Leucine-zipper-mediated homo- and hetero-dimerization of GIT family p95-ARF GTPase-activating protein, PIX-, paxillin-interacting proteins 1 and 2

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ADP-ribosylation factor GTPase-activating proteins (ARFGAPs) of the G-protein-coupled receptor kinase interactor 1/p95 paxillin kinase linker/p95-ARFGAP Pak-interacting exchange factor paxillin-binding protein (APP)-1 family are multidomain proteins, which interact functionally with both ARF and Rac GTPases. These proteins are involved in the dynamic reorganization of adhesion and the cytoskeleton during cell motility. Our previous work [Di Cesare, Paris, Albertinazzi, Dariozzi, Andersen, Mann, Longhi and de Curtis (2000) Nat. Cell Biol. **2**, 521–530] has pointed out a role for p95-APP1 in the regulation of ARF6-mediated membrane recycling. These proteins include different domains, and are capable of interacting stably with proteins that are supposed to play a role in the regulation of actin dynamics and adhesion. They contain a coiled-coil region comprising a putative leucine zipper, predicted to be involved in dimerization. In the present study, we have investigated the possibility that these proteins form dimers. Our results show that p95-APP1 forms homodimers and may also form heterodimers with the other member of the family, p95 paxillin kinase linker/p95-APP2. Both homo- and heterodimerization are disrupted by mutation of two leucine residues in the coiled-coil region of p95-APP1. The N-terminal portion of p95-APP1, including the ARFGAP domain, three ankyrin repeats and the Pak-interacting exchange factor-binding region, are not required for dimerization. Evidence is presented for the existence of endogenous oligomeric complexes. The implication of dimerization/oligomerization in the functioning of these proteins is discussed.

Key words: cytoskeleton, Pak-interacting exchange factor, Rac.

INTRODUCTION

Members of the ADP-ribosylation factor (ARF) family of small GTPases are well-known regulators of membrane trafficking in the cell [1]. Previously, a co-operation has been proposed between ARF and Rho GTPases for the regulation of the actin cytoskeleton and adhesion. For example, activation of plasma-membrane-localized ARF6 contributes to cell spreading [2] and to Rac1-mediated formation of membrane protrusions and ruffles [3] by utilizing the common effector arfaptin2/partner of Rac 1 (POR1) [4]. The G-protein-coupled receptor kinase interactor (GIT)-1/p95 paxillin kinase linker (PKL)/p95-APP1 [where APP stands for ARF GTPase-activating protein (ARFGAP) Pak-interacting exchange factor (PIX) paxillin-binding protein] protein family [5] includes multidomain proteins that share an ARF-specific GAP, ARFGAP domain, a Spa2 homology domain (SHD) required for binding to the Rac/Cdc42 exchanging factor PIX [6] and a paxillin-binding domain in the C-terminal part of the molecule [7]. These proteins have the requisites to act as possible linkers between ARF and Rho GTPases during cell adhesion and actin reorganization. There are two mammalian members of this family, GIT1 [8] and GIT2 [9], which correspond to avian p95-APP1 [10] and PKL/p95-APP2 [7,11] respectively. These proteins have been considered to play a role in the regulation of adhesion and migration, and in the trafficking of recycling membranes [12]. P95-APP1 has been identified in our laboratory as part of a multiprotein complex interacting with GTP-bound Rac GTPases [10].

Given their complex structure and their probable role in the formation of multiprotein complexes, as well as their probable involvement in highly dynamic processes such as cell motility, it is believed that complex control mechanisms must be involved in the regulation of these proteins. A further level of complexity has been uncovered recently by the finding that PIX, a protein directly binding these ARFGAPs, may dimerize via a leucine zipper present at the C-terminus of the polypeptide [13]. The observation that p95-APP1 and PKL/p95-APP2 have a putative leucine zipper in the central part of the polypeptide has motivated us to test the possibility that these proteins may form dimers. We present here evidence that both proteins can dimerize when overexpressed in cells. Immunochemical analysis with an antibody specific for p95-APP1 indicates that the endogenous protein is also present as dimers.

EXPERIMENTAL

Plasmids

The pFLAG-p95-APP1 and pFLAG-p95-APP2 plasmids contained the cDNAs coding for the full-length p95-APP1 and p95-APP2 respectively. The pFLAG-p95-LZ mutant, in which

Abbreviations used: ARF, ADP-ribosylation factor; ARFGAP, ARF GTPase-activating protein; PIX, Pak-interacting exchange factor; APP, ARFGAP PIX paxillin-interacting protein; CHO, Chinese-hamster ovary; CEF, chicken embryo fibroblast; GIT, G-protein-coupled receptor kinase interactor; HA, haemagglutinin; mAb, monoclonal antibody; pAb, polyclonal antibody; PKL, p95 paxillin kinase linker; SHD, Spa2 homology domain.

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Leu^{448} and Leu^{455} have been substituted by two proline residues, was obtained by site-directed mutagenesis with the QuikChange™ site-directed mutagenesis kit (Stratagene), starting from the pFLAG-p95 plasmid, and using the primers 5′-GTGAACAACAGCCCGAGCGATGAGCTGCGCCGGCC-GCAGCGCGAGATC-3′ and 5′-GATCTCGCGCTGCGGCCGG-CGCAGCTCATCGCTCGGGCTGTTGTTCAC-3′. The pFLAG-p95-C2-LZ was obtained from the pFLAG-p95-LZ plasmid. DNAs corresponding to wild-type and mutant p95-APP1 were cloned into the pBK-haemagglutinin (HA) vector, derived from pBK-CMV (Stratagene), to obtain the pBK-HA-p95-APP1 and pBK-HA-p95-LZ plasmids respectively. The plasmids pFLAG-p95-C, pFLAG-p95-C2 and pFLAG-p95-C3 have been described elsewhere [14].

The pXJ40-HA-βPIX-C plasmid was obtained by PCR with the PIX-C5 (TGGATCCTCTGTGAGCAACCCCACCATC) and PIX-C3 (GAAGATCTGCGCCTATAGATTGGTCTCATCCC) oligonucleotides. The PCR fragment was then ligated to pXJ40-HA. The PXJ40-HA-βPIX-C-ΔLZ plasmid was prepared by digesting the pXJ40-HA-βPIX-C plasmid with *Hin*dIII and *Bgl*II to remove the C-terminal coiled-coil region, and by ligating the paired oligonucleotides ΔLZ1 (AGCTTACTGCACAAG-TGCAAAGACGAGGCAGACCCTGAACTCAAGTTCACGC-AAAGAGTCTGCTCCACAAGTGCCCGGGTAGA) and ΔLZ2 (GATCTCTACCCGGGCACTTGTGGAGCAGACTCTTTGC-GTGAACTTGAGTTCAGGGTCGCCTCGTCTTTGCACTTG-TGCAGTA) to introduce a stop codon.

Antibodies

The polyclonal antibody (pAb) SI-61 was raised against a peptide including an N-terminal cysteine followed by 28 amino acids corresponding to residues 470–497 of avian p95-APP1 (CRQQAVPPHPTPAPSERPEHGHPPGTAPL), specific for the chicken p95-APP1 protein. The pAb SI-64 was raised against the GST–P95-C fusion protein (including amino acids 347–740 of p95-APP1), prepared from bacteria transformed with the pGEX-p95-C construct [10]. Rabbits were immunized with the peptide conjugated to keyhole-limpet (*Diodora aspera*) haemocyanin or with the fusion protein. Other antibodies used in the present study were: anti-FLAG monoclonal antibody (mAb) M5 (Sigma), anti-HA mAb 12CA5 (Primm, Milano, Italy), anti-HA pAb HA-11 (BabCO, Richmond, CA, U.S.A.) and anti-paxillin and anti-PIX mAbs (Transduction Laboratories–Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Cell culture and transfections

Chicken embryo fibroblasts (CEFs) obtained from embryonic day-10 chicken embryos were prepared and cultured as described previously [15]. For biochemical analysis, transient expression of proteins was achieved by transfection of CEFs by the calcium phosphate technique using 10 μg of DNA $\cdot$ (plasmid)$^{-1}$ $\cdot$ (6 cm diameter plate)$^{-1}$. Cells were used for biochemical analysis 18–24 h after transfection. Chinese-hamster ovary (CHO) cells were cultured in α-minimal essential medium (MEM; Invitrogen, Carlsbad, CA, U.S.A.) with 10 % (v/v) foetal bovine serum and transfected with 5 μg of DNA $\cdot$ (plasmid)$^{-1}$ $\cdot$ (6 cm diameter plate)$^{-1}$ (2 plates/IP) with 20 μl/dish of 2 mg/ml LipofectAMINE™ (Life Technologies, Gaithersburg, MD, U.S.A.) in OPTI-MEM (Invitrogen) without serum. After 5 h, the medium was substituted with fresh culture medium with serum, and cultured overnight. COS7 cells were grown in Dulbecco's modified Eagle's medium (Cambrex Bio Science Verviers S.P.R.L., Verviers, Belgium) with 10 % serum and transfected with FuGENE (Roche), according to the manufacturer's instructions.

Immunoprecipitation and immunoblotting

Cells [2–3 plates $\cdot$ (construct)$^{-1}$ $\cdot$ (experiment)$^{-1}$] were extracted with lysis buffer [1 % (w/v) Triton X-100/20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.5 mM PMSF/1 mM sodium orthovanadate/10 mM NaF] 18–24 h after transfection. Protein (200–400 μg) from cell lysates was used for each immunoprecipitation. Lysates were clarified by centrifugation and precleared by incubation for 2 h with rotation at 4 °C with 25 μl of Protein A–Sepharose beads. The unbound material was immunoprecipitated with the indicated antibodies preadsorbed to 25 μl of Protein A–Sepharose (10 μg of anti-FLAG M5 mAb or 10 μl of 12CA5 with 3 μl of rabbit anti-mouse immunoglobulin from Sigma). Immunoprecipitates were washed four times with 1 ml of washing buffer [0.5 % Triton X-100/20 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.5 mM PMSF/1 mM sodium orthovanadate/10 mM NaF], and were analysed by SDS/PAGE and immunoblotted with the indicated antibodies. For the detection of primary antibodies, the blots were incubated with 0.2 μCi/ml of either ^{125}I-labelled anti-mouse immunoglobulin or ^{125}I-labelled Protein A (Amersham Biosciences, Piscataway, NJ, U.S.A.), washed and exposed to Amersham Hyperfilm-MP.

Velocity-gradient centrifugation

On each gradient, 300–600 μg of protein from lysate of transfected cells or 1–3 mg of protein from lysate of non-transfected cells or tissue were loaded. Samples were loaded on to 4.5 ml linear 5–20 % (w/v) sucrose gradients prepared in lysis buffer containing an anti-protease mixture, including antipain, chymostatin, leupeptin and pepstatin, each at a final concentration of 20 μg/ml. Gradients were centrifuged at 189 000 ***g*** for the indicated times at 4 °C in a SW 50.1 rotor (Beckman Coulter Inc., Fullerton, CA, U.S.A.). Fractions (0.4 ml) were collected and the pellet was resuspended in 0.4 ml of lysis buffer. Protein standards (0.4 mg of each: BSA, 66 kDa; transferrin, 76 kDa; aldolase, 158 kDa; catalase, 252 kDa; ferritin, 440 kDa; all from Pharmacia Corp., Peapack, NJ, U.S.A.), diluted to 0.3 ml in lysis buffer, were run on sucrose gradients as described above. For protein denaturation, chicken brain lysates were corrected to 0.5 % SDS, boiled for 5 min, and then adjusted to 0.25 % SDS with lysis buffer, before loading on to the sucrose gradients.

Gel filtration

Three 6 cm dishes of COS7 cells, transfected with either pFLAG-p95-APP1 or pFLAG-p95-LZ, were extracted with 0.5 % Triton X-100 lysis buffer. Each type of lysate (200 μl) was sequentially run on a Superose 12 column (Amersham Biosciences) equilibrated in lysis buffer. Eluted fractions (0.3 ml each) were analysed for total protein by the Bio-Rad protein assay kit (Bio-Rad) and by immunoblotting to detect the overexpressed proteins. Molecular-mass markers in lysis buffer were run on the same column.

RESULTS AND DISCUSSION

Identification of p95-APP1 homodimers

Analysis of the amino acid sequences of chicken p95-APP1 and p95-APP2 indicated the presence of a coiled-coil region

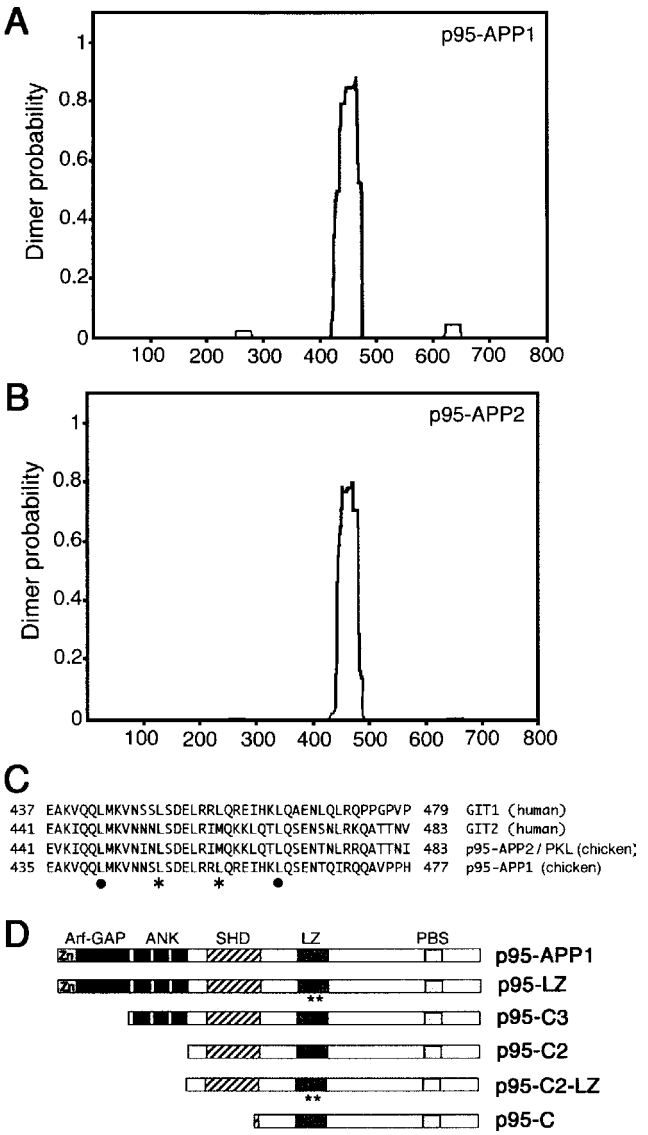

Figure 1 The leucine-zipper domain of p95-APP1 and p95-APP2 is predicted to mediate dimer formation

p95-APP1 (**A**) and p95-APP2 (**B**) amino acid sequences were analysed using the Multicoil program, which predicts the presence of coiled-coils and the probability for dimer formation based on pairwise residue interactions. (**C**) Alignment of the predicted dimerization regions of GIT family proteins shows characteristically placed leucine residues (● and ∗). The two leucine residues mutated in p95-APP1 to obtain the p95-LZ protein are indicated by asterisks. (**D**) Wild-type and mutant p95-APP1 utilized in the present study. Asterisks indicate the two-point mutations in the leucine-zipper domain (LZ; Leu^{448} and Leu^{455} have been mutated into two proline residues). ANK, ankyrin repeats; PBS, paxillin-binding subdomain.

between residues 421–474 of p95-APP1 and 432–482 of p95-APP2 (Figure 1). Analysis by the Multicoil program [16] indicated that both proteins have a high probability to form homodimers via this region (Figures 1A and 1B). These regions are highly conserved in the mammalian homologues GIT1 and GIT2 respectively (Figure 1C). In fact, analysis by the Multicoil program confirmed that both mammalian counterparts have a high probability to form dimers (results not shown). A putative leucine zipper can be predicted within this region of p95-APP1. To test for homodimerization of p95-APP1, we co-expressed the protein tagged to two distinct epitopes HA-p95-APP1 and FLAG-p95-APP1. Immunoprecipitation with the anti-FLAG mAb M5, followed by immunoblotting with the anti-HA

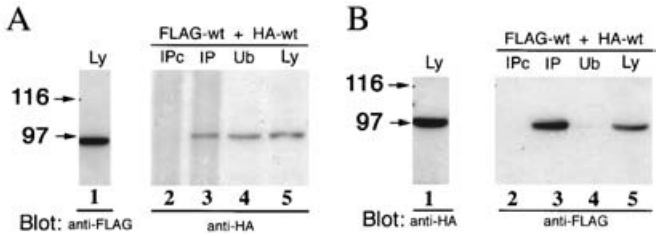

Figure 2 Identification of 95-APP1 dimers

CEFs were co-transfected to express FLAG-p95-APP1 and HA-p95-APP1. The lysate was divided into two aliquots. After preclearing with Protein A–Sepharose beads, the two aliquots of lysate were immunoprecipitated with anti-FLAG (**A**) or anti-HA (**B**) antibody respectively. Lysates (Ly), preclearings with Protein A–Sepharose beads (IPc), immunoprecipitates (IP) and unbound material (Ub) were analysed by immunoblotting with the indicated antibodies. Molecular-mass markers (kDa) are indicated on the left-hand side.

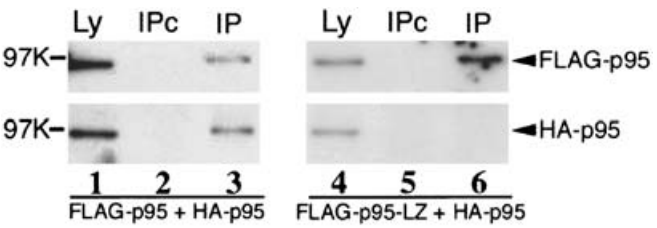

Figure 3 The leucine zipper of p95-APP1 is required for dimerization

CEFs were co-transfected to co-express FLAG-p95-APP1 with HA-p95-APP1 (lanes 1–3) or FLAG-p95-LZ with HA-p95-APP1 (lanes 4–6). After preclearing with Protein A–Sepharose beads (IPc), lysates (Ly) were incubated with beads coated with mAb anti-FLAG (IP). Each sample was prepared in duplicate from the same lysate. After SDS/PAGE, one set of filters (upper blots) was utilized for immunoblotting with the anti-FLAG mAb to detect the FLAG-p95-APP1 (lanes 1–3) and FLAG-p95-LZ (lanes 4–6) polypeptides. The second set of filters (lower blots) was utilized for immunoblotting with the anti-HA pAb to detect the HA-p95-APP1 polypeptide.

antibody, revealed the co-precipitation of the two proteins from lysates of co-transfected cells (Figure 2A). On the other hand, it was possible to co-immunoprecipitate a FLAG-p95-APP1–HA-p95-APP1 complex from lysates of co-transfected CEFs with anti-HA antibodies (Figure 2B). These results show the reconstitution of homocomplexes between the two transfected proteins.

To test whether the putative leucine zipper of p95-APP1 could be responsible for such an interaction, we prepared the p95-LZ mutant, in which two of the four leucine residues of the putative zipper motif (Leu^{448} and Leu^{455}; Figure 1C) were substituted by two proline residues. These mutations may interfere with the association of the polypeptide chains with each other. Accordingly, in contrast with the results obtained for cells overexpressing the two wild-type proteins (Figure 3, left panel), the HA-tagged protein could not be detected in the anti-FLAG immunoprecipitate from the lysate of cells co-expressing the wild-type HA-p95-APP1 with the mutant FLAG-p95-LZ protein (Figure 3, right panel). Therefore an intact leucine-zipper motif is necessary to form homocomplexes. Results also show that it is sufficient to disrupt the leucine zipper in just one of the chains to prevent the association with the wild-type polypeptide.

Identification of p95-APP1 dimers by sucrose velocity gradients and gel filtration

Analysis using the Multicoil program and the results presented above suggest that the identified homocomplexes corresponded to p95-APP1 dimers. To confirm this hypothesis, we have utilized velocity-gradient centrifugation to establish the approximate mass

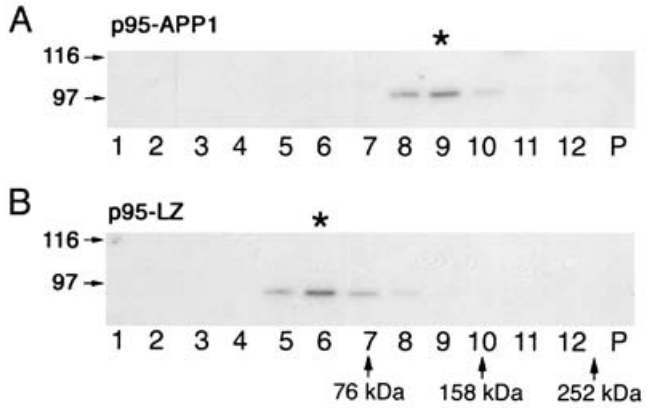

Figure 4 Analysis of the migration of p95-APP1 on velocity gradients

Lysates from CEFs transfected with either wild-type p95-APP1 (**A**) or p95-LZ (**B**) were loaded on top of 5–20 % sucrose velocity gradients as described in the Experimental section and centrifuged at 189000 ***g*** for 16 h. Fractions were analysed for the distribution of the p95 proteins by immunoblotting with the anti-FLAG antibody. The fraction number is indicated at the bottom of each lane. Fraction 1 corresponds to the top of the gradient and P represents the pellet. The peaks of distribution of the three molecular-mass markers transferrin, aldolase and catalase are indicated by arrows in the lower blot. Asterisks identify the peak fraction for the distribution of the p95 polypeptides in the two gradients.

of the identified complexes. For this purpose, lysates from CEFs transfected with either the wild-type or the leucine-zipper mutant of p95-APP1 were loaded on to linear 5–20 % sucrose gradients and centrifuged at 189000 ***g*** for 16 h. Analysis of the distribution of proteins along the gradients showed a peak at fraction 9 for the wild-type p95-APP1 protein (Figure 4A). This peak was close to the peak of migration of the molecular-mass marker aldolase (158 kDa). In contrast, the mutant p95-LZ protein had a distribution peak similar to that of the 66 kDa marker BSA (Figure 4B). These findings revealed a clear-cut difference in the migration patterns in the gradients of the wild-type and mutant proteins, and the difference was consistent with the wild-type p95-APP1 behaving as a dimer. On the other hand, the protein mutated in the leucine zipper behaved as a monomer.

A difference in mass was also detected by gel filtration on a Superose 12 column loaded with extracts from COS7 cells transfected with either the wild-type or the mutant p95-LZ protein (Figure 5). A clear-cut difference in the elution profiles of the two proteins could be detected, with the wild-type protein eluting faster (peak at fraction 8) when compared with the mutant protein (peak at fractions 13–14). These findings confirmed the requirement of an intact leucine zipper for p95-APP1 oligomerization.

The N-terminal part of p95-APP1 is not involved in dimerization

The N-terminal part of p95-APP1 includes the ARFGAP domain, three ankyrin repeats and the Spa2 homology domain (SHD) required for PIX binding (Figure 1D). We verified whether any of these domains was essential for dimerization. COS7 cells were co-transfected with a combination of HA-tagged full-length p95-APP1 together with a full-length or truncated FLAG-tagged p95 polypeptide, and used for studying co-immunoprecipitation. This analysis showed that partial or complete removal of the N-terminal part of the molecule did not affect its ability to form complexes with the full-length protein (Figure 6). Moreover, analysis by sucrose velocity gradients showed that the truncated p95-C2 polypeptide migrated further in the gradient when compared with the p95-C2-LZ protein, in which the leucine

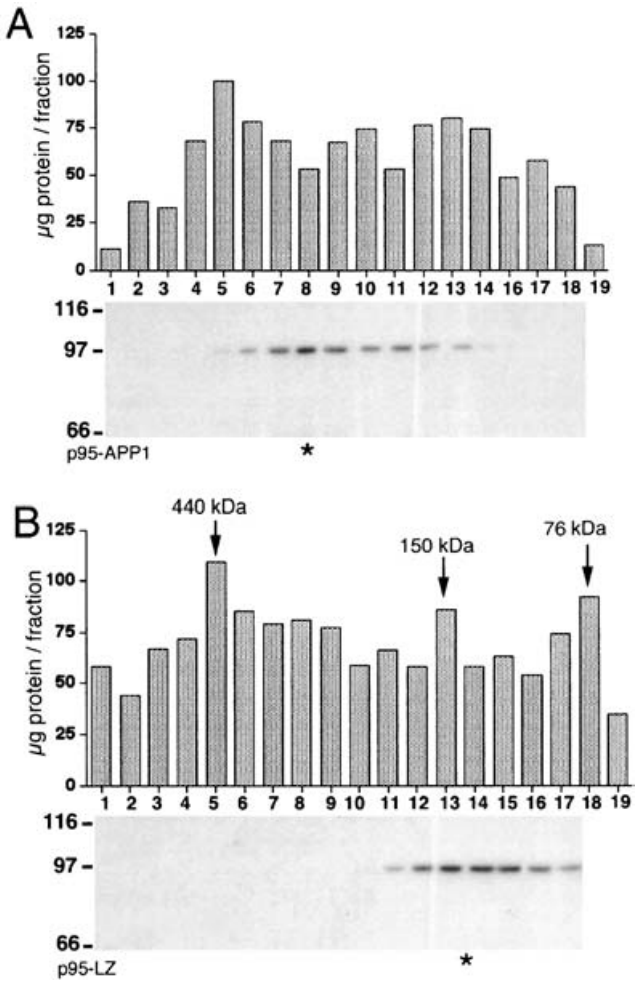

Figure 5 Analysis of p95-APP1 and p95-LZ by gel filtration

Lysates from COS7 cells transfected with either wild-type p95-APP1 (**A**) or p95-LZ (**B**) were loaded on to a Superose 12 column, and eluted with lysis buffer. Fractions (0.3 ml) were collected and analysed for the distribution of total protein, and of the p95 polypeptides using the anti-FLAG antibody. Fraction 1 corresponds to the first fraction in which protein detection began during elution. Asterisks identify the peak fractions for the distribution of the p95 polypeptides in the eluates. Peaks of distribution of the molecular-mass markers are indicated by arrows in (**B**).

zipper had been mutated (Figure 7), thus indicating that truncated p95-C2 was capable of forming homodimers.

Identification of heterodimeric p95-APP1–p95-APP2 complexes in transfected cells

Since analysis using the Multicoil program had suggested a high probability of dimer formation also for p95-APP2 (Figure 1B), we utilized the velocity-gradient centrifugation technique to compare the behaviour of p95-APP1 and p95-APP2. CHO cells were transfected with either wild-type p95-APP1 or wild-type p95-APP2. Each extract was loaded on to a 5–20 % sucrose gradient, which was then centrifuged at 189000 ***g*** for 16 h. The finding that both wild-type proteins migrated at similar positions along the gradients (Figure 8) confirmed the prediction that p95-APP2, similar to p95-APP1, is capable of forming homodimers.

We then tested whether heterodimers could also be induced in cells co-expressing both proteins. Analysis by co-immunoprecipitation from CHO cells overexpressing HA-p95-APP1 and

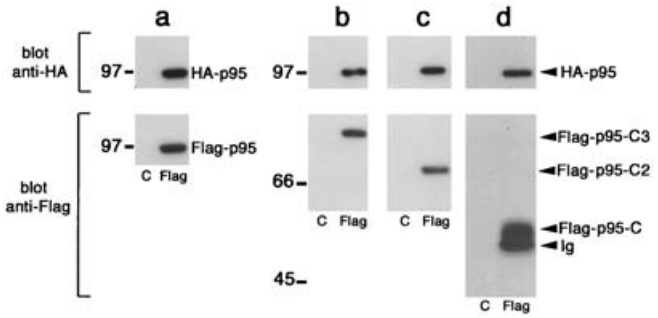

Figure 6 The N-terminal portion of p954-APP1 is not required for dimerization

COS7 cells were co-transfected to express HA-p95-APP1 together with one of the following: FLAG-p95-APP1 (**a**), FLAG-p95-C3 (**b**), FLAG-p95-C2 (**c**) or FLAG-p95-C (**d**). Cell lysates were immunoprecipitated with the anti-FLAG mAb. In each panel, the left-hand side lane shows the blot on beads preincubated with the lysate in the absence of the anti-FLAG antibody (C), the right-hand side lane shows the blot of immunoprecipitation with anti-FLAG (Flag). Upper blots show the co-precipitated full-length HA-p95-APP1 detected with anti-HA antibody; lower blots show the immunoprecipitated FLAG-labelled proteins. Molecular-mass markers are indicated on the left-hand side of the blots.

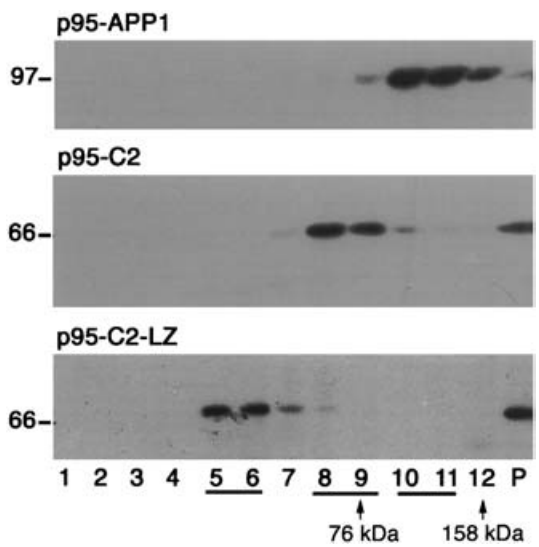

Figure 7 The truncated p95-C2 polypeptide may form dimers

CEFs were transfected to express full-length p95-APP1 (upper panel), p95-C2 (middle panel) or p95-C2-LZ (lower panel). Lysates were loaded on to top of 5–20 % sucrose velocity gradients as described in the Experimental section and centrifuged at 189 000 ***g*** for 20 h. Fractions were analysed for the distribution of the p95-derived polypeptides by immunoblotting with the anti-FLAG antibody. The fraction number is indicated at the bottom of each lane. Fraction 1 corresponds to the top of the gradient and P represents the pellet. The peaks of distribution of the molecular-mass markers transferrin and aldolase are indicated by arrows in the lower blot. Underlined fractions identify the peak fractions for the distribution of the p95 polypeptides in the three gradients.

FLAG-p95-APP2 indicated that these proteins could associate with each other efficiently in the cell (Figure 9, left panels). The involvement of the leucine zipper in the formation of these heterocomplexes was demonstrated by the finding that the wild-type p95-APP2 protein could not associate with the p95-APP1-LZ polypeptide mutated at Leu^{448} and Leu^{455} (Figure 9, right panels). These findings show that disruption of one of the two leucine-zipper motifs is sufficient to prevent heterodimerization.

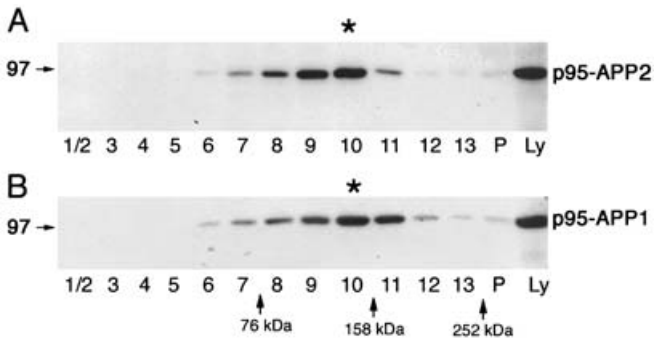

Figure 8 Analysis of the migration of p95-APP2 on velocity gradients

Lysates from CHO cells transfected with either wild-type p95-APP2 (**A**) or p95-APP1 (**B**) were loaded on to top of 5–20 % sucrose velocity gradients as described in the Experimental section and centrifuged at 189 000 ***g*** for 16 h. Fractions were analysed as described in Figure 4. The asterisks mark the peak fraction for the distribution of the p95 polypeptides in the two gradients. Ly, lysate.

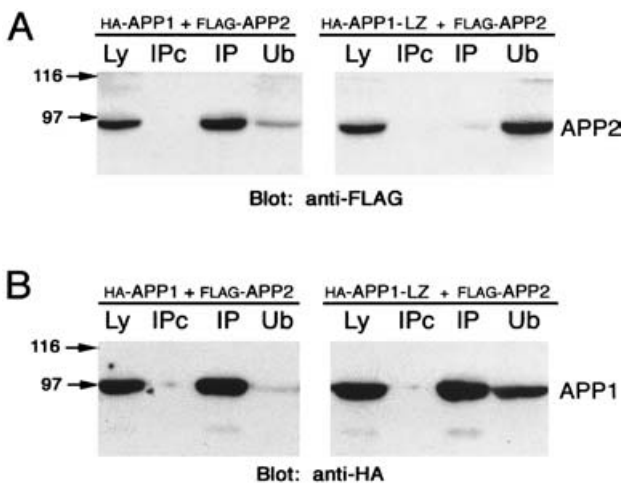

Figure 9 P95-APP1 and p95-APP2 form heterocomplexes

CHO cells were co-transfected to express HA-p95-APP1 and FLAG-p95-APP2 (left panels) or HA-p95-APP1-LZ (HA-APP1-LZ) and FLAG-p95-APP2 (right panels). After preclearing with Protein A–Sepharose beads (IPc), lysates (Ly) were immunoprecipitated with the anti-HA mAb 12CA5 (IP). Ub, unbound fractions after immunoprecipitation. Immunoprecipitations were performed in duplicate. One set of samples was analysed by immunoblotting with the anti-FLAG antibody to detect the FLAG-p95-APP2 polypeptide (**A**), whereas a second set was analysed with anti-HA antibodies to detect the HA-p95-APP1 and HA-p95-APP1-LZ polypeptides (**B**).

Identification of distinct endogenous p95-APP1–PIX complexes

We then decided to test whether the endogenous p95-APP1 protein was present as dimers in cells. For this purpose, a number of pAbs were raised against the avian p95-APP1 polypeptide. In the present study, we have utilized pAb SI-61, raised against a peptide corresponding to the amino acids 470–497 of chicken p95-APP1 and pAb SI-64, raised against the GST-p95-C fragment corresponding to a fusion protein including the C-terminal region (amino acids 346–740) of p95-APP1. This region is the least conserved between the two members of this family of ARFGAPs, showing only 53 % identity at the amino acid level. Sera from rabbits, immunized with either the peptide or the GST-p95-C protein, were characterized by immunoprecipitation and immunoblotting on lysates from embryonic chicken brain, where p95-APP1 is expressed abundantly. Of the two antibodies, SI-64 worked better in immunoblotting (results not shown)

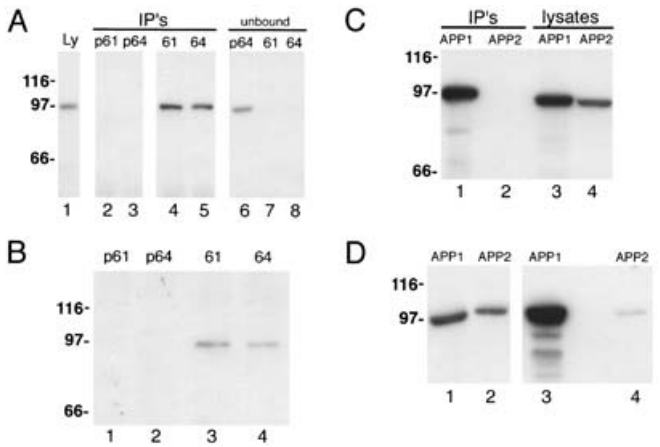

Figure 10 Characterization of the anti-p95-APP1 antibodies

(**A**) Aliquots (1 mg protein) of chicken embryonic-day-14 brain lysate were used for immunoprecipitation with 10 μl of either pre-immune (lanes 2 and 3) or immune (lanes 4 and 5) sera SI-61 (lanes 2 and 4) and SI-64 (lanes 3 and 5). Half of the unbound fractions after immunoprecipitation with pre-immune SI-64, and immune SI-61 and SI-64 sera were loaded in lanes 6–8 respectively. The blot was incubated with antiserum SI-64 to detect the endogenous p95-APP1 protein. (**B**) Aliquots (1 mg protein) of CEF lysate were used for immunoprecipitation with 10 μl of either pre-immune (lanes 1 and 2) or immune (lanes 3 and 4) sera SI-61 and SI-64. After blotting, the filter was incubated with SI-64 to detect the endogenous p95-APP1 protein. (**C**) Lysates from CHO cells, overexpressing FLAG-p95-APP1 or FLAG-p95-APP2, were immunoprecipitated with 10 μl of SI-61 antiserum. Immunoprecipitates (lanes 1 and 2) and 100 μg of aliquots of the corresponding lysates (lanes 3 and 4) were utilized for immunoblotting with the anti-FLAG mAb. (**D**) Aliquots (100 μg) of lysates from CHO cells overexpressing FLAG-p95-APP1 or FLAG-p95-APP2 were immunoblotted with anti-FLAG mAb (lanes 1 and 2) or with pAb SI-64 (lanes 3 and 4).

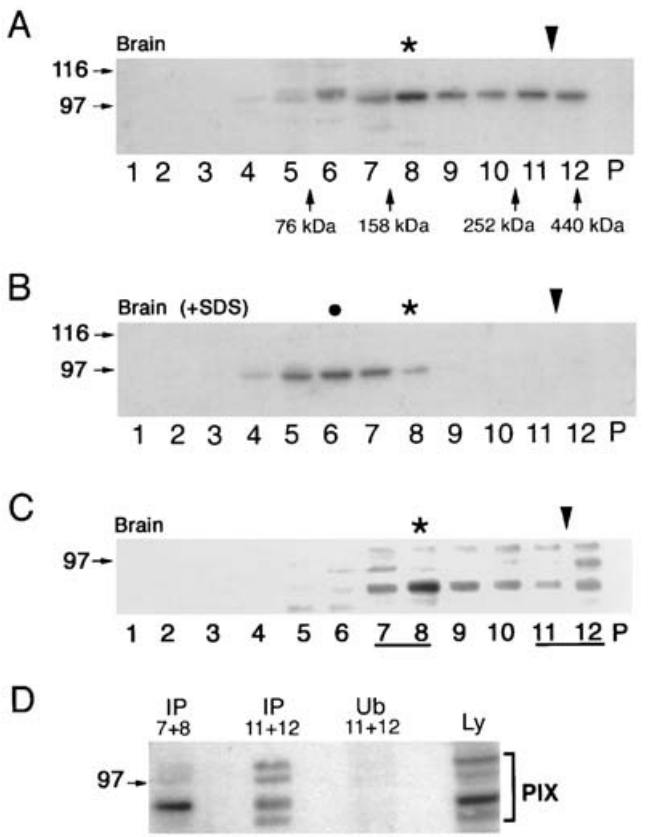

Figure 11 Analysis of the endogenous p95-APP1 by velocity gradients

Chicken brains from day 14 embryos were lysed with 1 % Triton X-100. Aliquots of the untreated lysate (**A**) and of lysate denatured by boiling with SDS (**B**) were loaded on to 5–20 % sucrose gradients. After centrifugation at 189 000 ***g*** for 10 h, gradient fractions were analysed for the distribution of the endogenous p95-APP1 protein by using the anti-p95 pAb SI-64. Aliquots of the fractions from gradient A were also analysed by immunoblotting with anti-PIX pAb (**C**). Pools of fractions 7 + 8, and 11 + 12 (underlined in **C**) from the gradient loaded with non-denatured lysate were immunoprecipitated with anti-p95 pAb SI-61, and analysed by immunoblotting with an anti-PIX pAb (**D**). Ub, unbound fraction after immunoprecipitation. Asterisk and arrowhead in (**A**) indicate the position of the two peaks for p95-APP1, which are also reported for comparison (**B**, **C**). The dot in (**B**) indicates the peak of distribution of the denatured p95-APP1 polypeptide.

and recognized a 95 kDa band in E14 chicken brain lysates (Figure 10A). Both SI-61 and SI-64 antibodies were capable of immunoprecipitating both efficiently and specifically a band of the same molecular mass from the brain lysate (Figure 10A). A band with the same molecular mass could also be immunoprecipitated by both antibodies from lysates of CEFs (Figure 10B); the intensity of the bands immunoprecipitated from the same amount of total protein of either brain or CEF lysate indicated that CEFs expressed lower levels of endogenous p95-APP1. We then tested the specificity of the two antibodies for p95-APP1. CHO cells were transfected with pFLAG-p95-APP1 or pFLAG-p95-APP2. Immunoprecipitation from lysates of transfected cells showed that the overexpressed p95-APP1 could be efficiently immunoprecipitated by the SI-61 serum, whereas p95-APP2 was not recognized by this antibody (Figure 10C). Aliquots of the lysates from the transfected cells were also tested for immunoblotting with the SI-64 antibody. This serum clearly recognized p95-APP1, whereas only a faint band was recognized in the lysate overexpressing p95-APP2 (Figure 10D). It should be noted that this band also includes, if not exclusively, the endogenous p95-APP1 expressed by these cells.

Based on the finding that p95-APP1 is abundantly expressed in embryonic avian brain, lysates from E14 chicken brain were loaded on to 5–20 % sucrose gradients and centrifuged at 189 000 ***g*** for 10 h (Figure 11A). It is interesting to observe that the overexpressed p95-APP1 distributed with a single peak (before the marker aldolase; Figure 4A), whereas two peaks could be observed for their endogenous protein in brain; a first peak localized just after the peak of aldolase (Figure 11A, asterisk) and a second peak towards the bottom of the gradient (Figure 11A, arrowhead). As expected, boiling the lysate with SDS before loading the gradient disassembled the complexes, leading to a shift in the distribution towards the top; under denaturing conditions, the endogenous p95-APP1 ran as a single peak closer to the 66 kDa marker BSA (Figure 11B).

The finding that the distribution of the endogenous p95-APP1 from embryonic brain showed two peaks on the gradient suggested the possibility of the existence of endogenous complexes, including other proteins interacting with p95-APP1. We have shown that p95-APP1 interacts with paxillin and PIX [10]. We therefore analysed the distribution of these two proteins in the fractions of the gradient shown in Figure 11(A). We found that, evidently, the distribution of paxillin along the gradient did not co-localize with that of p95-APP1, with most of the protein being located in the upper part of the gradient (results not shown).

We then analysed the distribution of the endogenous PIX by using an anti-PIX mAb, which recognized different bands by immunoblotting on brain lysates (Figure 11D). The same pattern was also recognized by a rabbit pAb raised recently in our laboratory (results not shown) against the same PIX fragment utilized for the production of the pAb described in [6]. Moreover, the pattern recognized in chicken brain by the two antibodies was very similar to that recognized in rat brain lysate by the anti-PIX pAb [6]. These results indicate that endogenous PIX existed in different forms in chicken brain, as in mammalian brain, and that

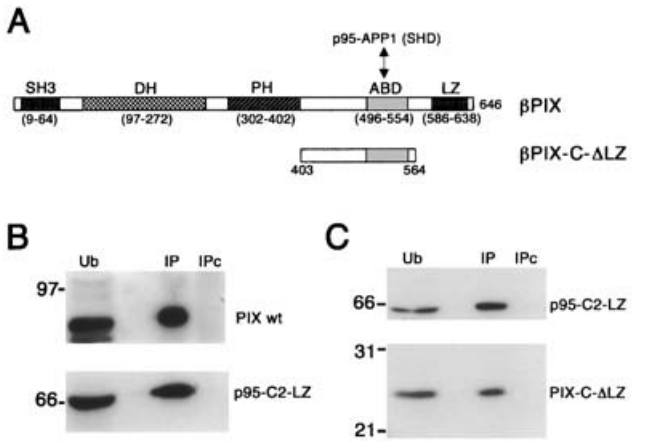

Figure 12 Leucine-zipper (LZ) motifs are not required for the association of p95-APP1 with βPIX

(**A**) PIX constructs utilized in the present study. COS7 cells were co-transfected to express FLAG-p95-C2-LZ with either HA-PIX-wt (**B**) or HA-PIX-C-ΔLZ (**C**). After preclearing with Protein A–Sepharose beads (IPc), cell lysates were used for immunoprecipitation with anti-FLAG mAb (IP). Aliquots of the unbound fractions after immunoprecipitation (Ub) were also loaded on to the gels. Filters were cut and immunoblotted with anti-HA pAb to detect wild-type PIX and PIX-C-ΔLZ, and with anti-FLAG mAb to detect p95-C2ΔLZ. SH3, Src homology 3; DH, Dbl homology; PH, pleckstrin homology.

PIX distributed along the gradient in a fashion similar to p95-APP1, with two peaks around fractions 8 and 11–12 respectively (Figure 11C). Immunoprecipitation with the anti-p95-APP1 SI-61 pAb of two pools corresponding to fractions 7 + 8 and 11 + 12 respectively showed that both peaks from the gradient contained p95-APP1–PIX complexes (Figure 11D). No detectable paxillin was found in the two immunoprecipitates (results not shown). Altogether, these results indicate that the endogenous p95-APP1 exists in distinct complexes with PIX. One interpretation of our results is that p95-APP1 and PIX may both exist *in vivo* as heterotetramers, distributing in the upper peak, and as larger oligomeric complexes that may account for the peak distributing towards the bottom of the gradient.

Leucine-zipper motifs are not required for the interaction between p95-APP1 and βPIX

Recently, it has been reported that one of the proteins interacting with GIT family ARFGAPs, the Rac exchanging factor βPIX, can form homodimers via a C-terminal leucine zipper [13]. It may be considered that p95-APP1 utilizes the leucine-zipper motif also to dimerize with other proteins, βPIX being a candidate for heterodimerization. On the other hand, previous studies have shown that p95-C, a truncated mutant of p95-APP1 lacking the SHD, but including the putative leucine-zipper domain, is not capable of interacting with βPIX. In contrast, the p95-C2 protein, which differs from p95-C for the presence of the SHD interacts efficiently with βPIX [10]. These findings indicated that the leucine-zipper motifs of these proteins were not sufficient to allow heterodimerization. In the present study, we verified whether the presence of the leucine-zipper motif was necessary for the SHD-mediated association of βPIX with p95-APP1. For this purpose, we have investigated the association of wild-type and mutant PIX proteins (Figure 12A) with the p95-C2-LZ polypeptide. P95-C2-LZ has the SHD and a mutated leucine-zipper motif (Figure 1D). Co-precipitation experiments from lysates of co-transfected COS7 cells showed that the wild-type βPIX polypeptide could associate with p95-C2-LZ (Figure 12B). Moreover, a short truncated PIX-C-ΔLZ polypeptide including the ARFGAP-binding region responsible for the association with the SHD of the GIT family ARFGAPs [17], but lacking a large N-terminal region and the C-terminal leucine-zipper motif was still capable of associating with p95-C2-LZ. These findings show that the association of βPIX with p95-APP1 is independent of the presence of leucine-zipper motifs. Moreover, since neither p95-C2-LZ nor PIX-C-ΔLZ may form homodimers, these results indicate that homodimerization is not an essential prerequisite for the SHD-mediated interaction between p95-APP1 and βPIX.

Conclusions

The leucine-zipper domain is an α-helical structure formed by several heptad repeats of hydrophobic residues, usually leucine and isoleucine, that are commonly found in nuclear transcription factors, and its role in promoting the homo- and heterodimerization of these proteins has been well characterized [18,19]. Leucine-zipper domains are also present in many other proteins, including protein kinases, adaptors and cytoskeletal proteins, although their function in these molecules has been studied less extensively. For the Rac exchanging factor βPIX, the reported dimerization via the C-terminal leucine zipper has been identified to play a role in the formation of membrane ruffles in NIH3T3 cells [13]. In the present study, we have shown that the leucine-zipper domain of p95-APP1 and PKL/p95-APP2 can mediate the formation of homo- and heterodimers, and that the N-terminal portion of the molecule, including the ARFGAP domain, the three ankyrin repeats and the SHD is not required for homodimerization. Moreover, by analysis using antibodies specific for p95-APP1 we have some evidence for the existence of endogenous p95-APP1 oligomers. In agreement with our findings, another laboratory has recently reported the identification of GIT family oligomers based on the C-terminal portion of GIT proteins [20]. To our knowledge, these are the first reports of GAPs for small GTPases that are capable of dimerizing via leucine-zipper motifs. It is known that both members of this family of ARFGAPs interact with βPIX via the SHD. The ability of these ARFGAPs to dimerize introduces a further degree of complexity in the understanding of the function and regulation of these proteins. One could envisage different possibilities concerning the way ARFGAP and βPIX dimers may interact (Figure 13). The first hypothesis is that each ARFGAP dimer would interact with one βPIX dimer; in this case, either of the two ARFGAP polypeptides would interact with one of the two polypeptides of the same βPIX dimer (Figure 13A). Alternatively, one could think that each ARFGAP dimer interacts with two different βPIX dimers, which in turn may interact with two distinct ARFGAP dimers (Figure 13B). This would result in the formation of larger multimeric complexes including ARFGAPs and βPIX dimers. In this direction, our results on the endogenous proteins (Figure 11) suggest that larger p95-APP1–βPIX complexes may exist in the cell, which could have functional relevance.

It has been postulated that the GIT family ARFGAPs and βPIX are part of complexes involved in the organization of adhesion and actin cytoskeleton during migration [21]. Moreover, we have demonstrated that the interaction between p95-APP1 and βPIX induces the recruitment of these complexes at large endocytic recycling structures [14]. One hypothesis is that these complexes may contribute to the endocytosis/recycling of membranes and of associated proteins required for actin organization during cell motility [12]. In this direction, it has been shown that these ARFGAPs may affect endocytosis [22]. Moreover, GIT proteins

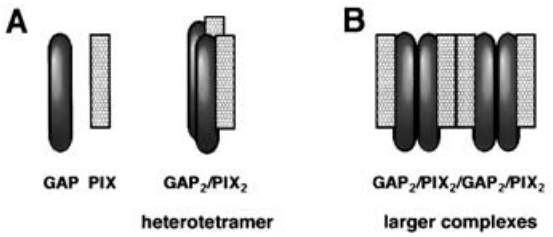

Figure 13 Model for the organization of the p95-APP1–PIX complexes

(**A**) Each ARFGAP dimer interacts with one βPIX dimer; in this case, each of the two ARFGAP polypeptides would interact with one of the two polypeptides of the same βPIX dimer. (**B**) Alternatively, each ARFGAP dimer interacts with two different βPIX dimers, which in turn may interact with two distinct ARFGAP dimers. This may lead to the formation of large multimeric complexes.

have GAP activity for different ARF proteins *in vitro*, including ARF6 [23], and our recent results indicate a specific role of p95-APP1 as an ARF6 GAP *in vivo* [24]. These proteins may be part of the machinery, which regulate ARF action during membrane dynamics. For p95-APP1, one could speculate that the formation of larger complexes at endocytic/recycling membranes may play a role in ARF6-mediated membrane endocytosis/recycling. Further studies will be required to understand the role of dimerization/oligomerization and its regulation in the function of these proteins and of the associated molecules during cell motility.

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