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POTENTIAL INTERACTION BETWEEN ANNEXIN VI AND A 56-kDa PROTEIN KINASE IN T CELLS

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Annexins belong to a large family of calcium-dependent phospholipid binding proteins known to undergo post-translational modifications such as phosphorylation. Physiological function of each annexin is still unclear since they may partipate in signal transduction. We have tested the presence of annexins in a T cell line (Jurkat) and studied their phosphorylation by protein tyrosine kinases of the src family. Among annexins I, II, V and VI found in Jurkat cells, annexin VI was shown to be phosphorylated *in vitro* by $p56^{lck}$ and annexins I and II by $p60^{src}$. We could not detect the phosphorylation of A-VI *in vivo*, even after cell stimulation. However, a 56-kDa phosphoprotein was found to be associated with A-VI after T cell activation. This 56-kDa protein shares some characteristics with $p56^{lck}$.

Annexins form a group of at least 13 different but structurally related proteins [1,3]. They all have the capacity to bind Ca^{2+} as well as negatively charged phospholipids (PL) in a Ca^{2+}-dependent manner. These proteins are characterized by a similar structure with 4 or 8 repeats of a 70 amino-acid conserved core and differ by a variable N-terminal domain. Annexin cores share 40%-60% amino acid sequence identity and contain the Ca^{2+} and PL binding sites. The N-amino-terminal domain contains phosphorylation sites for different protein kinases such as protein kinase C (PKC) and protein tyrosine kinases (PTKs) as well as protein-protein interaction domains. It is speculated that these unique sites are specific and responsible for the different functions of the annexins. Annexin I (A-I) is the major cellular substrate for phosphorylation by the tyrosine kinase activity associated with the epidermal growth factor (EGF) and insulin- receptors [4,5] *In vitro,* A-I is phosphorylated by $pp60^{c\text{-}src}$, the polyoma

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Abbreviations: PKC : protein kinase C, PTK : protein tyrosine kinase, PL : phospholipid, TcR : T cell receptor, EGF : epidermal growth factor.

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middle T/pp60$^{c\text{-}src}$ complex and pp50$^{v\text{-}abl}$ [6]. Annexin II (A-II) is the major substrate *in vivo* for pp60$^{v\text{-}src}$ [7]. Interestingly, annexin VI (A-VI), which contains a putative site for tyrosine phosphorylation [8], has never been shown to be phosphorylated on tyrosine residues. Moreover, A-I [9,10] and A-II [11-13] were also shown to be phosphorylated *in vivo* by protein kinase C.

Annexins seem to be involved in various biological processes, but their precise functions *in vivo* have not yet been established. However, their binding to components of the cytoskeleton, and their phosphorylation by several protein kinases, suggest the involvement of annexins in signal transduction during cell proliferation and/or differentiation. Therefore, we have studied the role of annexins during T cell activation by analysing their relationships with protein kinases in these cells. Members of the annexin family have been identified in T cells [14-16]. A-VI expression appeared to be developmentally regulated in T lymphocyte differentiation [17] and its function may be regulated growth-dependently by a post-translational modification [18].

We have sought to identify candidate substrates for PTKs during T cells activation by stimulation of the T cell antigen receptor/CD3 complex (TCR/CD3). The earliest biochemical event following stimulation of the TCR is the activation of PTKs which results in the phosphorylation of cellular substrates [19]. Activation of the TCR/CD3 stimulates the activity of the major PTKs expressed in T cells: lck, fyn and Zap-70 [20]. These PTKs were shown to be associated with other proteins: p56^{lck} with CD4, CD8, IL-2 receptor, pp32; p59^{fyn} with TCR and Zap-70 with the tyrosine-phosphorylated ζ chain. These events preceed activation of phospholipase C, rise in intracellular calcium levels and activation of protein kinase C [21].

The aim of this study was to test the possibility for annexins to be substrates for PTKs of the src family in Jurkat cells, and analyse their role in signal transduction through the TcR/CD3 complex. We showed that among the annexins tested, only A-VI was a substrate for p56^{lck} *in vitro*. However, A-VI was not phosphorylated *in vivo*, but our results identified an association with a 56 kDa protein kinase sharing some characteristics with p56^{lck}.

Methods

Materials: A Jurkat cell line derived from clone J77-6.8 was used in all experiments [22]. Rabbit polyclonal antibodies raised against annexins were developped in the laboratory except MC2, a polyclonal anti-A-VI antibody kindly provided by Dr. S.E. Moss (UCL, London). The rabbit polyclonal anti-p56^{lck} antibody was raised against an N-terminal peptide [22].

In vitro phosphorylation assay of annexins by p56^{lck} and p60^{src}: Annexins were purified from human placenta by a procedure described elsewhere [23]. Kinase assays were performed with p56^{lck} prepared as an immune complex [24] and with purified p60^{src} (Oncogene Science). Purified annexins were incubated at 30°C with the p56^{lck} immune complex in 30 ml kinase buffer: 50 mM Pipes pH 7.5, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM PMSF, 1 mM sodium orthovanadate and 100 units/ml aprotinin. The reaction was initiated by adding 2 µCi of [γ-^{32}P] ATP (5,000 Ci/mmol, Amersham), and 4.5 mM unlabeled ATP (ATP mix). After 20 min, samples were centrifuged 5 min at 15,000 rpm to pellet p56^{lck} immune complex. Sample buffer (1% SDS, 5% glycerol, 2.5% β-mercaptoethanol, 30 mM Tris/HCl pH 6.8, 0,001% bromophenol blue and 1 mM sodium orthovanadate) was added to the supernatant. For p60^{src}, annexins were incubated with 2 units of purified kinase in 50 mM HEPES, 0.1 mM EDTA, 0.015% NP40 and the reaction started with ATP mix containing 30 mM $MgCl_2$. The reaction

was stopped after 30 min with the addition of sample buffer. After boiling, phosphorylated proteins were resolved by SDS-PAGE, the gel dried and exposed to KODAK XAR film at -70 °C using intensifying screens. In some experiments in order to test the autophosphorylation of $p56^{lck}$, the assay was performed by measuring ^{32}P incorporation from [γ-^{32}P] ATP into immune complexes as described above except that the reaction was initiated with 30 µCi of [γ-^{32}P] ATP (5000 Ci/mmol; Amersham). Reactions were terminated by the addition of 2X SDS sample buffer and proteins were resolved on 10% polyacrylamide gels followed by autoradiography.

Immunoprecipitation of annexins from [^{35}S] methionine labelled Jurkat cells: Jurkat cells (2.10^6/ml) were metabolically labelled for 5 hours with 500 µCi/ml of [^{35}S]-methionine (1000 Ci/mmol, NEN) in methionine-free RPMI medium supplemented with 5% dialyzed fetal calf serum. At the end of the incubation period, cells were pelleted at 10000 g for 5 sec, washed twice in ice cold phosphate buffered saline and lysed in a buffer containing: 10 mM Tris-HCl pH 7.4, 50 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 5 mM EGTA, 1 mg/ml bovine serum albumine, 1 mM phenylmethylsulfonyl fluoride, 100 units/ml aprotinin and 1 mg/ml each of antipain, leupeptin and pepstatin. Lysates were precleared for 1 hour at 4°C with 1% (vol/vol) fixed Staphylococcus aureus bacteria (Pansorbin; Calbiochem, La Jolla, California). The respective polyclonal antibodies were added (1/100 dilution) and allowed to react overnight prior to collecting the immune complexes with protein A Sepharose. Immune complexes were washed 3 times with lysing buffer and three times with phosphate buffer saline (PBS). Immunoprecipitates were analyzed on a 10% SDS-polyacrylamide gel. The gel was treated 30 min with Amplify (Amersham), dried and labelled bands visualized by autoradiography at -70°C.

Immunoprecipitation following kinase assay: Immune complex kinase reactions performed as described above were boiled in 100 µl of buffer containing: 1% SDS, 150 mM NaCl, 10 mM Tris HCl, pH 7.4 for 10 min, diluted with 0.9 ml of buffer containing: 1% Triton X-100, 50 mM NaCl, 1mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 30 mM pyrophosphate, 50 mM NaCl, and 10 mM Tris-HCl, pH 7.4 and incubated with protein A-Sepharose to remove immunoglobulin molecules. After a 10 min centrifugation at 15000 rpm, the supernatants were incubated with non-immune rabbit serum or anti-lck serum. The resulting immunoprecipitates were resolved on 10% polyacrylamide gels and subjected to autoradiography.

Immunoprecipitation of annexins from [^{32}P]o-phosphate-labelled Jurkat cells: Cells (10^7/ml) were incubated in phosphate-free RPMI medium supplemented with 5% dialyzed fetal calf serum for 30 min and then labelled for 3 hours with 500 µCi/ml [^{32}P]o-phosphate (8500-9120 Ci/mmol, NEN). Prior to terminating the incubation, cells were treated for the indicated periods of time with anti-CD3 (mAB IoT3, dilution 1/200, Immunotech). Cells were washed with cold phosphate buffer saline (PBS) containing 100 mM sodium orthovanadate and immunoprecipitation was performed as described above except that the lysing buffer was supplemented with 1 mM sodium orthovanadate, 25 mM sodium fluoride, 5 mM paranitrophenyl phosphate, 10 mM sodium pyrophosphate. Phosphoproteins were analyzed on 10% SDS-polyacrylamide gels followed by autoradiography at -70°C.

Tryptic peptide mapping: In vivo [^{32}P]o-phosphate-labelled proteins of interest were excised from gels, rehydrated in 200 ml of a 50 mM Tris-HCl buffer pH 8.8 and digested with 50 µg TPCK-treated trypsin (EC 3,4,21,4, Sigma) at 37°C for 18 hours. The phosphopeptides were analyzed on 40% polyacrylamide gels as described [25]. Exposure time was 7 days at 70°C.

Results and Discussion

Detection of annexins in Jurkat cells: The expression of members of the annexin family was assessed by performing immunoprecipitation experiments from [^{35}S]-methionine-labelled Jurkat cells and using specific antibodies against 4 different annexins. Figure 1 showed that these cells expressed A-I (lane 1), A-II (lane 2), A-V (lane 3) and A-VI (lane 4). A-I migrated

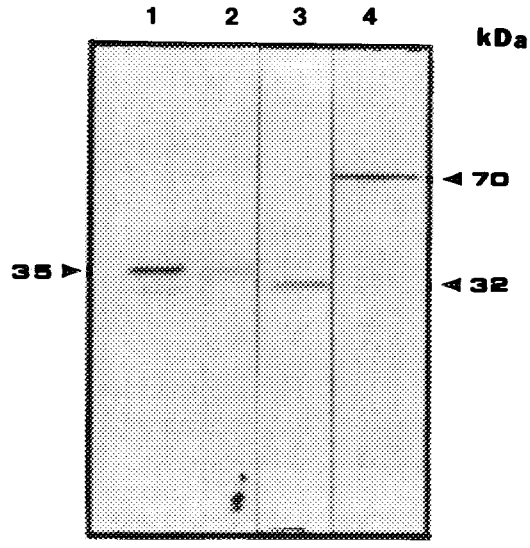

Figure 1. Immunoprecipitation of annexins from [^{35}S] methionine labelled Jurkat cells.
Cytosols from [^{35}S] methionine labelled cells were used to immunoprecipite annexins with anti-A-I (lane 1), anti-A-II (lane 2), anti-A-V (lane 3), and anti-A-VI (lane 4) antibodies. Immunoprecipitated proteins were then analysed by SDS-PAGE (10% gels), followed by autoradiography at -70 °C. Molecular masses (in kDa) of annexins are indicated by arrows.

as a doublet at 35 and 33 kDa (lane 1), the latter form being probably a proteolytic fragment of the native form as already described [6,10,26]. The apparent molecular weight of A-II (lane 2), A-V (lane 3) and A-VI (lane 4) were respectively 35, 32 and 70 kDa.
Since A-V and A-VI have already been dectected in T cells, the most important point of this experiment was to identify A-I and A-II in Jurkat cells.

Annexins are substrates *in vitro* for protein tyrosine kinases: Annexins were shown to be phosphorylated by PTKs (a): A-II is a major *in vivo* substrate for pp60^{src}, (b): A-I is a major cellular substrate for the EGF- and insulin- receptors, and is phosphorylated *in vitro* by different src kinases, (c): A-VI contains a similar phosphorylation site for a putative PTK. Therefore, we tested the ability of annexins expressed in Jurkat cells to be phosphorylated by p56^{lck} (a major PTK of the src family present in Jurkat cells), and first analysed if purified annexins could be substrates *in vitro* for p60^{src} (Fig. 2A) and p56^{lck} (Fig. 2B).
Fig. 2A showed that p60^{src} phosphorylated *in vitro* both A-I (lane 3) and A-II (lanes 4-6), but not A-V (lane 7) and A-VI (lane 8). Lane 1 (no substrate) and lane 2 (enolase) were added in the reaction as controls. The autophosphorylation of p60^{src} at 60 kDa could be observed in each lane.
Fig. 2B revealed that among A-I (lane 1), A-II (lane 2), A-V (lane 3) and A- VI (lane 5), only A-VI was phosphorylated by p56^{lck}. Several minor phosphoproteins in the 35 kDa range were observed (lane 5) and probably represented proteolytic fragments of A-VI. A minor 70 kDa-phosphoprotein appeared in lane 3, reflecting a slight contamination of purified A-V by A-VI

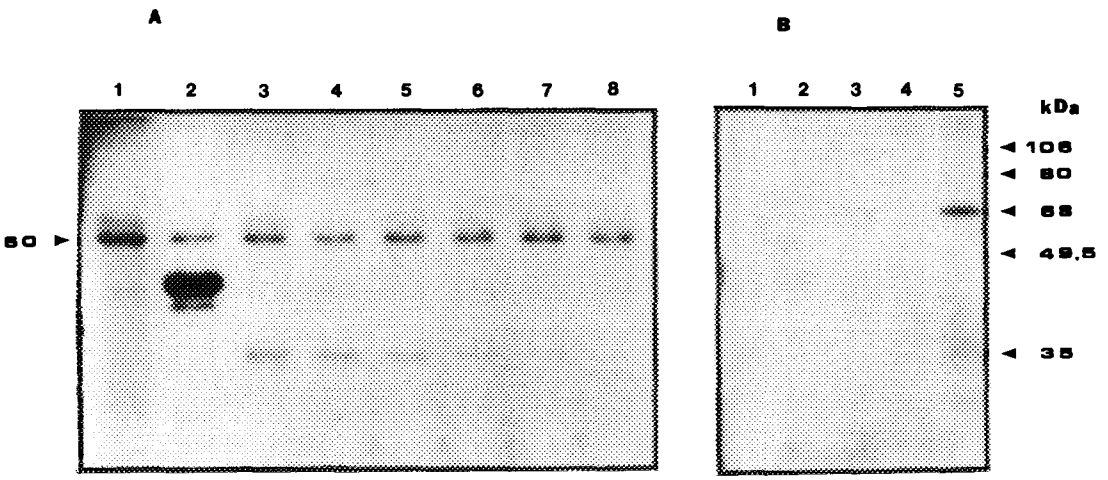

Figure 2. *In vitro* phosphorylation of annexins by protein tyrosine kinases. Purified annexins were incubated with purified $p60^{src}$ (panel A), or immunoprecipitated $p56^{lck}$ linked to protein A Sepharose (panel B), in the presence of [γ^{32}P]ATP as described in *Methods*. In the presence of $p60^{src}$ (panel A). lane 1: no substrate, lane 2: + enolase, lane 3: + 1 µg A-I, lanes 4-6: + 1, 0.5 and 0.1 µg of A-II, respectively, lane 7: + 1 µg A-V, lane 8: + 1 µg A-VI. In the presence of $p56^{lck}$ (panel B). lane 1: + 1 µg A-I, lane 2: + 1 µg A-II, lane 3: + 1 µg A-V, lane 4: no substrate, lane 5: + 1 µg A-VI. Phosphoproteins were analysed by SDS-PAGE (10% gels), followed by autoradiography at -70 °C. Arrows indicate the position of $p60^{src}$ (autophosphorylation) and molecular-mass standards are shown in kDa.

due to their close elution during the purification steps [23]. As a control, no substrate was added in lane 4. On the contrary, we did not observe the autophosphorylation of $p56^{lck}$ (Fig. 2A) because the immunecomplex was discarded after centrifugation and only the supernatant was analysed. The phosphorylation of A-VI (Fig. 2B, lane 5) was not due to a contaminating kinase in the purified annexin preparation, because the same sample failed to be phosphorylated by $p60^{src}$ (Fig. 2A, lane 8). In addition, the phosphorylation of A-VI was alcaline resistant (following a 2 hour treatment with 1 M KOH at 56°C) suggesting the presence of phosphotyrosine residues (data not shown).

In conclusion, these experiments showed that A-I and A-II are substrates *in vitro* for $p60^{src}$, and A-VI for $p56^{lck}$, two members of the src family of non-receptor PTKs. Both kinases did not phosphorylate the same annexins suggesting a substrate/kinase specificity. Since A-I and A-II were already shown to be substrates for PTK of the src family, this was the first demonstration that A-VI could be phosphorylated by a PTK. Therefore, this result suggested that A-VI could play a role as a substrate for $p56^{lck}$ during the intracellular signal transduction following T cell activation.

A 56-kDa phosphoprotein co-immunoprecipitates with A-VI: We then tested the ability of A-VI to be phosphorylated by $p56^{lck}$ in Jurkat cells. Therefore, we performed two sets of immunoprecipitations.

First we developped A-VI immunoprecipitations followed by an *in vitro* kinase assay allowing the autophosphorylation of the kinase as described in methods (Fig. 3A). Immunoprecipitation

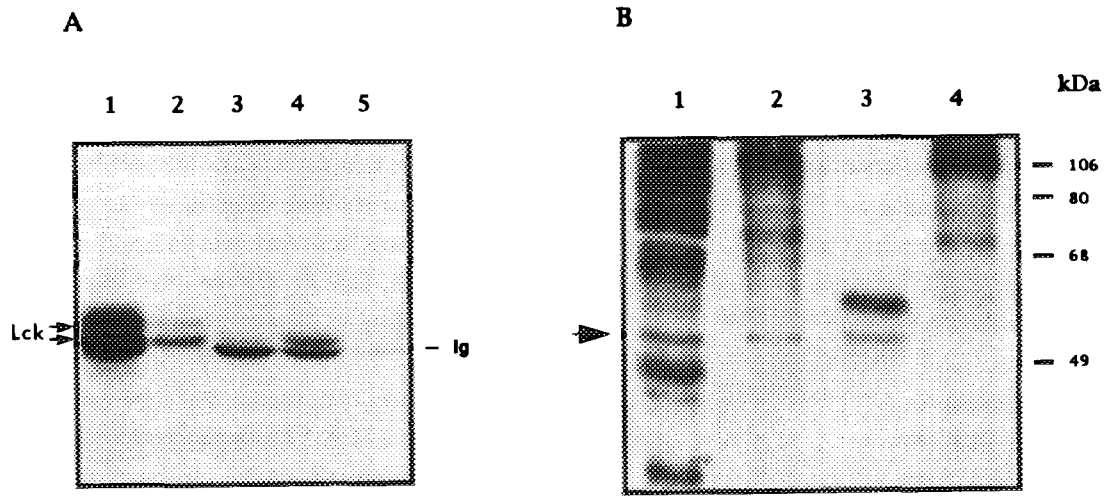

Figure 3. Coimmunoprecipitation of a 56-kDa protein with A-VI in Jurkat cells.
Panel A. $p56^{lck}$ (lanes 1, 2) or AVI (lanes 3,4) was immunoprecipitated from anti-CD3 treated (lanes 2,4,5) or untreated Jurkat cells (lanes 1,3), followed by an *in vitro* kinase assay as described in *Methods*. An immunoprecipitation using a pre-immune serum was performed as control (lane 5). Arrows indicated the positions of $p56^{lck}$ and immunoglobulins (Ig). All lanes were derived from the same SDS-PAGE but lanes 1 and 2 were under exposed in order to visualize the doublet of $p56^{lck}$. **Panel B.** *In vivo* [^{32}P]o-phosphate labelled cells were activated using anti-CD3 antibodies. A-VI (lanes 1, 2) was then immunoprecipitated using 2 different polyclonal antibodies (VIa: lane 1, MC2: lane 2). $p56^{lck}$ was also immuoprecipitated (lane 3) and a non-immune serum was used as a control (lane 4). Immunoprecipites were then analysed by SDS-PAGE. The arrow indicated the position of the 56 kDa phosphoprotein, and molecular-mass standards are shown in kDa on the right.

of $p56^{lck}$ from unstimulated (Fig. 3A, lane 2) or anti-CD3 stimulated cells (Fig. 3A, lane 1) revealed the autophosphorylation of the kinase which migrated as a doublet. The phosphorylation of the upper band increased upon anti-CD3 activation, reflecting increased serine phosphorylation in the amino terminus of $p56^{lck}$ [27]. Immunoprecipitation of A-VI from control (Fig. 3A, lane 3) or anti-CD3 stimulated (Fig. 3A, lane 3) cells did not detect A-VI phosphorylation after kinase assay. However, a phosphoprotein of 56 kDa appeared in the A-VI immunoprecipitates derived from activated cells (Fig. 3A, lane 4) compared to unstimulated cells (Fig. 3A, lane 3). An immunoprecipitation using a non immune serum, followed by a kinase assay did not reveal this protein (Fig. 3A, lane 5). In lanes 3-5, a phosphoprotein correponding to the heavy chain of immunoglobulins was detected (indicated by "Ig" in Fig. 3A), but its phosphorylation was not affected by CD3 stimulation (Fig. 3A, lane 3 versus lane 4). This experiment revealed the presence of a 56 kDa protein kinase activity associated with the A-VI immunoprecipitates from CD3 stimulated Jurkat cells. This phosphoprotein could be a substrate for the associated kinase, or the kinase itself. Since our experiment was performed under conditions allowing kinase autophosphorylation, this 56 kDa phosphoprotein could therefore reflect the autophosphorylated protein kinase. This associated kinase did not phosphorylate A-VI under these experimental conditions and Western blot experiments failed to identify the presence of annexin VI in $p56^{lck}$ immunoprecipitates. One possibility could be that the amount of annexin VI associated was under the limit of detection by this technique.

Second, immunoprecipitation experiments were performed from cells that were metabolically labelled with [^{32}P]o-phosphate, and stimulated by anti-CD3 antibodies (Fig. 3B). By using two different polyclonal antibodies raised against annexin-VI [VIa (Fig. 3B, lane 1) and MC2 (Fig. 3B, lane 2)], no A-VI phosphorylation could be detected. This was not due to a lack of A-VI immunoprecipitated, because Western blot analysis of the same samples revealed the presence of A-VI (results not shown). Interestingly, other phosphoproteins were detected on the gel (Fig. 3B, lanes 1, 2), and one of them (indicated by an arrow) was recovered with both anti-A-VI antibodies but not with the pre-immune serum (Fig. 3B, lane 4). On the other hand, immunoprecipitation of p56^{lck} revealed 2 bands corresponding to the autophosphorylated kinase (figure 3B, lane 3).

In conclusion, A-VI was not phosphorylated in Jurkat cells under our experimental conditions (Fig. 3B). However, a 56 kDa phosphoprotein was associated in A-VI immunoprecipitates derived from activated Jurkat cells (Fig. 3A, B). This protein could represent the autophosphorylated form of a protein kinase (Fig. 3A) which migrated at the same position than the lower form of the autophosphorylated p56^{lck} (Fig. 3A, B).

Phosphotryptic peptide mapping experiment: In order to test if the 56 kDa phosphoprotein from A-VI and p56^{lck} immunoprecipitates (showed by an arrow in Fig. 3B) was the same protein, we compared their phosphotryptic peptide maps (Fig. 4). The results

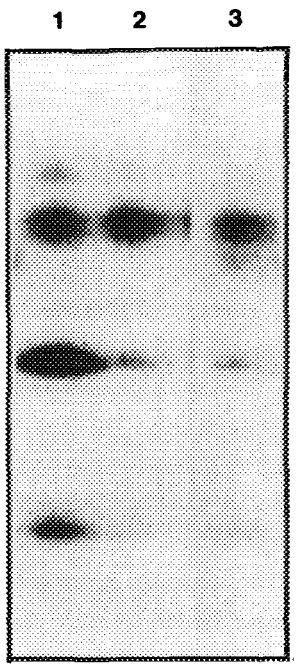

Figure 4. Comparison by tryptic peptide mapping of the 56-kDa phosphoproteins.

The 56-kDa phosphoproteins (indicated by an arrow in Fig. 3B, lanes 1, 2 and 3) were subjected to tryptic digestion as described in *Methods*. Phosphoproteins that have been obtained with anti-p56^{lck} (lane 1) or anti A-VI sera (VIa: lane 2, MC2: lane 3) were analysed on 40% acrylamide gels. Exposure time was 7 days for lane 3.

showed that phosphopeptides obtained from $p56^{lck}$ (lane 1) and A-VI [A-VIa (lane 2) and MC2 (lane 3) antibodies] immunoprecipitations were similar. These results indicated that the 56 kDa phosphoprotein which co-immunoprecipited with A-VI shared a similar phosphotryptic map with the lower migrating form of the autophosphorylated $p56^{lck}$.

Conclusion: Our results show that annexin-VI coprecipitates with a 56 kDa protein kinase having characteristics common with $p56^{lck}$ under conditions where protein-protein interactions remain intact. The finding that annexin-VI is associated with $p56^{lck}$, but is not phosphorylated on tyrosine, indicates that the SH2 (src homology 2) domains of $p56^{lck}$ do not likely participate in this interaction. More, annexin-VI does not contain proline rich domains and therefore does not likely interact with the lck-SH3 (src homology 3) domain.
In addition, a calcium dependent mechanism can also be excluded because the annexin VI/$p56^{lck}$ association is detected even in the presence of EGTA. Our data suggest that annexin-VI could interact not only with $p56^{lck}$ but with other proteins in a putative transducing complex. This association may be important *in vivo* since annexins are known to bind to negatively charged phospholipids of the plasma membrane and to elements of the cytoskeleton. Recently, A-VI has been involved in endocytotic processes [28] and was shown to bind to calspectin (brain spectrin) in a calcium and phospholipid-dependent manner [29]. Annexin VI could function as a link between the membrane located tyrosine kinase $p56^{lck}$ and intracellular signal transducing elements.

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