Mapping the site of interaction between annexin VI and the p120^{GAP} C2 domain

Andrew Chow, Debra Gawler*

School of Biomedical Sciences, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, Yorkshire, UK

Received 21 September 1999

Abstract Annexin VI is a Ca^{2+}-dependent membrane and phospholipid binding protein. It mediates a protein-protein interaction with the Ras p21 regulatory protein p120^{GAP}. In this study we have mapped the binding site of GAP within the annexin VI protein. Using Far Western overlay binding assays and cell lysate competition studies we have mapped the site of interaction to the inter-lobe linker region; amino acids 325–363. Finally, using a GST fusion protein corresponding to this linker region we have demonstrated that cellular loading of the fusion protein into Rat-1 fibroblasts by electroporation blocks the interaction and co-immunoprecipitation of annexin VI and GAP.

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

1. Introduction

Annexins are a family of structurally related Ca^{2+}-dependent phospholipid and membrane binding proteins. To date, at least 13 have been identified and cloned [1,2]. The crystal structure of both bovine and human annexin VI have been determined [3,4] and from these studies and molecular modelling it is apparent that the protein consists of two disk-shaped lobes and an inter-lobe connecting region. Each lobe contains four structural repeats of approximately 70 amino acid residues and the lobes are positioned perpendicular to each other. Each repeat forms a structural domain comprised of five α-helices and a number of Ca^{2+} binding sites have been proposed within loops located between specific helices within these repeat structures. Intriguingly, Ca^{2+} binding is predicted to occur on a convex surface of the protein which is proposed to bind to cellular membranes [4].

Although much is known about the structure of annexin VI, the precise cellular functions of the protein have yet to be established. The protein displays a wide tissue expression profile and is detectable in cells at abundant levels associated with the plasma membrane and internal membrane systems [5–8]. A range of cellular functions have been proposed for the protein including $Ca2^{+}$ and K^{+} conductance modulation, [9], Ca^{2+} homeostasis [10] and cellular growth and proliferation [11,12].

Recently, we have shown that annexin VI interacts directly with a key Ras regulatory protein p120-GTPase activating protein (p120^{GAP}) [13]. In addition, we have established that it is the conserved region 2 (C2) domain within GAP which interacts with annexin VI. We and others have demonstrated that C2 domains within proteins have the ability to bind negatively charged phospholipids in a Ca^{2+}-dependent manner [14,15] and the C2 domain within GAP has the ability to co-bind to phospholipid vesicles and cellular membranes in a Ca^{2+} and annexin VI dependent manner [16]. Our observations have led us to propose that the annexin VI-GAP interaction may play a role in the ability of GAP to translocate and membrane associate in response to intracellular Ca^{2+} elevation [16,17].

Here we have attempted to define the region within annexin VI where the C2 domain of GAP interacts. As a result of this study it will be possible to design both mutant proteins and smaller peptides corresponding to stretches within the interaction region in order to selectively disrupt this protein-protein interaction. Thus, by designing such tools, the cellular consequences of such a disruption may be evaluated and a cellular signalling function ascribed to the annexin VI protein.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were of analytical grade. Restriction endonucleases were purchased from New England Biolabs (Herts, UK), glutathione-agarose beads, anti-GST antibodies, thrombin, X-Omat Kodak X-ray film were purchased from Sigma. Factor Xa was purchased from Promega. 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). Anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight markers were purchased from Bio-Rad. Anti-GAP antibodies were purchased from UBI.

2.2. Construction, expression and purification of GST fusion proteins

GST fusion proteins encoding amino acids 606–648 of human GAP (GSTGAP C2) and amino acids 1–673 of human annexin VI (GSTannexin VI$^{1-673}$) were constructed as previously described [13]. cDNA encoding GSTannexin VI$^{1-673}$ was digested with *Bgl*II to yield fragments corresponding to amino acids 118–292, 292–421 and 421–529 of human annexin VI. Fragments were subcloned into a *Bam*HI digested pGEX-3X vector to yield the GST fusion proteins GSTannexin VI$^{118-292}$, GSTannexin VI$^{292-421}$ and GST annexin VI$^{421-529}$. A cDNA fragment encoding for amino acids 383–673 of annexin VI was PCR amplified using oligonucleotide primers: 5′-AAGACACAATCATCGATAT CATCA-3′ and 5′-CTACTACT AGGGCCCCT AGTCCTCACC-3′. The 3′-reverse primer contained an *Apa*I

*Corresponding author. Fax: (44) (113) 2334228.
E-mail: d.j.gawler@leeds.ac.uk

Abbreviations: GAP, GTPase activating protein; C2, conserved region 2; EGTA, ethylene glycol-bis(β-amino ethyl ether)-*N*′*N*′*N*′,*N*′-tetra-acetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; FBS, foetal bovine serum ; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence

0014-5793/99/$20.00
PII: S0014-5793(99)01336-8

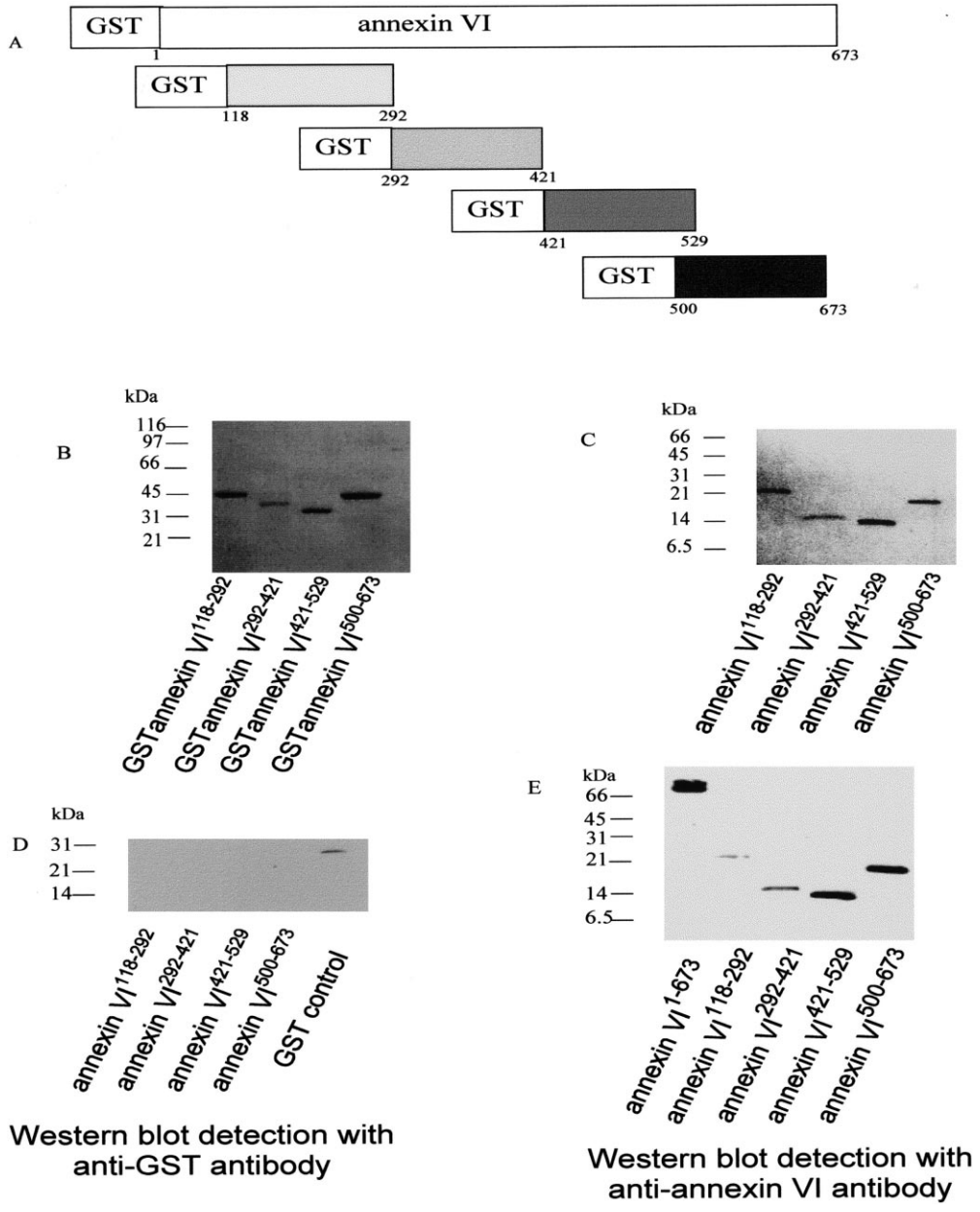

Fig. 1. Expression and detection of GST fusion protein constructs. A: Schematic diagram representing the amino acid boundaries for the GSTannexin VI fusion proteins. B: Coomassie-stained SDS-polyacrylamide gel of the purified GSTannexin VI fusion proteins. C: Coomassie-stained SDS-polyacrylamide gel of Factor Xa cleaved annexin VI protein fragments. D: Cleaved fragments were not detected with anti-GST antibodies. E: Cleaved annexin VI fragments were detected with anti-annexin VI antibodies.

restriction site to facilitate subcloning (underlined). The PCR product was digested with *Apa*I, Klenow blunted and subcloned into a *Sma*I digested pGEX-3X vector. The vector containing insert was then digested with *Bam*HI and re-ligated to yield GSTannexin VI$^{500-673}$. Annexin VI$^{325-363}$ was amplified by PCR from GSTannexin VI$^{1-673}$ using the following primers; 5′-TGATGATGAGGATCCGATGCT GCTGGCCAT CTT-3′ (containing a *Bam*HI site, underlined) and 5′-TCATATCAGAATTCCTTCAGCTC TACTCGGGCCA-3′ (containing an *Eco*RI site, underlined). The PCR product was *Bam*HI/*Eco*RI digested and subcloned into a *Bam*HI/*Eco*RI digested pGEX-2T vector, generating GSTannexin VI$^{325-363}$. Fusion protein constructs were verified by DNA sequencing.

2.3. Expression and purification of GST fusion proteins

All GST fusion proteins were expressed in XL1-Blue *Escherichia coli*, as described previously [14]. GSTGAP C2, GSTannexin VI$^{1-673}$, GSTannexin VI$^{421-529}$ and GSTannexin VI$^{325-363}$ were purified as previously described [13]. Bacterial cultures expressing GSTannexin VI$^{118-292}$and GSTannexin VI$^{500-673}$ were centrifuged at 3000×*g* for 15 min at 4°C and the pellet resuspended in 15 mM HEPES pH 7.5 containing 5 mM ethylene glycol-bis(β-amino ethyl ether)-*N*′*N*′*N*′,*N*′-tetra-acetic acid (EGTA), 1 mM phenylmethylsulfonyl fluoride (PMSF) and free Ca^{2+} at a concentration of 7 mM. The cells were sonicated on ice for 3×30s and centrifuged at 14 000×*g* for 30 min at 4°C. The pellet was washed twice in the calcium containing buffer and finally incubated on ice for 30 min following resuspension in 15 mM HEPES pH 7.5 containing 5 mM EGTA and 1 mM PMSF. Cells were centrifuged at 14 000×*g* for 30 min at 4°C. The supernatant was collected and purified on glutathione-agarose beads. A bacterial culture expressing GSTannexin VI$^{292-421}$ was centrifuged at

3000×*g* for 15 min at 4°C and the pellet resuspended in 25 mM Tris pH 7.5. Five miligram lysozyme was added and the resuspended pellet incubated shaking at 4°C for 30 min. Cells were sonicated and centrifuged as before. The pellet was washed with 25 mM Tris pH 7.5 containing 2% Triton X-100, incubated shaking at room temperature for 30 min in 25 mM Tris pH 7.5 containing 8 M Urea, 1% β-mercaptoethanol, 0.1% SDS and 1% glycerol. The solubilised protein was diluted 1:20 with ice cold 25 mM Tris pH 7.5, followed by centrifugation at 14 000×*g* for 15 min at 4°C.

2.4. Cleavage of GST fusion proteins

Annexin VI$^{1-673}$ was thrombin cleaved from GSTannexin VI$^{1-673}$ as previously described [16]. As appropriate recombinant annexin VI$^{1-673}$ was cleaved with V8 protease at a protease:protein (w/w) ratio of 1:20 for 2 h at 25°C and purified by electroelution. GSTannexin VI$^{118-292}$, GSTannexin VI$^{292-421}$, GSTannexin VI$^{421-529}$ and GSTannexin VI$^{500-673}$ were cleaved by incubating purified fusion protein with Factor Xa at a protease:protein (w/w) ratio of 1:50 for 4–6 h at 25°C with constant agitation.

2.5. Detection of Factor Xa cleaved annexin VI protein fragments

Cleaved fragments were resolved by SDS-PAGE, Western transferred and blots were blocked in 5% (w/v) skimmed milk powder in phosphate buffered saline (PBS). Blots were probed with either anti-annexin VI [13] or anti-GST antibodies diluted 1:200 (v/v) and 1:1000 (v/v), respectively in 5% (w/v) skimmed milk powder in PBS. Bound antibody was detected with goat anti-rabbit HRP conjugated secondary antibodies and visualised using enhanced chemiluminescence (ECL) and X-omat AR grey film.

2.6. Overlay assay with cleaved annexin VI fragments

Two-hundred nanomol of Factor Xa or V8 protease cleaved fragments were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were blocked in 5% (w/v) skimmed milk powder in PBS for 1 h and incubated with either 30 nM GST or 30 nM GSTGAPC2 as previously described [13]. Filters were then probed with anti-GST polyclonal antibodies and bound antibody detected with anti-rabbit-HRP and ECL.

2.7. Competitive displacement of annexin VI protein fragment binding with free annexin VI protein

Two-hundred nanogram (14 pmol) of purified annexin VI$^{292-421}$ (per gel lane) was resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Blots were blocked in PBS at 25°C for 1 h and incubated with 420 nM annexin VI$^{1-673}$, 30 nM GSTGAPC2 or 30 nM GSTGAPC2 and 420 nM annexin VI$^{1-673}$ at 25°C for 1 h with constant agitation. Bound fusion protein was detected with anti-GST polyclonal antibodies followed by probing with goat anti-rabbit-HRP antibodies and ECL.

2.8. Immunoprecipitation studies in Rat-1 fibroblasts/peptide competition assay

Rat-1 fibroblasts were maintained as previously described [13]. Prior to harvesting, cells were washed twice with PBS and lysed on ice with 20 strokes in a Dounce homogeniser. Cell suspensions were then centrifuged at 1000×*g* to remove nuclei and unbroken cells. Immunoprecipitation studies were essentially carried out as previously described [13] with the exception that cells were either lysed in RIPA buffer and cell lysates incubated with the antibody-bead complex in

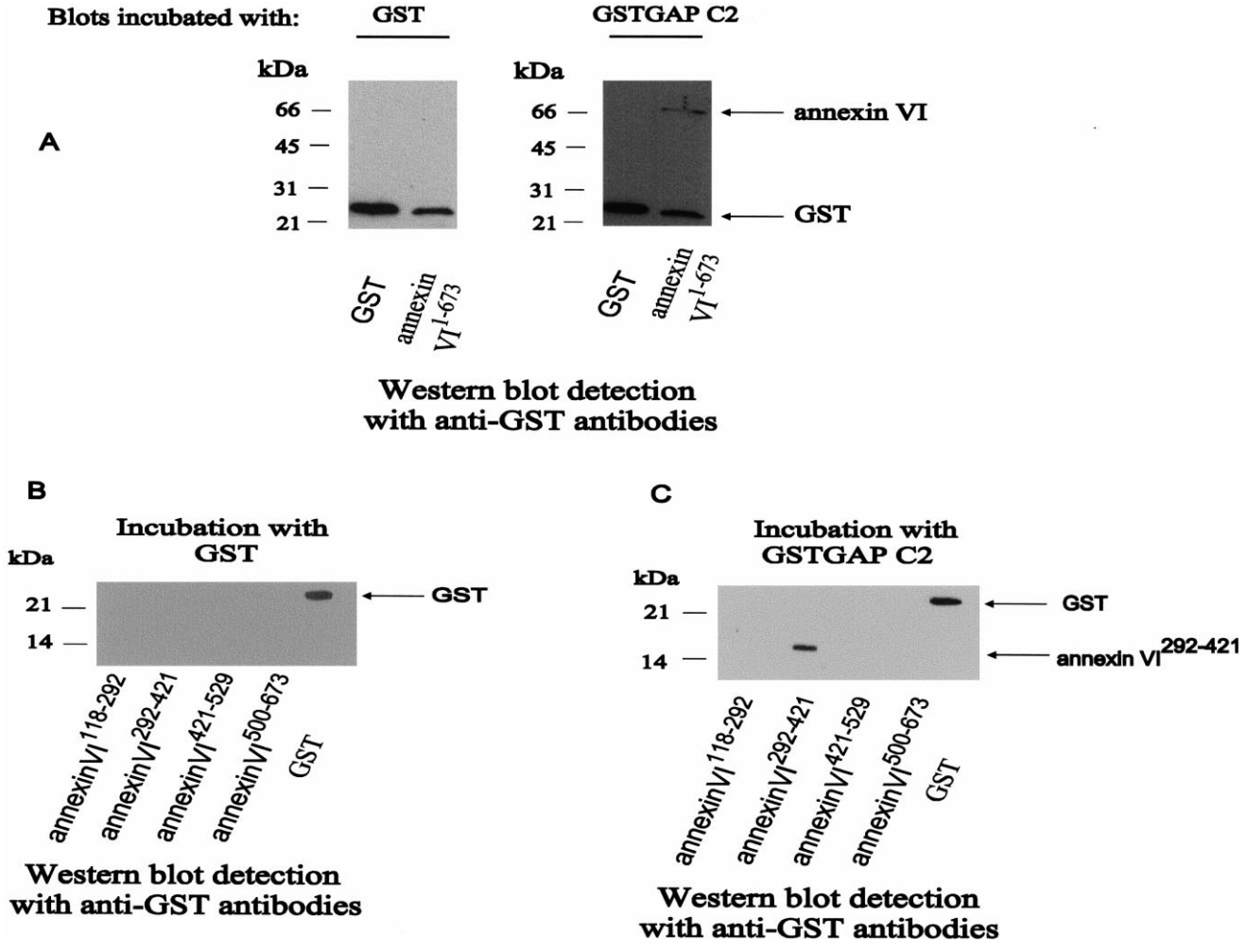

Fig. 2. A: Far Western overlay binding assay demonstrates recombinant full length annexin VI binds GSTGAP C2 and not GST. B: The annexin VI protein fragments do not bind GST. C: Annexin VI$^{292-421}$ binds to GSTGAP C2.

the presence or absence of either 0.42 μM GST, 0.42 μM GSTannexin VI$^{292-421}$ or else 0.54 μM GST or GSTannexin VI$^{325-363}$ loaded into cells by electroporation. Immunoprecipitated samples were analysed by SDS-PAGE, Western blotting and probed with anti-GAP and anti-annexin VI antibodies.

2.9. Electroporation of GSTannexin VI$^{325-363}$ into Rat-1 fibroblasts

Cells were harvested by trypsinisation. After supplementation with 10% (v/v) foetal bovine serum (FBS), fibroblasts were then washed twice with DMEM lacking FBS and resuspended to 1 ml with DMEM lacking FBS. The cells were then placed on ice for 10 min. 1 μM GST or GSTannexin VI$^{325-363}$ were then added to the cells and the resulting cell suspension transferred to an ice-cold sterile 0.4 mm electrode electroporation cuvette. A single pulse of 800 V was discharged across the cuvette and the cuvette incubated on ice for 10 min. The cells were gently pipetted into a 10 cm tissue culture plate and incubated with DMEM supplemented with 10% (v/v) FBS, 10 000 units/l penicillin, 10 000 μg/l streptomycin, 4 mM glutamine and allowed to recover overnight in a 5% CO_2 atmosphere prior to use.

3. Results

3.1. Construction and purification of GST annexin VI fusion proteins

In order to map the site of interaction within annexin VI where the GAP C2 domain binds, a series of GST annexin VI fusion proteins were first expressed in *E. coli* and purified to homogeneity. Fig. 1A shows a schematic diagram of the amino acid sequence boundaries for each fusion protein made. Each fusion protein contained between 100–170 amino acids of sequence corresponding to the human annexin VI protein sequence. Each fusion protein was expressed and analysed by SDS-PAGE (Fig. 1B) and then the GST sequence was cleaved free from the fusion protein by digestion with Factor Xa to yield purified annexin VI protein fragments (Fig. 1C). These protein fragments were then assayed by Western blotting for their ability to be detected by either anti-GST antibodies or anti-annexin VI antibodies. All purified annexin VI protein fragments were detected by anti-annexin VI antibodies (Fig. 1E) but no cross-reactivity with anti-GST antibodies was observed (Fig. 1D).

3.2. Far Western overlay binding assay for GAP C2 domain binding

First round screening for GAP C2 domain binding was undertaken using either full length annexin VI recombinant protein (Fig. 2A) or annexin VI protein fragments prepared and analysed as described above (Fig. 2B and C). Using a GST GAP C2 domain fusion protein and anti-GST antibodies to detect fusion protein binding, Far Western overlay binding was employed. Full length annexin VI or each annexin VI protein fragment was electrophoretically transferred from SDS-polyacrylamide gels onto nitrocellulose filters and after blocking, the filters were incubated with either GST control (Fig. 2A and B) or GSTGAP C2 domain (Fig. 2A and C)

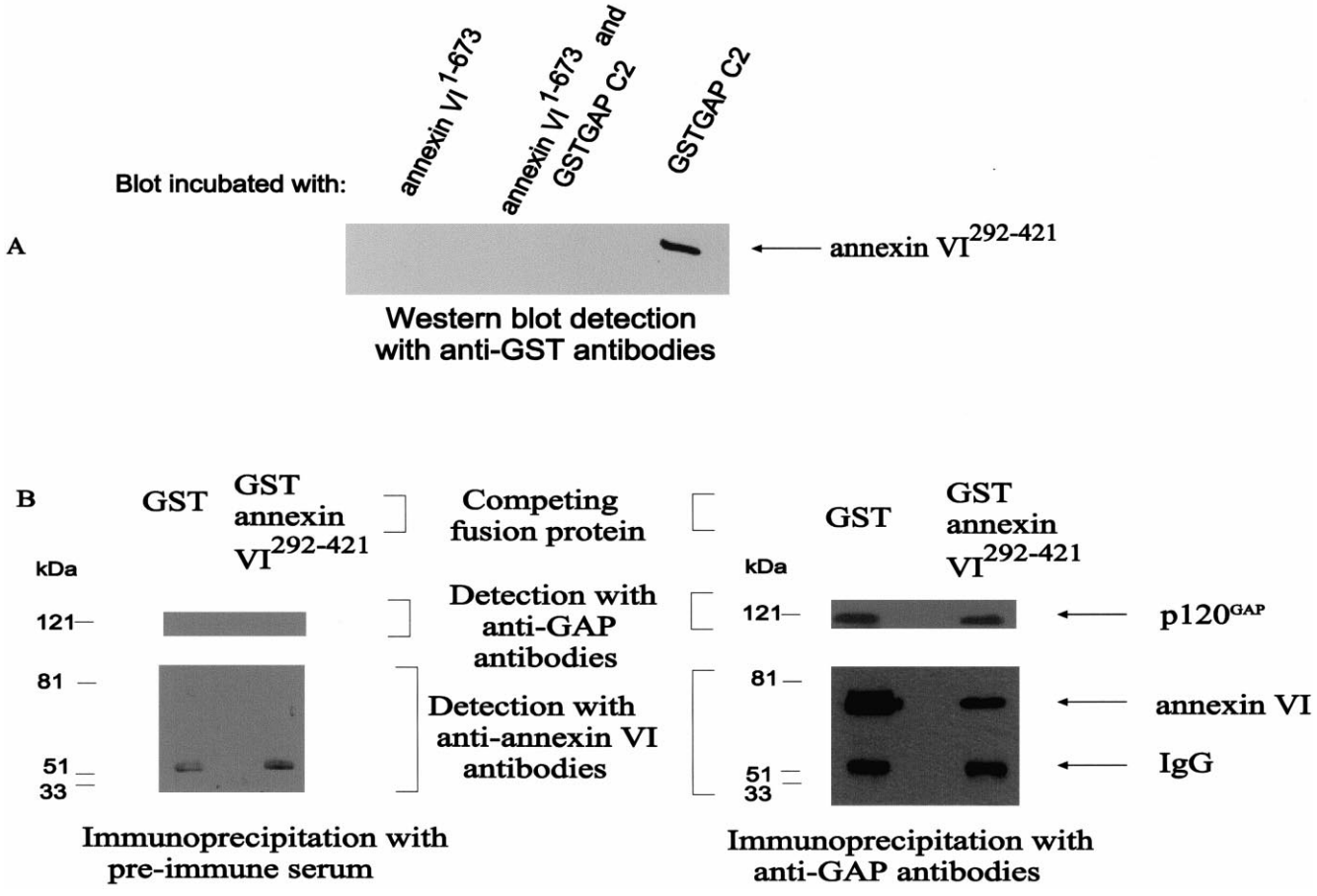

Fig. 3. A: Far Western overlay binding of annexin VI$^{292-421}$ to GSTGAP C2 in the presence and absence of recombinant annexin VI demonstrates a competitive displacement of annexin VI$^{292-421}$ with free annexin VI. B: GSTannexin VI$^{292-421}$ interferes with the co-immunoprecipitation of annexin VI and anti-GAP from Rat-1 fibroblast cell lysates.

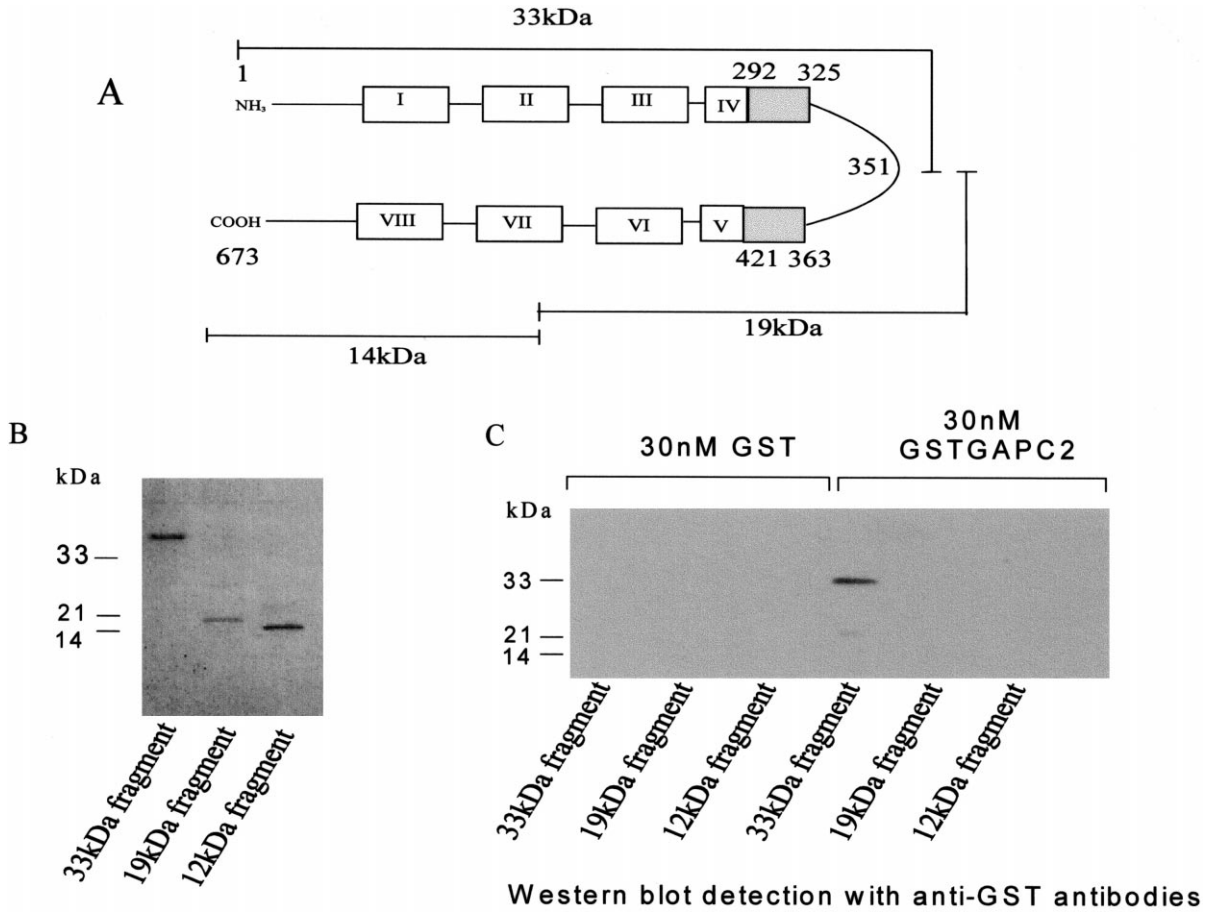

Fig. 4. A: Schematic diagram representing the V8 proteolytic fragments of annexin VI. B: Coomassie-stained SDS-polyacrylamide gel of purified V8 cleaved annexin VI fragments. C: The 33 kDa fragment binds to GSTGAP C2.

fusion proteins. GSTGAP C2 domain fusion protein was only detected as binding to the full length annexin VI protein and the annexin VI protein fragment corresponding to amino acid sequence 292–421 of human annexin VI.

3.3. Competition studies to confirm the GAP C2 domain interaction site

In order to confirm the importance of the annexin VI region 292–421 in the binding of the GAP C2 domain, two competition assays were employed. Firstly, using the protein fragment annexin VI$^{292-421}$ immobilised on the nitrocellulose filter, a Far Western overlay assay was undertaken in the presence or absence of full length recombinant annexin VI (Fig. 3A). When 30-fold molar excess annexin VI was present, the binding of the GAP C2 domain to the immobilised protein fragment was competed out. However, in order to establish whether this protein fragment could indeed interact with native cellular annexin VI protein, its ability to disrupt the interaction between full length GAP and annexin VI in Rat-1 fibroblast cell lysates was investigated. To do this, a GST control or GST annexin VI$^{292-421}$ fusion protein was included during immunoprecipitation of cell lysates with either pre-immune control sera or anti-GAP antibodies (Fig. 3B). Immunoprecipitated protein complexes were resolved by SDS-PAGE and separated proteins were Western transferred onto nitrocellulose filters. Blots were then probed for the presence of GAP and annexin VI proteins. GAP and annexin VI were specifically observed to co-immunoprecipitate when using anti-GAP antibodies and whilst the presence of the GST annexin VI$^{292-421}$ fusion protein did not significantly interfere with the ability of the anti-GAP antibodies to immunoprecipitate GAP protein from cell lysates (when compared with the GST protein control), the amount of co-immunoprecipitating annexin VI detected in the complex was significantly decreased (Fig. 3B). Indeed, scanning densitometry measurements estimated that the presence of the fusion protein decreased the amount of co-complexing annexin VI by approximately 70%.

3.4. V8 digestion studies

Molecular models based on X-ray crystallographic structural data for annexin VI indicated that the region containing amino acid sequence 292–421 corresponds to part of domain IV in lobe 1, the interconnecting 'linker' region and part of domain V within lobe two of the protein [3,4]. It was therefore necessary to further define the interaction site within the structural regions known to exist within the protein. To do this, protease digestion studies were undertaken. A schematic diagram of the structural units within annexin VI is shown in Fig. 4A and V8 protease-sensitive cleavage sites as defined by Ishitsuka et al. [18] are indicated. V8 digestion of recombinant human annexin VI was undertaken in order to produce char-

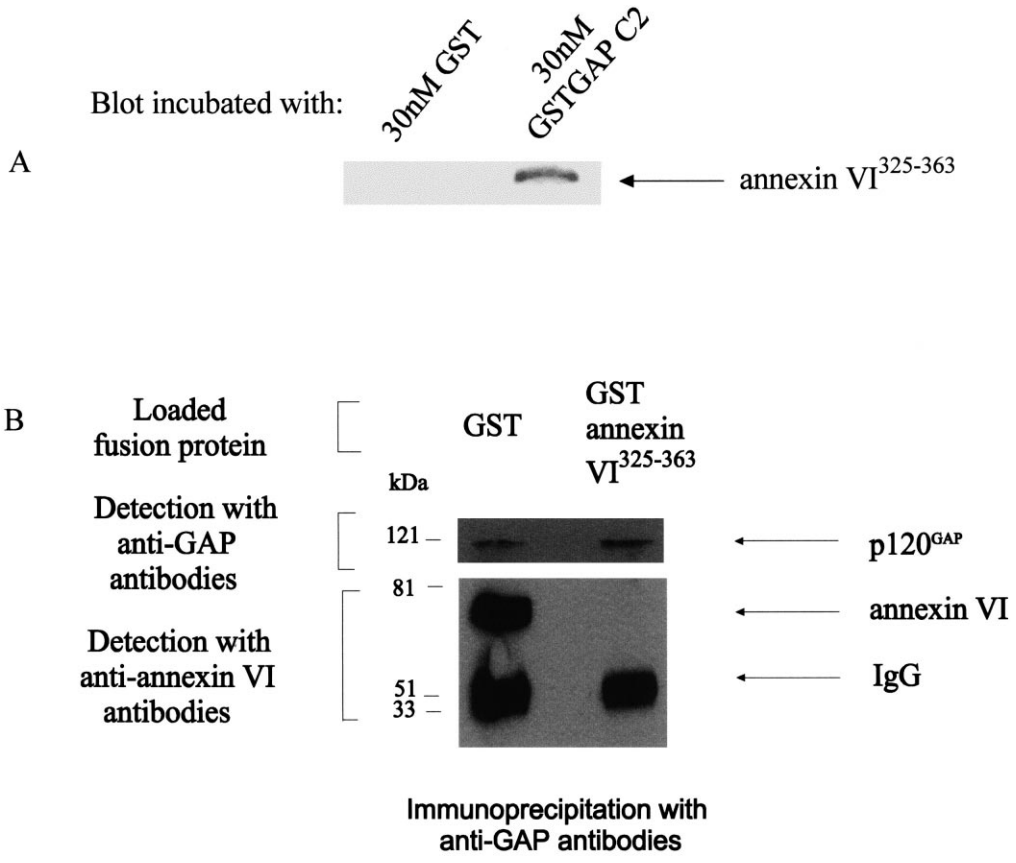

Fig. 5. A: Far Western overlay analysis demonstrates purified annexin VI$^{325-363}$ binds to GSTGAP C2 but not GST. B: Immunoprecipitation of GAP from Rat-1 fibroblasts loaded with GSTannexin VI$^{325-363}$ but not GST protein blocks the co-immunoprecipitation of annexin VI.

acteristic peptides derived from protein sequence within the already defined interaction site. Three characteristic 33, 19 and 12kDa proteolytic fragments previously described by Ishitsuka et al. were obtained (Fig. 4B). These protein fragments were Western transferred onto nitrocellulose filters and then subjected to Far Western overlay binding assay with either GST control or GST GAP C2 domain fusion proteins (Fig. 4C). The GST GAP C2 domain fusion protein was only detected as binding to the 33 kDa proteolytic fragment.

3.5. The annexin VI interconnecting 'Linker' region contains the GAP C2 domain binding site

Comparison of the overlapping region between the annexin VI$^{292-421}$ fragment and the 33 kDa V8 digestion peptide indicated that the GAP C2 domain interaction site is located between amino acid residues 292–351 (see Fig. 4A). However, since molecular modelling studies indicated that a discrete structural region known as the 'Linker' region is found between residues 325–363 of annexin VI, it was possible that the GAP C2 domain binding site could be located within this 'Linker' region. To test this, a GST fusion protein was constructed to correspond to this Linker region and the protein fragment was again liberated by cleavage from the fusion protein. GAP C2 domain interaction was tested for using firstly Far Western overlay binding assay (Fig. 5A) and cellular protein competition assay (Fig. 5B). The annexin VI$^{325-363}$ protein fragment was indeed found to bind to the GST GAP C2 domain fusion protein. Also, the GST annexin VI$^{325-363}$ fusion protein was able to remove the ability of cellular full length GAP to co-immunoprecipitate with cellular full length annexin VI protein when this fusion protein (but not GST control protein) was loaded into cells by electroporation (Fig. 5B). Hence, we have defined 27 amino acid residues (annexin VI$^{325-351}$) located within the annexin VI interlobe connecting region as being important for the binding of the GAP C2 domain to annexin VI in cells.

4. Discussion

In this study we have identified the structural region within annexin VI where the GAP C2 domain binds. We have mapped the interaction site to within 27 amino acid residues which are located within the inter-lobe linking region of the annexin VI protein. The results of this study strongly indicate that the interaction between GAP and annexin VI is a highly specific protein-protein interaction because all other annexin family members so far identified are characteristically comprised of one tetrad repeat of 70 amino acid residue domains whereas annexin VI is unique in that it is comprised of two such tetrad repeats co-joined by this inter-lobe linker region. Intriguingly, crystal structure data indicate that the first lobe is rotated about 90° relative to the second lobe when in the crystal structure and in solution. However, when bound to a lipid monolayer both lobes appear to be coplanar [19]. Thus, a significant change in conformation must occur upon binding of the protein to membranes and the inter-lobe linker region must display corresponding flexibility. How such a flexibility could be involved in its ability to interact with GAP or if

indeed whether the presence of this protein-protein interaction could influence the ability of annexin VI to rotate its lobes and interact with cellular membranes are clearly intriguing areas for further investigation.

Finally, if, as has been suggested previously this annexin structure has arisen as a result of a series of gene duplications and fusions during evolution [20,21], then such an evolutionary adaptation has presumably resulted in a corresponding cellular function associated with GAP and thereby possibly the Ras signalling pathway. It is now our aim to disrupt this protein-protein interaction and study the cellular consequences of this action.

Acknowledgements: This work was funded by the North of England Cancer Research Campaign (NECRC) and the Cancer Research Campaign [CRC] project Grant reference number SP2440/0201.

References

[1] Raynal, P. and Pollard, H.B. (1994) Biochim. Biophys. Acta 1197, 63–93.
[2] Liemann, S. and Lewit-Bentley, A. (1995) Structure 3, 233–237.
[3] Benz, J., Bergner, A., Hofmann, A., Demange, P., Gottig, P., Liemann, S., Huber, R. and Voges, D. (1996) J. Mol. Biol. 260, 638–643.
[4] Avila-Sakar, A.J., Creutz, C.E. and Kretsinger, R.H. (1998) Biochim. Biophys. Acta 1387, 103–116.
[5] Dedman, J.R. and Kaetzel, M.A., (1992) in: The Annexins (Moss, S.E., Ed.), pp. 125–137, Portland Press, London.
[6] Moore, P.B., Kraus-Friedmann, N. and Dedman, J.R. (1984) J. Cell Sci. 72, 121–133.
[7] Drust, D.S. and Creutz, C.E. (1991) J. Neurobiol. 56, 469–478.
[8] Owens, R.J., Gallagher, C.J. and Crumpton, M.J. (1984) EMBO J. 3, 945–952.
[9] Naciff, J.M., Behbehani, M.M., Kaetzel, M.A. and Dedman, J.R. (1996) Am. J. Physiol. 271, c2004–c2015.
[10] Gunteski-Hamblin, A.M., Song, G., Walsh, R.A., Frenzke, M., Boivin, G.P., Dorn II, G.W., Kaetzel, M.A., Horseman, N.D. and Dedman, J.R. (1996) Am. J. Physiol. 270, H1091–H1100.
[11] Theobald, J., Smith, P.D., Jacob, S.M. and Moss, S.E. (1994) Biochim. Biophys. Acta 1223, 383–390.
[12] Edwards, H.C. and Moss, S.E. (1995) Mol. Cell. Biochem. 149/150, 293–299.
[13] Davis, A.J., Butt, J.T., Walker, J.H., Moss, S.E. and Gawler, D.J. (1996) J. Biol. Chem. 271, 24333–24336.
[14] Gawler, D.J., Zhang, L.-J.W. and Moran, M.F. (1995) Biochem. J. 307, 487–491.
[15] Davletov, B.A. and Sudhof, T.C. (1993) J. Biol. Chem. 268, 26386–26390.
[16] Chow, A., Davis, A.J. and Gawler, D.J. (1999) Cell. Signal. 6, 443–451.
[17] Gawler, D.J., Zhang, L.-J.W., Reedijk, M., Tung, P.S. and Moran, M.F. (1995) Oncogene 10, 817–825.
[18] Ishitsuka, R., Kojima, K., Utsumi, H., Ogawa, H. and Matsumoto, I. (1998) J. Biol. Chem. 273, 9935–9941.
[19] Kawasaki, H., Avila-Sakar, A., Creutz, C.C. and Kretsinger, R.H. (1996) Biochim. Biophys. Acta 1313, 277–282.
[20] Smith, P.D. and Moss, S.E. (1994) Trends Genet. 241–246.
[21] Morgan, O. and Fernandez, M.P. (1997) Cell. Mol. Life Sci. 53, 508–515.