The endocytic protein intersectin is a major binding partner for the Ras exchange factor mSos1 in rat brain

Xin-Kang Tong1, Natasha K.Hussain1, Elaine de Heuvel1, Alexei Kurakin2, Elia Abi-Jaoude3, Christopher C.Quinn4, Michael F.Olson5, Richard Marais5, Danny Baranes3, Brian K.Kay2 and Peter S.McPherson1,3,6

1Department of Neurology and Neurosurgery, Montreal Neurological Institute and 3Department of Anatomy and Cell Biology, McGill University, Montreal, QC H3A 2B4, Canada, 2Department of Pharmacology, University of Wisconsin, Madison, WI 53706, 4Section of Neurobiology, Yale University School of Medicine, New Haven, CT 06510, USA and 5CRC Center for Cell and Molecular Biology, Institute of Cancer Research, London SW3 6JB, UK

© European Molecular Biology Organization 1263

Introduction

Src homology 3 (SH3) domains, 50–70 amino acid modules, mediate protein–protein interactions by binding specific proline-rich peptide sequences within cellular ligands. A well studied example of an SH3 domain-mediated interaction is the recruitment of the Ras guanine-nucleotide exchange factor, mammalian son-of-sevenless (mSos), to the plasma membrane by the adaptor Grb2 (Baltensperger et al., 1993; Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). mSos contains a proline-rich C-terminus that mediates its stable association with the SH3 domains of Grb2, a protein composed of two SH3 domains flanking a central SH2 domain (Lowenstein et al., 1992). Through its SH2 domain, Grb2 interacts with activated growth factor receptors either directly or through the adaptor protein SHC (Ceresa and Pessin, 1998), thereby recruiting mSos to the membrane where it, in turn, activates Ras (Aronheim et al., 1994; Quilliam et al., 1994). However, recent evidence suggests that mSos can be recruited to the membrane by additional mechanisms. Specifically, activation of Ras by mSos can occur in the absence of Grb–mSos interactions (Karlovich et al., 1995; McCollam et al., 1995; Wang et al., 1995), suggesting that other domains of mSos, such as its Dbl homology (DH) or pleckstrin homology (PH) domains, play important roles in mSos membrane targeting and activity (Chen et al., 1997; Qian et al., 1998), or that targeting of mSos to the membrane can be mediated through the actions of other adaptor proteins.

SH3 domain-mediated protein–protein interactions also function in vesicular trafficking, particularly in endocytosis (McPherson, 1999). For example, intersectin, a novel protein involved in clathrin-mediated endocytosis, contains two N-terminal Ephexin homology (EH) domains, a central helical region and five C-terminal SH3 domains (termed SH3A–E) (Guipponi et al., 1998; Yamabhai et al., 1998). Intersectin is homologous to Dap160 in Drosophila (Roos et al., 1993; Egan et al., 1993). Through its SH2 domain, Grb2 interacts with activated growth factor receptors either directly or through the adaptor protein SHC (Ceresa and Pessin, 1998), thereby recruiting mSos to the membrane where it, in turn, activates Ras (Aronheim et al., 1994; Quilliam et al., 1994). However, recent evidence suggests that mSos can be recruited to the membrane by additional mechanisms. Specifically, activation of Ras by mSos can occur in the absence of Grb–mSos interactions (Karlovich et al., 1995; McCollam et al., 1995; Wang et al., 1995), suggesting that other domains of mSos, such as its Dbl homology (DH) or pleckstrin homology (PH) domains, play important roles in mSos membrane targeting and activity (Chen et al., 1997; Qian et al., 1998), or that targeting of mSos to the membrane can be mediated through the actions of other adaptor proteins.

Keywords: clathrin/EH domain/endocytosis/Ras/SH3 domain
SH3 domains from the endocytic proteins endophilin I (de Heuvel et al., 1997; Ringstad et al., 1997), amphiphysin II (Ramjaun et al., 1997) and syndapin I (Qualmann et al., 1999), selectively inhibit late events of clathrin-coated vesicle formation involving membrane fission, intersectin SH3A was unique in its ability to block earlier stages (Simpson et al., 1999).

To follow up on this observation, we performed overlay assays with a glutathione S-transferase (GST)–SH3A fusion protein and identified an SH3A domain-specific binding partner of 170 kDa (p170). Analysis of the optimal ligand preference of the SH3A domain suggested that p170 was mSos1, a result confirmed by a variety of methods. Like intersectin, mSos1 is enriched in nerve terminals, and the two proteins strongly co-immunoprecipitate from embryonic brain extracts. Intersectin and Grb2 compete for binding to mSos1, and sucrose-density gradient analysis indicates that in brain, intersectin and mSos1 form a stable complex that primarily excludes Grb2. Interestingly, overexpression of the SH3 domains of intersectin strongly attenuates epidermal growth factor (EGF)-dependent GTP loading of Ras. Thus, it appears that intersectin may function as a scaffold molecule for protein components of both the endocytic machinery and signal transduction pathways.

Results
Identification of an intersectin SH3A domain-specific binding partner
In previous collaborative studies, we determined that the SH3A domain of intersectin is unique among a wide variety of SH3 domains from endocytic proteins, including the other SH3 domains of intersectin, in its ability to block intermediate steps leading to the formation of a sequestered clathrin-coated pit (Simpson et al., 1999). We thus performed overlay assays using a GST–SH3A fusion protein in order to identify specific SH3A-binding partners. The intersectin SH3A domain, as well as the SH3 domains from amphiphysin I and II and endophilin I, II and III, each interact with bands at 100 and 145 kDa (Figure 1). The 100 and 145 kDa bands are most likely to be dynamin and synaptojanin, respectively, which have been observed to interact with the SH3 domains used here in several studies (Micheva et al., 1997; Ramjaun et al., 1997; Yamabhai et al., 1998). Interestingly, SH3A also interacted with an additional band at 170 kDa (Figure 1). The 170 kDa band (p170) was readily detectable at concentrations of fusion protein as low as 100 ng/ml, but was not detected in overlay assays with the other four SH3 domains of intersectin nor with the SH3 domains from the amphiphysins or the endophilins, at the same concentrations (Figure 1 and data not shown).

GST–SH3A overlays of tissue extracts revealed that p170 was most enriched in brain, although significant levels were detected in all tissues tested, and in several tissues p170 was the only protein species identified (Figure 2A). Within the brain, subcellular fractionation revealed that p170 was present in both soluble and particulate fractions. Its overall distribution was similar to that of dynamin and synaptojanin (Figure 2B), proteins that are enriched in pre-synaptic nerve terminals (McPherson et al., 1994a), although a small pool of the protein was found in the P1 fraction, possibly in association with nuclei.

Identification of p170 as mSos1
SH3 domains interact with proline-rich peptides with the core sequence PXXP (where X is any amino acid). To define the ligand specificity of the intersectin SH3A
Intersectin–mSos1 interactions

Fig. 3. Identification of consensus-binding sites for SH3A in mSos1.
(A) The sequences of 12 peptides, affinity selected from a phage-displayed X6PXXPX6 peptide library (where X is any amino acid) using the SH3A domain from intersectin, are listed. The peptides define the SH3A-binding consensus sequence Pp(V/I)PPR, where p is typically proline. (B) Two putative ligand sites for the SH3A domain, PPVPPR, occur within human Sos1 at sequences between amino acids 1148 and 1161 and 1287 and 1300, and a third related site is found between amino acids 1208 and 1221, as indicated. (C) The segments of human Sos1 shown in (B) were fused to the N-terminus of secreted alkaline phosphatase and tested for binding to GST fusion protein encoding the individual SH3 domains of intersectin or to GST alone.

Fig. 4. Conformation of p170 as mSos1. Aliquots of antiserum C23 against mSos1, as well as normal rabbit sera (NRS), were precoupled to protein A–Sepharose beads. Precoupled beads were washed, incubated overnight at 4°C with an E18 rat brain cytosolic fraction and extensively washed the next day. The material specifically bound to the beads (top two blots labeled beads) was eluted and processed, along with an aliquot of the cytosolic extract (starting material, SM), for Western blot analysis with the anti-mSos1 antibody (top blot), or for SH3A domain overlay (middle blot). The proteins that did not bind to the beads (void) were also subjected to an SH3A domain overlay assay (bottom blot).

mSos1 (Figure 4, top panel) and the immunoprecipitated protein is strongly reactive in a GST–SH3A domain overlay (Figure 4, middle panel). Furthermore, immunodepletion of mSos1 from rat brain extracts depletes p170 (Figure 4, bottom panel), confirming that this interacting protein is mSos1. Moreover, the tissue distribution of mSos1 as determined by Western blotting (data not shown) closely parallels that of p170 (demonstrated in Figure 2A), suggesting that mSos1 is equivalent to p170 in all tissues tested.

Intersectin and mSos1 interact in neurons

To explore the potential interaction between mSos1 and intersectin in situ, we first performed immunoperoxidase staining to examine the regional distributions of the two proteins. Intersectin and mSos1 were expressed in discrete and highly overlapping neuronal populations in rat brain cortex, caudate and ventral pallidum (data not shown). Previously, it was demonstrated that Dap160, the Drosophila homologue of intersectin, was present in nerve terminals from third instar larvae (Roos and Kelly, 1998). Staining of 1-day-old neuron cultures from the CA3 region of hippocampus revealed that intersectin is broadly distributed in neurons including an enriched pool in growth cones (Hussain et al., 1999; data not shown). Interestingly, like intersectin, mSos1 is present throughout the hippocampal neurons and is enriched at the tip of their growth cones (Figure 5A). This enrichment is due to an increase in the density of mSos1 positive puncta in the growth cone area (Figure 5B) as well as an increase in the content of mSos1 per puncta (Figure 5C).
Fig. 5. Interaction of intersectin and mSos1 in situ. (A) mSos1 is found in puncta that are expressed throughout the cell body and neurites of hippocampal neurons maintained in culture for 1 day. The puncta are relatively homogeneous along the neurite but are enriched at growth cones (arrows). (B) A higher magnification image of the growth cone in (A) reveals that the density of mSos1 positive puncta is higher at the tip of the growth cone than along the neurite. (C) Color coding of fluorescent intensities of the area in (B) indicates that the intensity of individual mSos1 positive puncta is higher in the growth cone (red) than in other regions of the dendrite. Scale bar: (A), 10 μM; (B and C), 1.5 μM. (D) Aliquots of antisera 2173 and 2174 against intersectin, as well as pre-immune 2173 sera (NRS), precoupled to protein A–Sepharose beads, were incubated overnight at 4°C with an E18 rat brain cytosolic fraction. The beads were extensively washed and the material specifically bound to the beads was eluted and processed for Western blot analysis with polyclonal antibodies against mSos1 and intersectin. (E) As for (D) except that immunoprecipitations were performed from Triton X-100 solubilized membrane fractions. The antigens and their approximate molecular weights (kDa) are denoted with arrows on the right and left sides of the figure, respectively.

the enrichment of mSos1 in the intersectin immunoprecipitated samples relative to the starting material). mSos1 also strongly co-immunoprecipitates with intersectin from a Triton X-100 solubilized particulate fraction, suggesting that the proteins also interact at the membrane (Figure 5E).

**Intersectin competes with Grb2 for binding to mSos1**

To explore the relationship between mSos1, intersectin and Grb2, we immunoprecipitated mSos1 from soluble E18 rat brain extracts and performed Western blots with antibodies against the various proteins. Intersectin-l, intersectin-s and Grb2 all co-immunoprecipitate with mSos1 (Figure 6). However, whereas intersectin-l was enriched in the immunoprecipitate relative to the starting material, Grb2 was not (Figure 6), even though in overlay assays, GST–Grb2 and GST–SH3A fusion proteins interact equally well with blotted mSos1 (data not shown). One plausible explanation is that intersectin and Grb2 compete for binding to mSos1, and the greater co-immunoprecipitation of intersectin versus Grb2 is due to intersectin being more abundant than Grb2 in the brain.

mSos1 contains many SH3 domain-binding consensus sequences (i.e. PXXP). To determine if Grb2 and SH3A compete for binding to mSos1, GST–SH3A was used in
In vitro competition binding assays of intersectin SH3A domain and Grb2 to mSos1. E18 rat brain cytosolic fractions were separated on SDS–PAGE, transferred to nitrocellulose membranes, and strips of the membrane were processed for overlay assays with GST–SH3A at 200 ng/ml. The overlay assays also contained His6-tagged Grb2 at increasing molar ratios of Grb2 to SH3A ranging from 0:1 (control) to 100:1 as indicated at the bottom of the figure. An example of the overlay results is shown at the top of the graph. The intensity of the stained mSos1 band was determined by densitometry of the autoradiographs and was normalized to control. The bars represent the mean ± SEM from three separate experiments.

Overlay assays on E18 rat brain extracts in the presence of increasing concentrations of His6-tagged Grb2. Addition of Grb2 significantly reduced SH3A binding to mSos1 in a concentration-dependent manner, with binding completely eliminated at a molar ratio of 5:1 (Figure 7). Thus, Grb2 and SH3A may compete for binding to the same or overlapping site(s) in mSos1 although steric hindrance cannot be excluded. Previous mapping experiments have demonstrated that Grb2 binds to human Sos1 at three sites (PXXP cores at amino acids 1151–1154, 1212–1215 and 1290–1293) (Li et al., 1993; Rozakis-Adcock et al., 1993). The first and third of these sites are the same as those predicted by phage-display experiments to be ligand sites for the SH3A domain (Figure 3).

To explore this issue further, we infected undifferentiated PC12 cells with a recombinant adenovirus encoding the five tandem SH3 domains of intersectin fused to GFP (GFP–SH3A–E). Following expression of the construct, soluble cell extracts were prepared and processed for anti-GFP immunoprecipitation followed by blotting for co-immunoprecipitating mSos1. The anti-GFP antibody immunoprecipitates the GFP–SH3A–E fusion protein (which migrates with the expected molecular mass of 85 kDa) (Figure 8, middle panel) and leads to a strong co-immunoprecipitation of mSos1 (Figure 8, top panel). However, Grb2 is not detected in the immunoprecipitated samples (Figure 8, bottom panel), consistent with the observation that intersectin and Grb2 bind competitively to mSos1.

We next investigated the association of intersectin with mSos1 by subjecting a soluble rat brain protein extract to sucrose-density gradient centrifugation and analyzing the distribution of the proteins by Western blotting (Figure 9, top panels; the gradient was fractionated from the bottom such that lower fraction numbers correspond to a greater density of sucrose). Intersectin-l was most abundant in fraction 9, but was detectable even in the densest gradient fractions. Interestingly, mSos1 migrated on the gradients with a molecular mass that was similar, but not identical, to that of intersectin-l. Both intersectin-l and mSos1 exhibited higher native molecular masses than either
clathrin triskelia (~650 kDa) or the AP-2 heterotetramer (~300 kDa), the migratory positions of which were revealed by Western blots of the clathrin heavy chain or the α-subunit of AP-2, respectively. Intriguingly, Grb2 remained at the top of the gradient and was effectively separated from mSos1.

To confirm that the extremely large native molecular mass for mSos1 was due, at least in part, to its interactions with intersectin, we performed co-immunoprecipitation experiments from the gradient fractions (Figure 9, bottom panels). mSos1 could be co-immunoprecipitated with intersectin from each of the gradient fractions containing the two proteins. Overall, the distribution of mSos1 seemed to parallel more closely that of intersectin-l than intersectin-s. Whether this reflects a relatively higher affinity of intersectin-l versus intersectin-s for mSos1 or is due to the apparently greater abundance of intersectin-l in brain is not known. Regardless, these data suggest that in brain, a pool of the mSos1 protein is in a stable, large molecular weight complex that includes intersectin but that predominantly excludes Grb2.

**Overexpression of the SH3 domains of intersectin blocks Ras activation**

To determine if the SH3 domain-mediated interactions of intersectin with mSos1 might affect Ras activation, we measured the levels of Ras–GTP in cells with a GST fusion protein encoding the Ras-binding domain of Raf1. This fusion protein binds to Ras–GTP but has significantly weaker affinity for Ras in its GDP-bound form (de Rooij and Bos, 1997; Marais et al., 1998). In HEK-293 cells treated with EGF for 2 min, a substantial pool of the total cellular Ras binds to GST–Raf1 beads as detected by Western blots with a Ras monoclonal antibody, whereas little binding is detected in the absence of EGF stimulation (Figure 10, top panel). Overexpression of GFP has little effect on the ability of EGF to stimulate GTP loading on Ras (Figure 10, bottom panel; note that the ratio of Ras bound to the beads relative to that in the starting material is similar to that seen in the EGF-challenged non-infected cells in the top panel). In contrast, overexpression of the GFP–SH3A–E construct sharply attenuates EGF-activated GTP loading on Ras (Figure 10, bottom panel). There is no effect of the overexpression of either construct on the basal activity of Ras measured in the absence of EGF (data not shown).

**Discussion**

The identification and characterization of accessory proteins in endocytosis has received a great deal of attention in recent years. One of these proteins has been identified in *Drosophila*, *Xenopus* and mammals and is variously referred to as Dap160, intersectin, Ese and EHSH (Guipponi et al., 1998; Roos and Kelly, 1998; Yamabhai et al., 1998; Hussain et al., 1999; Okamoto et al., 1999; Sengar et al., 1999). This family of proteins contains two EH domains, followed by a putative helical region and four to five tandem SH3 domains. Intersectin is localized in part to clathrin-coated pits and interacts *in vivo* through its EH and SH3 domains with several proteins involved in clathrin-mediated endocytosis. In addition, intersectin interacts with another accessory component of the endocytic machinery, Eps15, through its coiled-coil region (Sengar et al., 1999). Thus, intersectin may function as a scaffolding protein in the assembly of clathrin-coated pits.

We have now identified mSos1 as a protein that interacts with the SH3A domain of intersectin. Previously, Leprince et al. (1997) had isolated amphiphysin II in a two-hybrid screen using the proline-rich C-terminus of human Sos1. In experiments reported here, we have found in a side-by-side comparison using overlay assays that the SH3A domain of intersectin can bind mSos1 better than the other four SH3 domains from intersectin or than the SH3 domains from amphiphysin I and II and endophilin I, II and III. These data suggest that intersectin can bind mSos1 with higher affinity than other SH3 domain-containing endocytic proteins but do not rule out a role for amphiphysin II in mSos1 function. Intersectin and mSos1 are both expressed at high levels in growth cones of developing neurons, and immunoprecipitation analysis from embryonic brain extracts confirms that the two proteins are associated *in situ*. In fact, sucrose-density gradients, coupled with co-immunoprecipitation analysis, suggest that in brain, intersectin and mSos1 are components of a large molecular mass protein complex that primarily excludes Grb2. Thus, the interaction of the SH3A domain with mSos1 appears specific and is likely to be biologically relevant.

Given that intersectin is involved in the formation of clathrin-coated pits, and that the intersectin SH3A domain interacts specifically with cellular targets that function early in the formation of a clathrin-coated bud (Simpson et al., 1999), it is interesting to speculate that mSos1 may also play a role in clathrin-coated pit formation, possibly through activation of Ras. Many vesicular budding events that are mediated by coat proteins are initiated by the activation of small GTP-binding proteins through the actions of guanine-nucleotide exchange factors (Schekman...
and Orci, 1996). Very recently, Nakushima et al. (1999) have demonstrated that overexpression of mutant forms of Ras, as well as the small GTP-binding protein Rap, which plays a major role in mediating downstream Ras function (Feig et al., 1996), blocks the internalization of the EGF receptor. Furthermore, mSos1 can activate Rac (Nimnal et al., 1998; Scita et al., 1999), which has been implicated in transferrin receptor endocytosis (Lamaze et al., 1996). Finally, it should be noted that the long form of intersectin, which is generated by alternative splicing in neuronal tissues, contains DH, PH and C2 domains (Guipponi et al., 1998; Hussain et al., 1999; Okamoto et al., 1999; Sengar et al., 1999). Comparison of the primary structure of the DH and PH domains with other proteins suggests that the long form of intersectin may be a guanine-nucleotide exchange factor for Rho. Further work is necessary to clarify the involvement of GTP-binding proteins in clathrin-mediated endocytosis.

Another possible function of the intersectin–mSos1 complex is to couple the molecular machineries for endocytosis and signal transduction. For example, it has been demonstrated that dynamin-dependent endocytosis of the EGF receptor is necessary for EGF-dependent activation of the MAP-kinase pathway (Vieira et al., 1996). The ability of insulin-like growth factor-1 (IGF-1) to activate the SHC/MAP-kinase pathway, but not the insulin receptor substrate 1 pathway, is also dependent on clathrin-mediated endocytosis of the IGF receptor (Chow et al., 1998). Furthermore, endocytosis of the β2-adrenergic receptor is necessary for coupling to MAP-kinase activation (Daaaka et al., 1998; Luttrell et al., 1999). Specifically, overexpression of a mutant form of β-arrestin, which prevents the β2-adrenergic receptor from targeting to clathrin-coated pits, blocks agonist activation of MAP kinase. Thus, it is possible that the clathrin-coated pit can function as a membrane microdomain, directing the assembly of signaling complexes, much as has been proposed for caveoli (Anderson, 1998). In fact, activation of the EGF receptor can lead to the formation of signaling complexes that include mSos1 and which are localized largely in endosomes (Di Guglielmo et al., 1994).

Given the evidence for a link between endocytosis and signaling, it is interesting to speculate that intersectin could play an important role in bringing together endocytic proteins such as dynamin, with signaling molecules such as mSos1. In fact, our data demonstrating that overexpression of the SH3 domains of intersectin functions in a dominant-negative manner to block EGF-dependent Ras activation strongly support a role for intersectin in cell signaling. Moreover, human intersectin has been found to interact by yeast two-hybrid screening with the protooncogene product, c-Cbl (Robertson et al., 1997), a guanine-nucleotide exchange factor for Rho. Further work is necessary to clarify the involvement of GTP-binding proteins in clathrin-mediated endocytosis.

Materials and methods

Antibodies
Polyclonal antibodies against intersectin were described previously (Hussain et al., 1999). Antibodies against clathrin (Simpsom et al., 1996) and α-adaptin (Robinson, 1987) were a generous gift from Dr. Margaret Robinson (Cambridge University). Polyclonal antibodies against mSos1 (C23) and GFP were purchased from Santa Cruz Biotechnology and Molecular Probes, respectively. Monoclonal antibodies against Grb2 and Ha-Ras were purchased from Transduction Laboratories.

Generation of fusion protein constructs
GST fusion protein constructs encoding the SH3 domains of amphiphasin I (David et al., 1994), amphiphasin II (Ramauna et al., 1997), endophilin I (Micheva et al., 1997), the individual SH3 domains of intersectin (Yamabhali et al., 1998) and full length Grb2 (McPherson et al., 1994b) were described previously. The SH3 domains of endophilin II and III were generated by PCR using full length cDNAs (Sparks et al., 1996b) as templates in PCR with Vent DNA Polymerase (New England Biolabs) and the following primer pairs: endophilin II, forward primer 5′-GCGGATCCGACCAGCTGCAAG (nucleotides 919–936) and reverse primer 5′-GGGGAATTCACATGCGAGCCAGGACCCAG (nucleotides 1104–1108); endophilin III, forward primer 5′-GGGGAATTCACATGCGAGCCAGGACCCAG (nucleotides 1104–1108). The resulting PCR products were subcloned into the corresponding BamHI–EcoRI and BamHI–SmaI sites of pGEX-2T (Pharmacia Biotech Inc.), respectively. For His6-tagged Grb2, the GST–Grb2 construct in pGEX2T (Pharmacia Biotech Inc.) and His6-tagged Ha-Ras were purchased from Transduction Laboratories.

Generation of recombinant adenovirus
A recombinant adenovirus encoding the five tandem SH3 domains of intersectin with an N-terminal GFP tag was produced using the system described previously (McPherson et al., 1998). For competition experiments, GST–SH3A endophilin I (Micheva et al., 1997), the individual SH3 domains of intersectin (Yamabhali et al., 1998) and full length Grb2 (McPherson et al., 1994b) were described previously. The SH3 domains of endophilin II and III were generated by PCR using full length cDNAs (Sparks et al., 1996b) as templates in PCR with Vent DNA Polymerase (New England Biolabs) and the following primer pairs: endophilin II, forward primer 5′-GCGGATCCGACCAGCTGCAAG (nucleotides 919–936) and reverse primer 5′-GGGGAATTCACATGCGAGCCAGGACCCAG (nucleotides 1104–1108); endophilin III, forward primer 5′-GGGGAATTCACATGCGAGCCAGGACCCAG (nucleotides 1104–1108). The resulting PCR products were subcloned into the corresponding BamHI–EcoRI and BamHI–SmaI sites of pGEX-2T (Pharmacia Biotech Inc.), respectively. For His6-tagged Grb2, the GST–Grb2 construct in pGEX2T was digested with BamHI plus EcoRI enzymes and the liberated insert was subcloned into pTmHisA (Invitrogen) using the same enzymes.

Tissue and subcellular distribution experiments
Various rat tissues were homogenized in buffer A (10 mM HEPES–OH pH 7.4, 0.5 M NaCl, 0.23 mM phenylmethylsulfonylfluoride, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin). Post-nuclear supernatants were obtained by centrifugation for 5 min at 80 000 g_{\text{max}} and were then separated into cytosolic and membrane fractions by ultracentrifugation at 205 000 g_{\text{max}} for 1 h at 4°C. Differential centrifugation of rat brain extracts leading to the defined subcellular fractions in Figure 2B was as described previously (McPherson et al., 1994a). Dissociated cell cultures were prepared from the CA3 and dentate regions of hippocampi from P1 rat pups as described (Hussain et al., 1999).

Overlay assays
Overlay assays with GST fusion proteins were performed as described (McPherson et al., 1994b). For competition experiments, GST–SH3A was mixed with increasing concentrations of His6-tagged Grb2 immediately prior to addition to the nitrocellulose transfers.

1269
**Immunoprecipitation analysis**

Cytosolic extracts, prepared as described above, were made to 1% in Triton X-100 and preclarred by incubation with protein A-Sepharose. The precleared samples were then incubated with various antibodies precoupled to protein A-Sepharose, and following an overnight incubation at 4°C, were washed three times in buffer A containing 1% Triton X-100 before the proteins specifically bound to the beads were eluted and processed for SDS-PAGE. In some cases, membrane fractions, generated as described above, were incubated for 30 min at 4°C in buffer A containing 1% Triton X-100, the samples were centrifuged at 205 000 g, for 1 h at 4°C, and the soluble supernatants were used for immunoprecipitation. For immunoprecipitations from infected cells, PC12 cells were incubated with recombinant adenovirus encoding GFP–SH3A–E in 6-well plates at a multiplicity of infection of 100. The medium was changed the next day, and the cells were left for an additional 48 h. The cells were then washed, scraped off the plate in buffer B (buffer A with 5 mM EGTA, 5 mM EDTA, 50 mM sodium fluoride, 20 mM sodium pyrophosphate and 1 mM sodium vanadate), lysed, Triton X-100 was added to 1%, and the extracts were centrifuged in a Beckman TLA 100.2 rotor at 75 000 rpm. for 15 min. The solubilized extracts were preclarred with protein A-Sepharose before the addition of anti-GFP antibody precoupled to protein A-Sepharose. Following a 2 h incubation, the samples were washed three times in buffer B containing 1% Triton X-100, and the proteins specifically bound to the beads were eluted and processed for SDS-PAGE.

**Sucrose-density gradient centrifugation**

Proteins from a rat brain cytosolic fraction were layered onto linear gradients of 5–20% sucrose prepared in buffer A. The gradients were centrifuged in a SW50.1 50 V39 vertical rotor (Sorvall) at 195 000 g for 2.5 h and were then collected into 19 fractions of 2 ml each from the bottom. Aliquots of each fraction were analyzed by SDS–PAGE and Western blotting. For immunoprecipitations, 750 μl of each fraction were made to 1% in Triton X-100 before the addition of protein A-Sepharose beads that had been precoupled to intersectin antibody. Following an overnight incubation at 4°C, the samples were washed three times in buffer A containing 1% Triton X-100, and the proteins specifically bound to the beads were eluted and processed for SDS-PAGE.

**Determination of SH3A binding specificity by phage display**

GST–SH3A was used to affinity select phage from a library of bacteriophage M13 displaying combinatorial peptides at the N-terminus of mature protein III. The library displayed peptides of the form X6PXXPX6, where X represents any of the 20 naturally occurring amino acids and P represents invariant proline residues (Sparks et al., 1996a). Three different 14mer peptides from mSos1 were fused to the N-terminus of human amphiphysin, a protein similar to the yeast proteins Rvs167 and Rvs161.

**Ras assays**

Recombinant adenoviruses encoding GFP–SH3A–E or GFP alone were added to HEK-293 cells plated on poly-L-lysine substrate in 10-cm² dishes at a multiplicity of infection of 100. The medium was changed the next day, and the cells were left for an additional 48 h. The cells were then transferred into serum-free medium (along with non-infected controls), and following an overnight incubation, were treated with 100 nM EGFr for 2 min at 37°C or were left untreated. The cells were then washed and immediately processed for Ras assays as described (de Rooij and Bos, 1997; Marais et al., 1998).

**Acknowledgements**

We would like to thank Dr Channing Der (University of North Carolina) for analysis of the intersectin-1-specific sequences, Dr Dengshun Miao (McGill University) for assistance with immunostaining of brain sections, Dr Margaret Robinson (Cambridge University) for the generous gift of antibodies to clathrin and AP-2, and Dr David Kaplan and Stephen Morris (Montreal Neurological Institute) for gifts of PC12 cells and recombinant GFP adenovirus, respectively. We would also like to thank Drs Felix Rintelen and Ernst Hafen (University of Zurich) and Dr John O’Bryan (National Institute for Environmental Health Sciences) for discussion and for sharing unpublished results. Finally, we thank Drs Wayne Sossin, John O’Bryan and Ted Fon for advice and discussion.

This work was supported by Medical Research Council of Canada Operating Grants MT-13461 to P.S.M. and MOP-36413 to D.B., and by grants from The Leukemia Society of America (New York, NY) and Novoalal Pharmaceutical Corporation (Research Triangle Park, NC) to B.K.K. The work was also supported by a research contract from BiochemPharma (Laval, QC) to P.S.M. C.C.Q. is supported by NINDS grant NS22807 to Dr Susan Hockfield of Yale University. N.K.H. is supported by a McGill University Faculty of Graduate Studies and Research Fellowship and P.S.M. is a Scholar of the Medical Research Council of Canada.

**References**


Intersectin–mSos1 interactions


Accepted January 6, 2000

Received September 29, 1999; revised November 24, 1999;