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Identification of a novel protein complex containing annexin VI, Fyn, Pyk2, and the p120^{GAP} C2 domain

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Abstract p120^{GAP} (RasGAP) has been proposed to function as both an inhibitor and effector of Ras. Previously we have shown that RasGAP contains a C2 domain which mediates both Ca^{2+}-dependent membrane association and protein–protein interactions. Specifically, three proteins have been isolated in a complex with the C2 domain of RasGAP; these are the Ca^{2+}-dependent lipid binding protein annexin VI (p70) and two previously unidentified proteins, p55 and p120. Here we provide evidence that p55 is the Src family kinase Fyn and p120 is the focal adhesion kinase family member Pyk2. In addition, in vitro binding assays indicate that Fyn, but not Pyk2 binds directly to annexin VI. Finally, co-immunoprecipitation studies in Rat-1 fibroblasts confirm that Fyn, Pyk2, annexin VI and RasGAP can form a protein complex in mammalian cells.

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Key words: Conserved region 2; GTPase activating protein; Ras; Annexin; Ca^{2+}

1. Introduction

p21^{ras} proteins play an important role in cellular proliferation and differentiation processes [1,2]. They transduce signals between receptor tyrosine kinases [3], (Gi/Gq) G protein coupled receptors [4] or src non-receptor tyrosine kinases [5] and downstream effectors including raf and phosphatidylinositol 3-kinases [6]. In common with all guanine nucleotide binding proteins, the biological activity of Ras proteins is dependent on the GTP/GDP bound state in which they reside [7]. This in turn is regulated by guanine nucleotide exchange factors and GTPase activating proteins (GAPs) respectively. A number of Ras selective GAPs have been identified [8–11] and primary sequence analysis indicates that many members display sequence similarity both within their GTPase activation domains (known as the GAP related domain or GRD) and also within a non-catalytic region called the C2 domain [8–12]. One such GAP which contains one copy of a C2 domain and is an important regulator of Ras activity is the p120^{GAP} (RasGAP).

C2 (conserved region 2) domains were originally identified in protein kinase C isoforms which become activated and membrane associated in response to Ca^{2+} elevation [13]. C2-like domains have also been identified in a wide range of Ca^{2+}-responsive proteins with vastly diverse cellular signalling functions [14]. The precise function of C2 domains remains unclear. However, we [15,16] and others [17,18] have demonstrated that C2 domains may function to direct protein–phospholipid and protein–cellular membrane interactions. In the case of p120^{GAP}, we have demonstrated that the C2 domain is important for its translocation from the cytoplasm to a membrane associated location in response to Ca^{2+} elevation [16]. However, many proteins contain two non-identical C2-like domains [9–11,19,20] and it is thus possible that C2 domains function only to direct membrane association events. It is, however, probable that C2 domains can function to mediate specific protein–protein interactions.

We have recently shown that RasGAP mediates a protein complex formation with three proteins [21]. We have identified one of these proteins, annexin VI, as interacting directly with the RasGAP C2-like domain and we have purified the other two proteins which form a protein complex with annexin VI and RasGAP. These proteins have estimated molecular weights of approximately 55 and 120 kDa, but so far their identities have remained undetermined [21]. In this study we have attempted to identify these proteins in order to gain clues for any further potential cellular signalling roles that RasGAP and annexin VI may play.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were of analytical grade. Restriction endonucleases were purchased from New England Biolabs Inc. (Herts, UK). Glutathione, glutathione-agarose beads, anti-GST (glutathione *S*-transferase) antibodies, thrombin, X-Omat Kodak X-ray film were purchased from Sigma. pGEX expression system was purchased from Pharmacia. Tissue culture media and supplements were purchased from Gibco BRL. Oligonucleotides for fusion protein construction and sequencing were synthesised by Genosys Biotech (Europe) Ltd.

Anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies and SDS–PAGE molecular weight markers were purchased from Bio-Rad. Rabbit anti-Fyn polyclonal antibodies, anti-myc, anti-Pyk2, anti-GAP and mouse anti-Fyn monoclonal antibodies were purchased from UBI. Anti-annexin VI antibodies were prepared as previously described [21]. The Marathon-Ready human brain cDNA library was purchased from Clontech laboratories Ltd. pCMV-Tag1 vector was purchased from Stratagene.

2.2. Immunoprecipitation studies

COS-1 cells and Rat-1 fibroblasts were grown as previously described [21]. Unless otherwise stated, cells were routinely used when they were 80–90% confluent. Cells were lysed and immunoprecipita-

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Abbreviations: GAP, GTPase activating protein; C2, conserved region 2; GST, glutathione *S*-transferase; Pyk2, proline rich tyrosine kinase 2; EGTA, ethylene glycol-bis(β-amino ethyl ether)-*N*′,*N*′,*N*′,*N*′-tetraacetic acid; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence

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tion samples were prepared using anti-Fyn polyclonal, anti-Pyk2, anti-GAP and mouse anti-Fyn antibodies as previously described [21].

2.3. Western blot detection of complexing proteins in cell lysates

Following SDS–PAGE resolution, proteins were Western transferred to nitrocellulose and membranes were blocked with 5% (w/v) skimmed milk. Blots were then probed by their incubation overnight at 4°C with either rabbit anti-Pyk2 (1 μg/ml), anti-GAP (1 μg/ml), anti-Fyn antibodies (2 μg/ml), or anti-annexin VI (1/1000 v/v dilution) antibodies. Bound antibodies were detected by incubation for 1 h with goat anti-rabbit HRP-conjugated secondary antibodies diluted 1/3000. Detection of secondary antibody binding was made using enhanced chemiluminescence (ECL) followed by visualisation on X-Omat AR grey film.

2.4. Construction of GST-Fyn

Human Fyn cDNA was isolated by first round PCR amplification using a Marathon-Ready human brain cDNA library as template and oligonucleotide primers: 5′-ATGGGCTGTGTGCAATGTAAGGAT-3′ and 5′-CTCGAATGCTTCACAGAGGAGGTT-3′. The PCR product obtained was then re-amplified using primers containing *Bam*HI and *Eco*RI restriction endonuclease sites: 5′-TGATGATGAGGATCCATGGGCTGTGTGCAATGTAAGGAT-3′ and 5′-TGATGATGAGAATTCCTCGAATGCTTCACAGAGGAGGTT-3′. This second round PCR amplified fragment was then digested with *Bam*HI and *Eco*RI and subcloned into *Bam*HI/*Eco*RI digested pGEX-2T vector. The identity and reading frame of the Fyn cDNA was verified by DNA sequencing of the pGEX-Fyn construct.

2.5. Expression and purification of the GST-Fyn construct

Protein expression was induced with 0.1 mM IPTG for 3 h at 37°C. The protein was purified on a non-denaturing 10% polyacrylamide gel from the inclusion body cell fraction. The protein band of expected fusion protein size was then excised and electroeluted. The isolated soluble protein was affinity purified on glutathione-agarose beads and then cleaved with thrombin at a protease:protein (w/v) ratio of 1:10 for 2 h at 25°C with constant agitation.

2.6. Purification of GAP C2 domain complexing proteins from sheep liver

Ca^{2+}-dependent membrane binding proteins were prepared from sheep liver homogenates as previously described [21] and, from this fraction, GAP C2 domain complexing proteins were affinity purified using a GSTGAP C2 domain ($GSTGAP^{606-648}$) fusion protein as previously described by us [21].

2.7. Annexin VI overlay binding assay

GSTGAP C2 domain complexing proteins purified from sheep liver as described above were eluted from glutathione-agarose beads with 15 mM free glutathione. Approximately 5 μg of total protein was resolved on SDS polyacrylamide gels and transferred to nitrocellulose filters. Filters were then blocked overnight with phosphate buffered saline (PBS) containing 5% (w/v) skimmed milk powder followed by their incubation in the presence or absence of 16 nM GST or GSTannexin VI at 25°C for 1 h. Bound fusion protein was detected with anti-GST polyclonal antibodies (1/1000 v/v dilution) followed by probing with goat anti-rabbit HRP antibodies and ECL.

2.8. Fyn overlay binding assay

300 ng of recombinant Fyn was subjected to SDS–PAGE and Western transferred to nitrocellulose membranes. Membranes were blocked with 5% (w/v) skimmed milk powder in PBS for 1 h and then incubated with either 16 nM GST or 16 nM GSTannexin VI at 25°C for 1 h. Bound fusion protein was detected with anti-GST polyclonal antibodies followed by probing with anti-rabbit HRP antibodies and ECL.

2.9. Transfection of COS cells with pCMV vector or pCMVPyk2 DNA

COS-1 cells were routinely diluted to approximately 20–25% confluence 3–6 h prior to their transfection. For each 10 cm plate of cells used, 4 μg of vector (pCMV-Tag1) or pCMVPyk2 DNA [26] was preincubated with 25 μl of lipofectin in a final volume of 4 ml of Opti-MEM medium for 15 min prior to transfection. The cells were washed three times with serum-free Opti-MEM and incubated for 12 h with the DNA–lipofectin mixture in a 5% CO_2 atmosphere at 37°C. The transfection mixture was then removed and replaced with Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum and the cells were incubated in a 5% CO_2 atmosphere at 37°C. Cells were harvested for immunoprecipitation studies no more than 24 h after transfection began, otherwise cell death was observed. Routinely between four and six plates of cells were pooled after transfection (approximately 2×10^6 cells in total) for each immunoprecipitation sample.

2.10. Western blot detection of myc-tagged proteins in COS cell lysates

Anti-Pyk2 or anti-GAP immunoprecipitated protein complexes from transfected COS cells were resolved by SDS–PAGE and Western transferred to nitrocellulose filters. Filters were probed with either anti-Pyk2 (1 μg/ml) or anti-myc (5 μg/ml) antibodies diluted in 5% skimmed milk/PBS solution. Bound antibodies were detected with goat anti-rabbit HRP-conjugated antibodies (1/1000 v/v dilution) followed by ECL.

3. Results

3.1. Affinity purification of the p55, p70, p120 complex using a GTGAPC2 domain fusion protein and the identification of p55 as an annexin VI binding protein

Our previous studies using far Western overlay assays had indicated that the RasGAP C2 domain interacts directly with annexin VI. However, no evidence had been obtained for the direct interaction of this C2 domain with either p55 or p120 in this protein complex. Thus, it was conceivable that annexin VI could in turn be mediating a protein–protein interaction with one or more of these proteins. So, in order to address this question, we purified proteins complexing with the C2 domain of RasGAP from sheep liver using a GSTGAP C2 domain affinity purification procedure as previously described by us [21]. We then resolved samples of the complexing proteins on SDS polyacrylamide gels (Fig. 1A) and transferred the purified complex proteins to nitrocellulose. The proteins were then assayed for their ability to bind to GST or GSTannexin VI fusion protein (Fig. 1B). We observed that the annexin VI

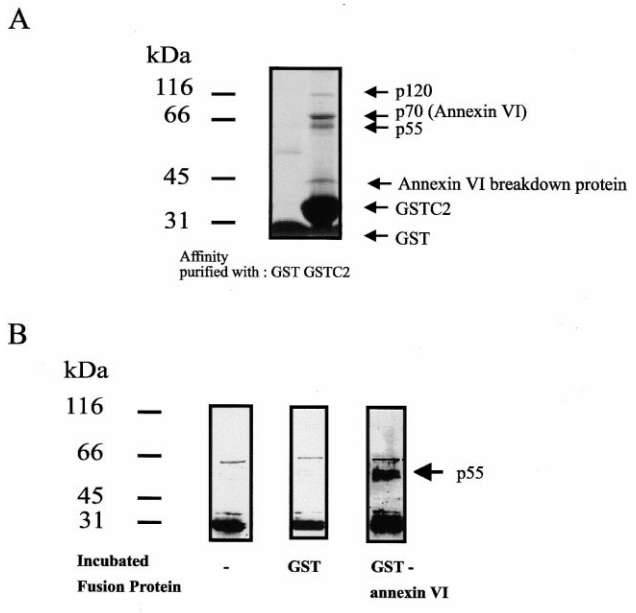

Fig. 1. Annexin VI binds to p55. A: Coomassie blue stained gel showing affinity purified GST and GSTGAP C2 complexing proteins from sheep liver homogenates. B: Far Western overlay binding assay demonstrates GSTannexin VI fusion protein specifically bound to p55.

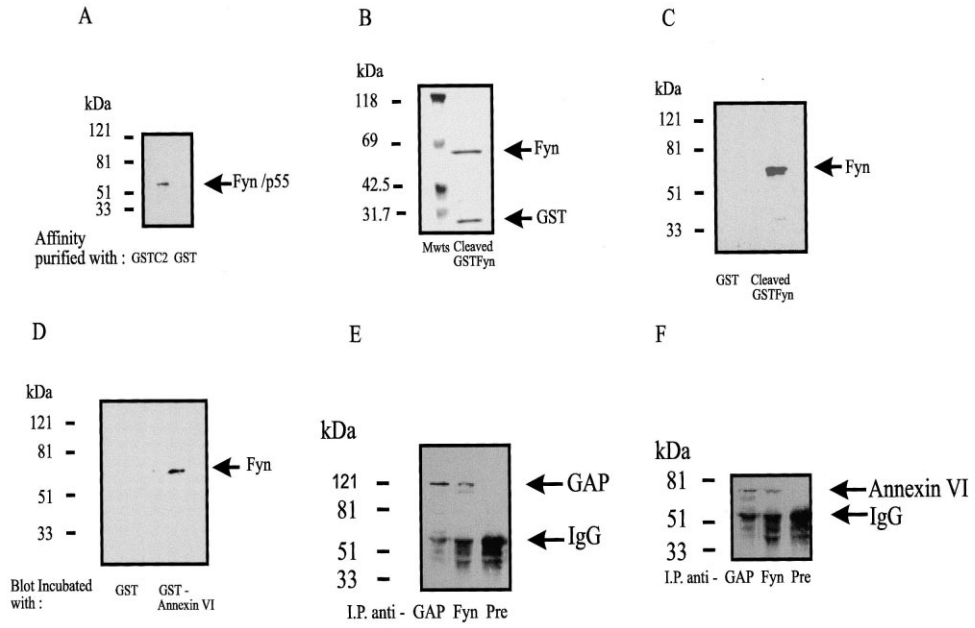

Fig. 2. p55 is Fyn. A: The 55 kDa GSTGAP C2 complexing protein is specifically detected with anti-Fyn antibodies. B: Coomassie stained SDS polyacrylamide gel showing purified recombinant Fyn (~59 kDa) and GST (27 kDa) after cleavage of GSTFyn with thrombin. C: Anti-Fyn antibodies detect recombinant cleaved GSTFyn, but not GST control protein. D: Far Western overlay binding assay demonstrates GST-annexin VI, but not GST, binds to recombinant Fyn protein. E: GAP and F: annexin VI are detected in anti-GAP and anti-Fyn but not pre-immune serum control immunoprecipitates from Rat-1 fibroblast cell lysates.

fusion protein specifically bound to the complexing proteins around the expected size for p55. Neither the anti-GST antibody, which was used to detect the fusion protein, or the control GST protein was detected as binding to p55 (Fig. 1B). It was also noted that a protein band of ~42 kDa is often additionally observed by Coomassie blue staining of gels (Fig. 1A), but since this protein is detected by annexin VI antibodies when these samples are Western blotted, we believe that this is a breakdown product of annexin VI (data not shown).

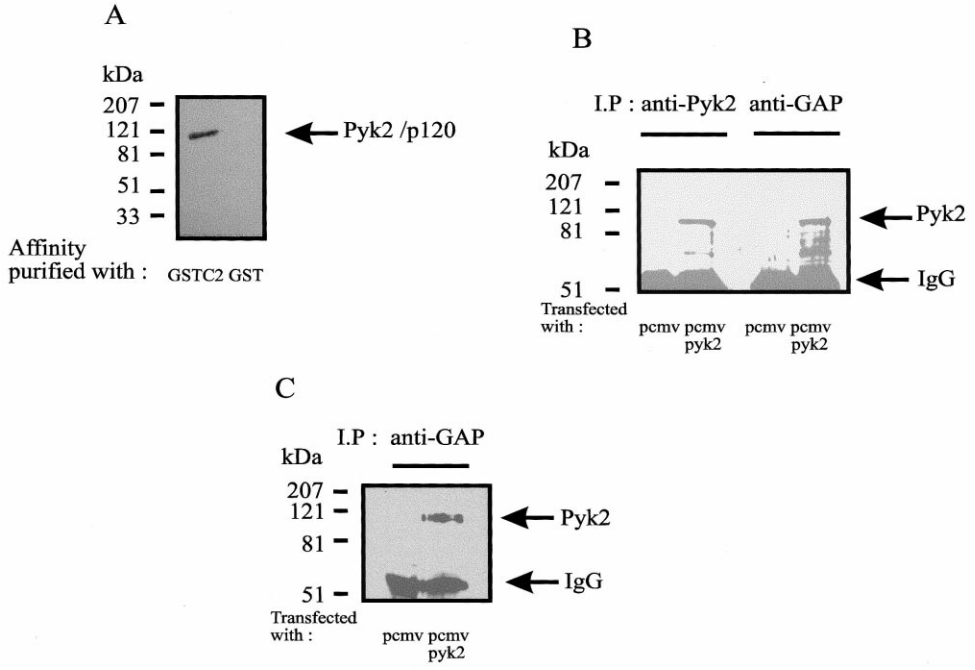

Fig. 3. Evidence that p120 is the proline rich tyrosine kinase Pyk2. A: Anti-Pyk2 antibodies specifically detect the 120 kDa GSTGAP C2 complexing protein. B: Confirmation of Pyk2 expression and GAP co-immunoprecipitation of Pyk2 in pCMV or pCMVPyk2 transfected COS cells. C: Anti-Myc antibodies detect Myc tagged Pyk2 protein from anti-GAP immunoprecipitates of pCMVPyk2, but not pCMV, transfected COS cells.

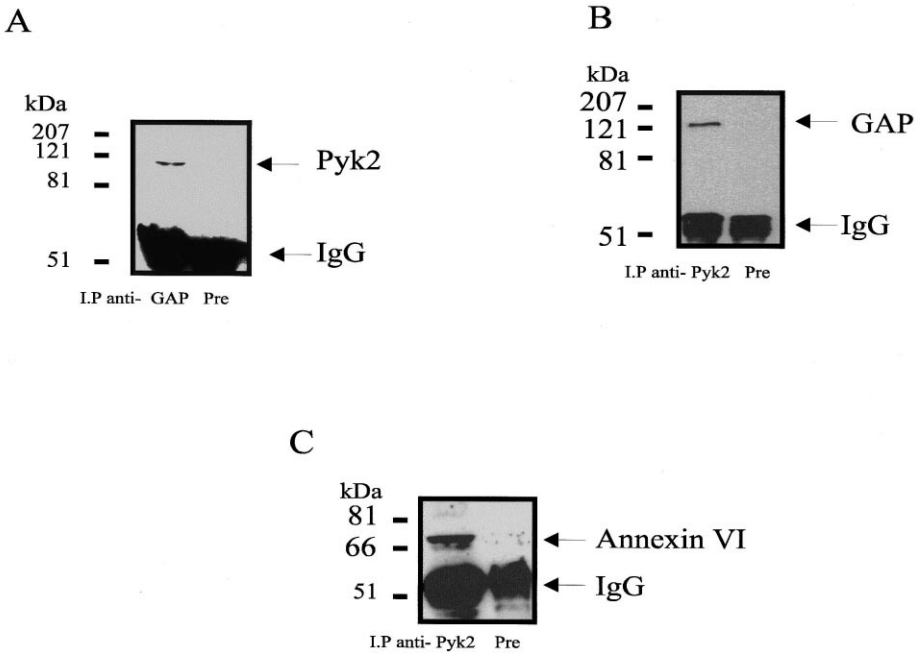

Fig. 4. Pyk2 simultaneously complexes with GAP and annexin VI in Rat-1 fibroblast cell lysates. A: Anti-GAP antibodies specifically co-immunoprecipitate Pyk2. B: Anti-Pyk2 antibodies specifically co-immunoprecipitate GAP. C: Anti-Pyk2 antibodies specifically co-immunoprecipitate annexin VI.

3.2. Identification of p55 as Fyn

In order to identify p55, proteins that had a similar apparent molecular weight and had previously been shown to interact with annexin VI were first considered. The Src family kinase Lck thus became a prime candidate since it has a molecular weight of 56 kDa and has been shown to interact with annexin VI in T cells [22]. However, Lck is predominantly expressed in lymphocytes and since we had observed reasonably high levels of p55 complexing with GAP in liver we reasoned that it was improbable that p55 was Lck. However, because Src family kinases are known to play an important role in the activation of the Ras signalling pathway and often have been demonstrated to functionally compensate for each other in cellular systems, we considered the possibility that p55 could be another Src family kinase. The protein Fyn has a molecular weight of 59 kDa (also a 56 kDa splice variant has been detected in some cell types), is a Src family kinase and displays a wide tissue expression profile [23]. Thus we tested the possibility that p55 could be Fyn. Western blots containing the GAP GSTC2 domain interacting proteins purified from sheep liver were probed with anti-Fyn antibodies and the presence of Fyn was indeed detected (Fig. 2A). In addition, the antibody was very specific since it only detected a protein of the expected ~55 kDa size even though this protein is not the most abundant protein in the protein complex (Fig. 1A). To further verify the identity of p55, purified recombinant Fyn was expressed as a GST fusion protein in *Escherichia coli*. The fusion protein was cleaved from the GST sequence to liberate soluble Fyn protein (Fig. 2B) and the identity of the recombinant Fyn protein was confirmed by Western blot analysis using anti-Fyn antibodies (Fig. 2C). Using this cleaved fusion protein in an overlay binding assay with GST control and GSTannexin VI fusion proteins we confirmed that annexin VI was indeed able to specifically bind to recombinant Fyn (Fig. 2D). Next, in order to test whether Fyn could co-complex with GAP and annexin VI in a mammalian cell system, we immunoprecipitated Fyn from Rat-1 fibroblast cell lysates using our anti-Fyn polyclonal antibody and anti-GAP and pre-immune serum antibodies as positive and negative controls respectively. After resolving immunoprecipitated proteins on SDS polyacrylamide gels, we firstly probed Western blots for the presence of RasGAP (Fig. 2E) and then re-probed for the presence of annexin VI (Fig. 2F). We were able to clearly detect both RasGAP and annexin VI as co-complexing in these anti-Fyn immunoprecipitated samples. Therefore, p55 is Fyn and it can interact with annexin VI to form a protein complex with GAP when annexin VI and RasGAP are interacting in cellular systems.

3.3. Identification of p120 as the proline rich tyrosine kinase Pyk2

We next reasoned that p120 could be a Fyn binding protein and thus have the ability to form a protein complex with annexin VI and RasGAP. Since a range of Src family kinases are known to interact with the focal adhesion kinase (FAK) family member Pyk2 in a Ca^{2+}-dependent manner and Pyk2 has an apparent molecular weight of 110–125 kDa [24,25], we considered the possibility that p120 could be Pyk2. To test this, using anti-Pyk2 antibodies and Western blotting, we firstly probed for the presence of Pyk2 in the GSTGAP C2 domain complexing proteins purified from sheep liver and were clearly and specifically able to detect Pyk2 (Fig. 3A). Next, in order to eliminate the possibility that our anti-Pyk2 antibody could be cross-reacting with the related kinase FAK (even though our antibody is raised to the stretch of amino acids corresponding to residues 726–863 of human Pyk2 which is uniquely found in Pyk2) we checked the specificity of our antibody. To do this, we transfected COS cells with the

mammalian expression vector $_p$cmv containing cDNA encoding Rat Pyk2, which contained an N-terminal myc epitope tag [26]. Using our anti-Pyk2 antibodies, and anti-GAP antibodies, we were able to immunoprecipitate and Western blot detect the heterologously expressed Pyk2 from $_p$cmvPyk2 but not from $_p$cmv vector control transfected cells (Fig. 3B). Similarly by anti-GAP immunoprecipitation and anti-myc Western blot detection we were able to demonstrate that GAP was indeed co-immunoprecipitating with the myc-tagged Pyk2 protein (Fig. 3C). Thus, p120 is Pyk2 and it can co-complex in COS cells with RasGAP.

3.4. Co-immunoprecipitation studies in Rat-1 fibroblasts; Pyk2 forms a complex with RasGAP and annexin VI in mammalian cells

Finally, we looked for evidence for the co-complexing of Pyk2 with GAP and annexin VI in rat fibroblasts (Fig. 4). By immunoprecipitating complexed proteins with either anti-GAP antibodies (Fig. 4A), or anti-Pyk2 antibodies (Fig. 4B,C) we were indeed able to detect the presence of Pyk2 in a complex with RasGAP and annexin VI in cell lysates.

4. Discussion

In this study we have successfully identified the proteins p55 and p120 which were previously shown to form a protein complex with the $p120^{GAP}$ C2 domain [21]. Our rationale was to identify these proteins in order to obtain clues as to the signalling functions of annexin VI and RasGAP. From our studies presented here and our previous work [21,27] we conclude that the C2 domain interacts with annexin VI, which in turn interacts with Fyn. Thus, annexin VI appears to be a 'bridging' protein for protein complex formation. We speculate that Pyk2 in turn may interact with Fyn since others have shown that a range of Src family kinases have been identified as co-complexing with Pyk2 [28,29]. Also, a Src SH2 domain binding site has been identified as a tyrosine residue (Y402) in rat Pyk2 [28].

Since Fyn and Pyk2 are both important tyrosine kinases associated with the regulation of p21 Ras proteins, and C2 domains have been proposed to function as Ca^{2+} sensors in signalling proteins [30] we can at this stage only speculate that the signalling role of this protein complex may well be intimately linked with a Ca^{2+} mediated regulation of p21 Ras activity. Certainly, other workers have demonstrated that elevation of intracellular Ca^{2+} levels either by ionophore treatment or by stimulation of GPCRs coupled to PLC-β activation via Gq or Gi proteins can result in Ras [4] and MAP kinase [24,31,32] activations. Hence, this novel protein complex may have a role to play in the convergence of tyrosine kinase and G protein coupled receptor/Ca^{2+} signalling pathways to Ras. The elucidation of the signalling role of this protein complex will be the subject of our future work, as will the more detailed investigation of the interaction mechanisms and mapping of sites of interactions within these proteins.

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