively *eps8* (Scita *et al.*, 2001, and this study). This suggests that possible alterations in *eps8* null mice might be confined to those tissues or organs that do not show redundant expression of other family members, such as the brain. The lack of gross morphological defects in this organ (Scita *et al.*, 2001, and our unpublished results), however, points to the possibility that more subtle phenotypes, such as behavioral deficits, might be linked to *eps8* removal. We are currently investigating this possibility.

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