Structural requirements for annexin I–S100C complex-formation

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S100C is a member of the S100 family of EF-hand-type Ca^{2+}-binding proteins which are thought to bind to and thereby regulate the activity of cellular target proteins in a Ca^{2+}-dependent manner. An intracellular ligand for S100C is the Ca^{2+}/phospholipid-binding protein annexin I and we show here that complex-formation is mediated through unique domains within S100C and annexin I. Using a proteolytically truncated annexin I derivative as well as a number of N-terminal annexin I peptides in liposome co-pelleting and ligand-blotting assays we map the S100C-binding site to the N-terminal 13 residues of annexin I. Similar analyses employing recombinantly expressed S100C mutants reveal that residues D91 to I94 in the unique C-terminal extension of this S100 protein are indispensable for annexin I binding. Interaction between S100C and an N-terminal annexin I peptide containing a tryptophan at position 11 can also be monitored by fluorescence emission spectroscopy after tryptophan excitation. This analysis indicates that the local environment of the tryptophan in annexin I becomes less aqueous on S100C binding, suggesting a hydrophobic nature of the protein–protein interaction. Thus the structural basis of the annexin I–S100C complex-formation probably resembles to a large extent that of the well-characterized annexin II–p11 interaction.

INTRODUCTION

Intracellular Ca^{2+} signalling, i.e. the response to transient elevations of cytoplasmic Ca^{2+} levels, is mediated by proteins capable of binding Ca^{2+}. Such intracellular Ca^{2+}-binding proteins can be tentatively divided into two groups: those containing a Ca^{2+}-binding motif of the helix–loop–helix type, the so-called EF-hand, and those binding Ca^{2+} ions through other types of sequence elements. S100 proteins are a family of small (approx. 10 kDa) dimeric proteins belonging to the former group (for reviews see [1,2]), and annexins, soluble proteins that interact Ca^{2+}-dependently with cellular membranes, are part of the latter group of non-EF-hand-type Ca^{2+}-regulated proteins (for reviews see [3–5]). Although it is generally assumed that these Ca^{2+}-binding proteins exert their function by binding to and thereby modulating the activity or structure of cellular targets in a Ca^{2+}-dependent manner, increasing evidence suggests that at least some S100 proteins and annexins can form pairs thereby regulating one another.

p11 (S100A10) was the first S100 protein shown to bind to a member of the annexin family, the tyrosine kinase substrate annexin II (for a review see [6]). Complex-formation leads to an annexin II_2p11_2 heterotetramer which displays different biochemical properties and shows an altered intracellular location when compared with monomeric annexin II [7–10]. Thus p11 is thought to regulate via direct binding the intracellular function of annexin II which has been implicated in membrane transport steps during endocytosis and/or Ca^{2+}-regulated secretion (for reviews see [3,11]).

Complex-formation between annexin II and p11 is mediated through the N-terminal 14 amino acids of annexin II which form an amphipathic α-helix with the hydrophobic side representing the prime p11 contact [12,13]. The 14 residues are part of the N-terminal domain of annexin II which is unique within the annexin family and precedes a protein core domain containing the annexin-type Ca^{2+}- and phospholipid-binding sites. The core domain is a typical annexin unit built of four so-called annexin repeats showing structural similarity to the repeats found in other annexins (for a review see [4]). The annexin II-binding site on the p11 molecule resides in a cluster of hydrophobic residues present in the so-called C-terminal extension [14]. This region is unique within the S100 proteins and follows the F-helix of the second EF-hand. Typically, all S100 proteins are composed of two EF-hands separated by a short linker and preceded and followed by unique N- and C-terminal extensions respectively [2]. It is generally assumed that Ca^{2+} binding to the EF-hands leads to an increased hydrophobicity of the extensions which is in turn required for target protein binding. p11, however, has lost the ability to bind Ca^{2+} because of deletions and substitutions in the conserved EF-hand loops and is thought to be frozen in a state competent for annexin II binding (for a review see [6]).

The Ca^{2+}-independent p11 binding to annexin II contrasts with the Ca^{2+}-regulated interaction within two other S100–annexin pairs described recently, the calcyclin (S100A6)–annexin XI and the S100C–annexin I complexes [15–17]. While the structural requirements underlying the p11–annexin II interaction have been characterized in detail, less is known about the calcyclin–annexin XI and S100C–annexin I interactions. In both cases the S100-binding sites reside in the unique N-terminal domains of the annexins [17,18]. The annexin-binding site, however, has only been mapped in the case of calcyclin where it appears to be contained within the N-terminal extension [19].

Complex-formation between S100C and annexin I is of particular interest as it could affect the phosphorylation of annexin I by the epidermal growth factor (EGF) receptor kinase at Tyr-20 in the N-terminal domain of the molecule [20]. This phosphorylation occurs in multivesicular bodies, which are endocytic structures involved in sorting the EGF receptors away from recycling plasma-membrane receptors, and phosphorylated annexin I is thought to play a role in this process [21]. To understand the structural basis of the regulation of annexin I by S100C, we characterized the annexin I–S100C interaction in more detail. By employing different methods, including ligand-blotting, liposome co-pelleting and fluorescence spectroscopy, we show that the N-

Abbreviations used: DTT, dithiothreitol; EGF, epidermal growth factor; EGS, ethylene glycol bis(succinimidyl succinate).

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terminal 13 amino acids of annexin I, including a correctly processed N-terminus, harbour the S100C-binding site. In S100C we identify a sequence in the C-terminal extension which is essential for annexin I binding.

MATERIALS AND METHODS

S100C cDNA cloning and construction of mutants

The cDNA of porcine S100C was amplified by PCR using as template a λgt11 cDNA library from porcine lung (Clonetech) and oligonucleotide primers, the sequences of which were based on the cDNA sequence of porcine S100C [22]. The forward primer C1 introduced an *Eco*RI cleavage site upstream of the ATG start codon (5′-CGTACCCTGCTGAATTCCGACATG-GCAAAAAGACCCACAG), and the reverse primer C2 introduced a *Hin*dIII cleavage site downstream of the stop codon (5′-CAGGCCCAAAGGAAGCTTAGATTTACTTCTGGGT-AGACTTAA). After gel purification, the amplified cDNA was digested with *Eco*RI and *Hin*dIII and ligated into the pBluescript SK(+) vector (Stratagene), linearized with the same enzymes. The cDNA was sequenced by the dideoxy method [23] using the T7 sequencing kit (Pharmacia). For bacterial expression the S100C cDNA was cloned in the bacterial expression vector pET23a (Novagen). To obtain optimal expression of S100C without amino acids fused to the authentic N-terminus the T7 tag of the pET23a vector was removed by digestion with *Eco*RI and *Nde*I. The tag-less linear vector containing the S100C cDNA insert was gel-purified, blunted by treatment with mung bean exonuclease, phosphorylated and religated. The distance between the ribosomal binding site and the ATG start codon of the S100C cDNA in the resulting plasmid pET S100C was 7bp, as determined by dideoxy sequencing.

To map the annexin I-binding site in S100C, two constructs truncated in the 3′ part of the protein-coding region were generated by PCR. The T7 standard primer (Stratagene) was used as forward primer. The reverse primers, which introduced a *Hin*dIII cleavage site 3′ of the novel stop codon, were as follows: K95stop (5′-CTTAGATTTAAAGCTTGGTAGATTAAATA-AAGGAGTC) and D91stop (5′-CTTCTGGGTAGACAAG CTTAAGGATTAATGCAAGCAAGCTATG). PCR was carried out with *Pfu* polymerase (Stratagene) using pET S100C as a template. The products were gel-purified, digested with *Xba*I and *Hin*dIII and cloned into the appropriately linearized pET23 vector for bacterial expression. All mutant cDNAs were fully sequenced using the dideoxy method.

Expression and purification of S100C

Expression of wild-type and mutant S100C was achieved by transforming *Escherichia coli* BL21 (DE3)pLysS cells (Novagen) with the following plasmids: pET S100C, pET S100C (K95stop), pET S100C (D91stop). Cells containing the expression plasmids were grown at 37 °C in 200 ml of Luria broth containing 150 μg/ml ampicillin and 34 μg/ml chloramphenicol. Expression was induced by addition of 0.4 mM isopropyl β-D-thiogalactoside at late exponential phase. After 4 h the cells were harvested by centrifugation and resuspended in 20 ml of 20 mM imidazole/HCl, pH 7.5, containing 300 mM NaCl, 1 mM EGTA, 2 mM dithiothreitol (DTT) and 5 μg/ml leupeptin (Sigma). The cells were lysed by repeated (3 ×) freezing and thawing followed by sonication. After clarification of the lysate by centrifugation (100000 ***g***; 30 min; 4 °C), it was adjusted to a final concentration of 5 mM $CaCl_2$ and applied to a phenyl-Sepharose CL4B column (Pharmacia) equilibrated with 25 mM imidazole/HCl, pH 7.5, containing 150 mM NaCl, 5 mM $CaCl_2$ and 1 mM DTT. The column was washed with 25 mM imidazole/HCl, pH 7.5, containing 150 mM NaCl, 1 mM $CaCl_2$ and 0.25 mM PMSF and Ca^{2+}-dependently bound proteins were eluted with 25 mM imidazole/HCl, pH 7.5, containing 150 mM NaCl and 1 mM EGTA. Fractions containing S100C were pooled, adjusted to 1 mM DTT/2 mM NaN_3 and stored at 4 °C. The purified proteins were identified by SDS/PAGE [24] and by N-terminal sequence analysis using an automated gas-phase sequenator (Knauer model 810).

Liposome pelleting and ligand blotting

S100C was co-pelleted with annexin I in a Ca^{2+}-dependent liposome pelleting assay [25]. To prepare liposomes, a chloroform/methanol extract of bovine brain (Sigma) was dissolved in chloroform, dried in a N_2 stream and sonicated in buffer A (50 mM imidazole/HCl, pH 7.4, 150 mM NaCl). Binding studies were carried out in a total volume of 150 μl of buffer A containing 0.25 mM $CaCl_2$, 50 μg of liposomes, 5 μg of annexin I and 5 μg of S100C. After incubation of the mixture at room temperature for 20 min, liposomes were pelleted by centrifugation at 200000 ***g*** for 15 min. Proteins bound to the liposomes were eluted by resuspending the pellet in buffer A containing 10 mM EGTA and incubation for 20 min at room temperature. Liposomes were pelleted again (200000 ***g***; 15 min) and the supernatant containing the released proteins was analysed by SDS/PAGE [24].

The ligand-blotting assay was carried out with annexin I purified from pig lung, porcine annexin I treated with trypsin at an enzyme/substrate ratio of 1:150 for 20 min, and a protein extract from *E. coli* cells expressing porcine annexin I. Proteins were subjected to electrophoresis in SDS/12.5 % polyacrylamide gels [26] and transferred to nitrocellulose [27] using 50 mM Tris/50 mM boric acid as transfer buffer. Free binding sites were blocked with blocking buffer [5 % (w/v) milk powder in TBS (20 mM Tris/HCl, pH 7.4, 150 mM NaCl)] and the membrane was subsequently incubated for 1 h with 5 μg/ml S100C in blocking buffer containing 0.5 mM $CaCl_2$ or 10 mM EGTA. After extensive washing with TBS containing 0.5 mM $CaCl_2$, bound S100C was detected using a polyclonal S100C antibody which was raised in rabbits against the recombinant protein. Peroxidase-coupled anti-rabbit IgGs (Dako) were employed as secondary antibodies and detected using the enhanced chemiluminescence system (Amersham-Buchler).

Synthetic peptides

Peptide synthesis based on Fmoc chemistry was carried out on an automated synthesizer (model 9050; Milligen). Peptides were left on the resin and the Fmoc group was released by treatment with dimethylformamide. Subsequently, the free N-terminus was acetylated with acetic anhydride/*N*-ethyldi-isopropylamine for 30 min. Removal of the protection groups was then achieved by treatment with 95 % trifluoroacetic acid/2.5 % ethanedithiol/2.5 % anisole. Peptides were purified by reverse-phase HPLC on a preparative column (Vydac 218TP 1022). After being washed with 30 ml of solvent A (0.1 % trifluoroacetic acid in water), peptides were eluted with a 270 ml linear gradient of 10–60 % solvent B (90 % acetonitrile in 0.1 % trifluoroacetic acid) at a flow rate of 9 ml/min. Absorbance was monitored at 214 nm and peaks were collected manually. Peptides were characterized by MS (MALDI compact III; Kratos Analytical) and by N-terminal sequence analysis of a non-acetylated aliquot (see above). The purified peptides were lyophilized and stored at −20 °C. Filter-coupled peptides corresponding to different N-terminal annexin I sequences were synthesized by the spot method [28] using a

multiple-peptide synthesizer (AMS 422; Abimed). S100C binding to the immobilized peptides was analysed by subjecting the peptide-containing filters to the ligand-blotting assay.

Miscellaneous techniques

Annexin I was purified from pig lung as described by Glenney et al. [29]. Recombinant porcine annexin I was isolated from transformed *E. coli* BL21 (DE3)pLys cells carrying the porcine annexin I cDNA cloned into the bacterial expression vector pKK223-3 (Pharmacia). The cDNA was obtained by immunoscreening of a porcine lung cDNA library in λgt11 (Clonetech) with annexin I antibodies. Bacterially expressed annexin I was recovered from the soluble protein fraction and purified by Ca^{2+}-dependent liposome binding as described previously [29a].

Chemical cross-linking was carried out with ethylene glycol bis(succinimidyl succinate) (EGS; Sigma). S100C was dialysed against 25 mM imidazole/HCl, pH 7.5, containing 150 mM NaCl, 1 mM EGTA and 1 mM DTT and incubated at a concentration of 0.4 mg/ml with 1 mg/ml EGS for 30 min at room temperature. The reaction was stopped by chlorofom/methanol precipitation [30] and the products were analysed by SDS/PAGE [24].

Analytical gel filtration was performed using a Superose S12 column connected to the Smart system (Pharmacia). The running buffer contained 25 mM imidazole/HCl, pH 7.5, 150 mM NaCl and 1 mM EGTA. Fractions were collected and analysed by SDS/PAGE [24].

For fluorescence analysis, wild-type (wt) S100C and the D91 stop mutant were dialysed against 50 mM Tris/HCl (pH 7.95)/100 m KCl, and the lyophilized annexin I peptide comprising amino acids 1–18 (with the single tryptophan at position 11) was dissolved in the same buffer. Then 10 μM wt– or D91stop–S100C was mixed with an equimolar amount of the annexin I peptide, and fluorescence emission spectra were recorded on a Fluoromax spectrofluorimeter (Spex) with the excitation wavelength set at 295 nm. Subsequently $CaCl_2$ was added to a final concentration of 0.75 mM and the spectra were recorded again.

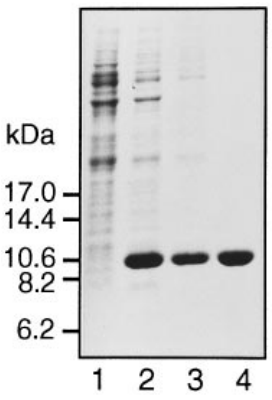

Figure 1 Purification of recombinant S100C

S100C was purified from *E. coli* cells transformed with the pETS100C expression plasmid and fractions of the purification protocol were analysed by SDS/PAGE. Lane 2 shows a total protein extract from bacteria expressing S100C whereas the equivalent extract from the parental non-transformed bacteria is shown in lane 1. High-speed centrifugation of the extract shown in lane 2 yielded a soluble protein fraction containing S100C (lane 3). This fraction was applied to a phenyl-Sepharose column in the presence of Ca^{2+}, and bound S100C was eluted by the addition of EGTA (lane 4). Molecular masses are indicated on the left. Note the strong expression of the recombinant S100C (approx. 10 kDa) which can be purified using a single chromatographic step.

RESULTS

Specific binding of S100C to the N-terminal domain of annexin I

To employ biochemical and spectroscopic assays for analysing the interaction between S100C and annexin I in more detail, we first developed a bacterial expression system for S100C. The cDNA encoding porcine S100C was isolated via PCR amplification and cloned into a modified pET expression vector in which the S100C start codon was moved close to the ribosomal binding site. This resulted in high-level S100C expression in appropriately transformed bacteria, with S100C representing the predominant protein in the total bacterial lysate (Figure 1). S100C was purified from the soluble protein fraction by Ca^{2+}-

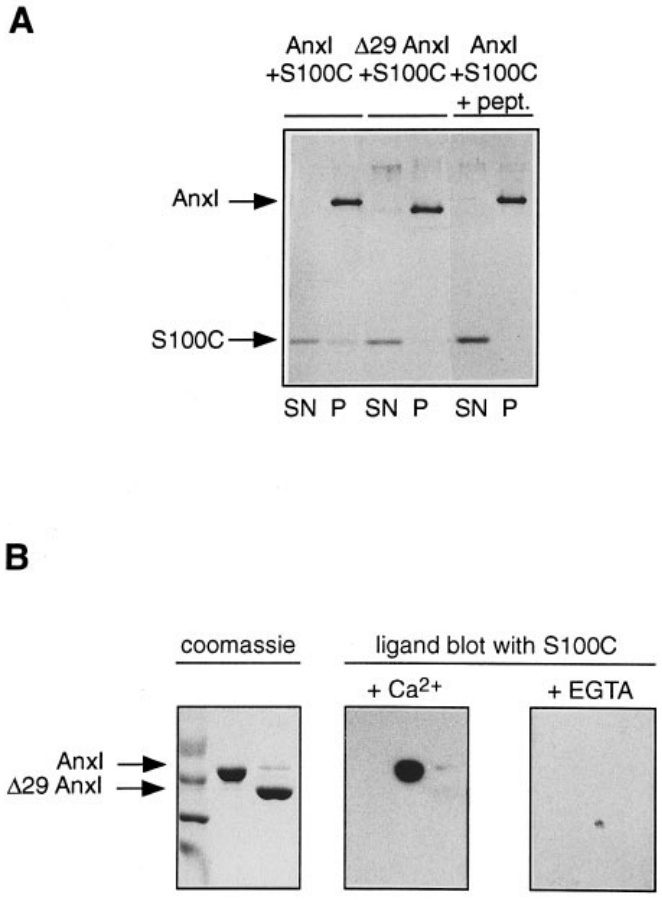

Figure 2 Annexin I–S100C interaction revealed by liposome co-pelleting and ligand blotting

(**A**) shows a liposome co-pelleting assay. S100C was incubated in the presence of Ca^{2+} with liposomes and annexin I (AnxI) or an N-terminally truncated annexin I generated by limited proteolysis (Δ29AnxI). After pelleting of the liposomes by high-speed centrifugation, the supernatant (SN) containing unbound proteins was collected. Ca^{2+}-dependently bound proteins (shown in P) were released by incubating the pellet in an EGTA-containing buffer. The fractions were analysed by SDS/PAGE. Note that S100C was co-pelleted together with annexin I, while Δ29 annexin I fails to induce the pelleting of S100C. When an N-terminal peptide of annexin I (Ac1–18) is present in this assay, co-pelleting is also inhibited (AnxI + S100C + pept.). Ligand blots are shown in (**B**). Annexin I and Δ29 annexin I were subjected to SDS/PAGE and the proteins were either stained with Coomassie Blue or transferred to nitrocellulose membranes. The membranes were incubated with a solution containing S100C in the presence of Ca^{2+} or EGTA. Bound S100C was detected with a specific antibody.

Table 1 Binding of S100C to immobilized annexin I peptides

Filter-bound peptides comprising different sequences of the N-terminal domain of annexin I were subjected to ligand blotting using porcine S100C. Ligand binding to the peptide spots was visualized using an antibody directed against S100C and positive signals were scored '+'. The sequences of the different peptides are given in the one-letter code.

Peptide no.	Position in annexin I	Sequence	Binding of S100C
1	1–25	AMVSEFKLQAWFIDNEEQEYIKTVK	+
2	1–18	AMVSEFKLQAWFIDNEEQ	+
3	1–13	AMVSEFKLQAWFI	+
4	13–25	IDNEEQEYIKTVK	−

dependent hydrophobicity chromatography on phenyl-Sepharose (Figure 1). This single step resulted in a more than 95% pure protein as judged by SDS/PAGE and N-terminal sequence analysis.

Annexin I binding of the recombinant S100C was first analysed by a liposome co-pelleting assay. Here, we exploited the ability of annexin I to bind Ca^{2+}-dependently to phospholipid liposomes, which can be pelleted by centrifugation. Thus, this assay allows the identification of interacting proteins which are co-pelleted with liposomes in an annexin I-dependent manner. Whereas S100C alone is unable to bind to the liposomes (not shown), the presence of annexin I purified from porcine lung results in co-pelleting of a fraction of the S100C (Figure 2A). This binding critically depends on the presence of the N-terminal domain of annexin I since an annexin I core generated by limited tryptic cleavage fails to induce the co-pelleting (Figure 2A). The tryptic annexin I core, which continues to bind efficiently to the liposomes, was shown by N-terminal sequence analysis to start at Gly-30. Moreover, a synthetic peptide comprising the N-terminal 18 residues of porcine annexin I with an acetylated N-terminal alanine (Ac1–18) inhibits S100C co-pelleting when included in the assay (Figure 2A). The peptide does not interfere with liposome binding of annexin I, indicating that the N-terminal 18 residues are probably involved in the S100C but not the liposome interaction. Liposome co-pelleting also reveals that the annexin I binding is specific for S100C, as other S100 proteins (S100α, S100L, S100P, p11) fail to be recovered from the annexin I–liposome pellet (not shown).

Liposome co-pelleting does not distinguish between Ca^{2+}-dependent and Ca^{2+}-independent S100C–annexin I interaction since Ca^{2+} is a prerequisite to the establishment of the annexin I–phospholipid binding. To analyse the Ca^{2+}-dependence of S100C binding to annexin I, we chose the ligand-blotting assay which had previously been employed to visualize S100–annexin interactions [15,16,31]. Porcine annexin I or the tryptic protein core starting at residue 30 (Δ29AnxI) was immobilized on nitrocellulose membranes after SDS/PAGE and then treated with a solution containing the recombinant S100C in the presence or absence of Ca^{2+}. Subsequently, bound S100C was detected with a specific antibody raised against bacterially expressed S100C protein. Figure 2(B) clearly reveals the Ca^{2+}-dependent binding of S100C which is completely lost upon removal of the N-terminal 29 residues of annexin I.

The combined results show that the N-terminal 29 residues of annexin I are essential for S100C binding but do not allow the conclusion that they contain the entire binding site. To address this point and to map the S100C-binding site more precisely, we performed direct peptide-binding experiments. Peptides corresponding to different regions within the N-terminal 25 residues of annexin I were generated in a filter-bound form with an acetylated N-terminal residue by following a spot-synthesis protocol [28]. The filter with the immobilized peptides was then subjected to ligand blotting with recombinant porcine S100C in the presence of Ca^{2+}. The results show that synthetic peptides are capable of binding S100C and that the S100C-binding site is found within the N-terminal 13 residues of annexin I (Table 1).

Location of the annexin I-binding site in the unique C-terminal extension of S100C

To map the annexin I-binding site in S100C we generated C-terminally fore-shortened S100C derivatives through bacterial expression of the respective mutant cDNAs (Figure 3). The proteins were purified according to the protocol developed for wild-type S100C, i.e. by exploiting their Ca^{2+}-dependent binding to phenyl-Sepharose, and then tested for annexin I binding. We concentrated on the C-terminal domain as this region is unique within a given member of the S100 family and thus likely to be involved in member-specific and not general S100 functions. Moreover, the annexin II-binding site in another S100 protein, p11, was mapped to its C-terminal extension [14]. The two truncation mutants, K95stop and D91stop, were first compared

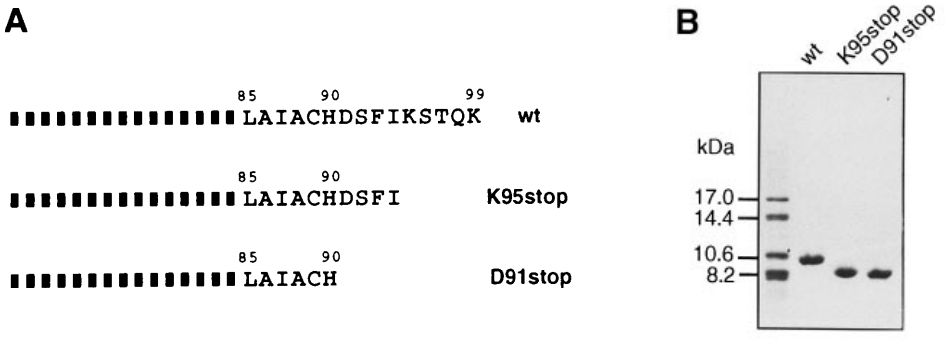

Figure 3 S100C mutant proteins

Sequences of the C-terminal extension of wild-type S100C (wt) and the two C-terminally truncated derivatives (K95stop, D91stop) are given in (**A**). The mutant proteins were expressed in *E. coli* and purified as shown for wt–S100C in Figure 1. An SDS/polyacrylamide gel of the purified proteins is shown in (**B**). Markers of the molecular masses indicated were included in the left lane.

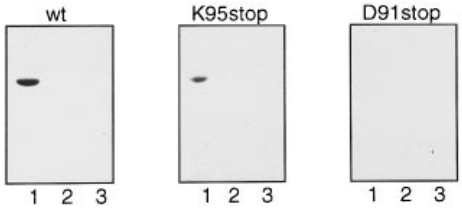

Figure 4 Ligand-blotting assay using mutant S100C derivatives

Porcine annexin I (lane 1), the proteolytic Δ29 annexin I derivative (lane 2) and an *E. coli* lysate containing recombinantly expressed porcine annexin I (lane 3) were subjected to SDS/PAGE and then transferred to nitrocellulose membranes. The membranes were incubated in the presence of Ca^{2+} with a solution containing wt–S100C (wt), K95stop–S100C (K95stop) or D91stop–S100C (D91stop). Bound S100C proteins were detected with a specific antibody. Note the binding of wt– and K95stop–S100C to annexin I which is not observed for the D91stop mutant. The binding depends on a properly processed N-terminal domain of annexin I since it is not seen with Δ29 annexin I and the bacterially expressed protein.

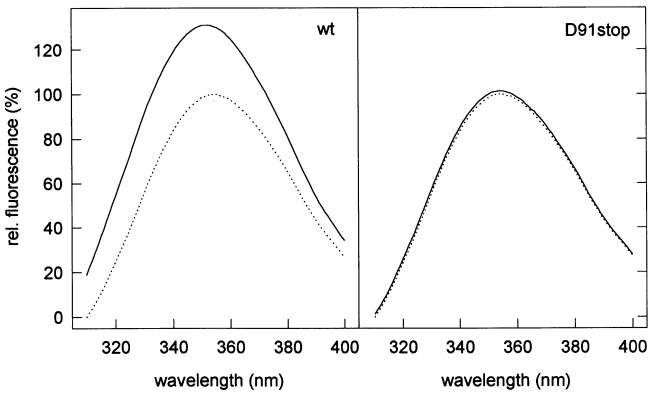

Figure 5 Fluorescence emission spectroscopy of an N-terminal annexin I peptide (Ac1–18) in the presence of wt– or D91stop–S100C

Equimolar amounts of the Ac1–18 peptide (which corresponds to the N-terminal annexin I sequence and contains a single tryptophan at position 11) and wt– or D91 stop–S100C were mixed in the absence of Ca^{2+}, and fluorescence emission was recorded after excitation at 295 nm (· · · ·). Subsequently $CaCl_2$ was added to a final concentration of 0.75 mM and the spectra were recorded again (——). Note the Ca^{2+}-dependent increase in Ac1–18 fluorescence observed in the presence of wt– but not D91stop–S100C.

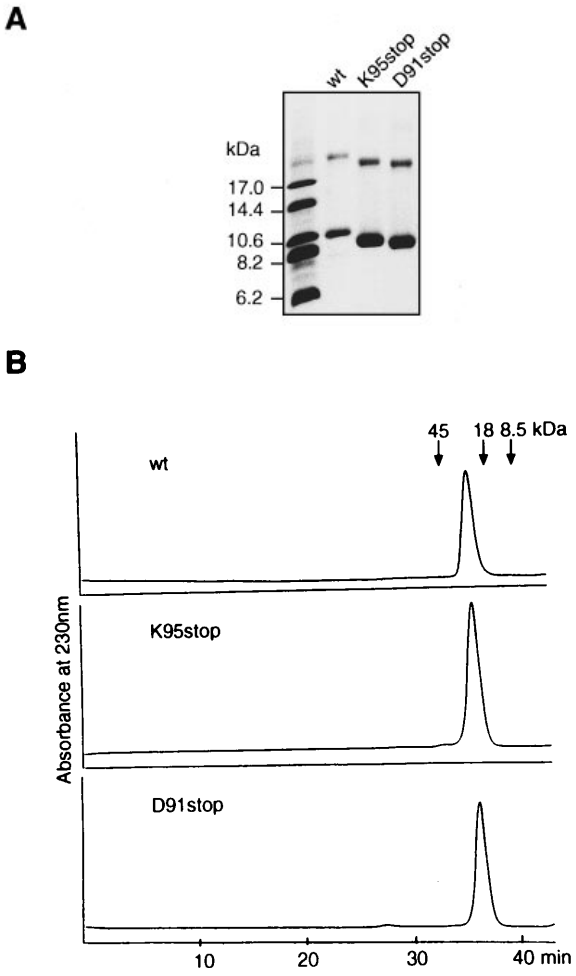

Figure 6 S100C dimerization as revealed by chemical cross-linking and analytical gel filtration

Chemical cross-linking of wild-type S100C (wt) and the C-terminally truncated mutant derivatives (K95stop, D91stop) is shown in (**A**). The purified proteins were incubated at a concentration of 0.4 mg/ml with 1 mg/ml EGS for 30 min and products of the reactions were analysed by SDS/PAGE. A molecular-mass standard was included in lane 1. Analytical gel filtration of wt–, K95stop– and D91stop–S100C is shown in (**B**). The analysis of the purified proteins was carried out on a Superose S12 column. The respective protein peaks are given as a function of elution time. Migration positions of the marker proteins egg albumin (45 kDa), myoglobin (18 kDa) and ubiquitin (8.5 kDa) are indicated. Note that all S100C derivatives show an almost identical elution profile with the protein peak migrating at a position corresponding to a globular protein of around 20 kDa.

with wild-type (wt)–S100C in the ligand-blotting assay using as immobilized binding targets porcine annexin I, the tryptic core of porcine annexin I (Δ29AnxI) and a bacterial lysate containing recombinantly expressed porcine annexin I. Figure 4 shows that K95stop–S100C remains capable of interacting with annexin I. This interaction is observed only with intact annexin I from porcine lung and not with the bacterially expressed protein, thus further strengthening the importance of a correctly processed, i.e. acetylated, N-terminus. The binding to porcine annexin I is, however, completely lost in the D91stop mutant. This result shows that amino acid residues 91–94 (DSFI) of S100C are necessary for the binding of S100C to immobilized annexin I.

Binding of S100C to the N-terminal domain of annexin I was also analysed in solution by fluorescence spectroscopy. The N-terminal annexin I peptide Ac1–18, which contains a single tryptophan at position 11, was mixed with S100C, and tryptophan fluorescence was induced by excitation at 295 nm. Since S100C lacks tryptophan the emission spectrum recorded solely reflects the signal from the Ac1–18 tryptophan. Figure 5 (left panel) shows this fluorescence spectrum recorded in the absence or presence of 0.75 mM Ca^{2+}. In the absence of Ca^{2+} the fluorescence maximum resides at 356 nm and the spectrum is indistinguishable from that of Ac1–18 alone (not shown). Addition of Ca^{2+} induces a shift in the emission maximum to 352 nm and a significant increase in fluorescence intensity (Figure 5, left panel). This effect can be specifically attributed to the presence of S100C, as the spectrum of Ac1–18 alone is not affected by Ca^{2+} (not shown). Thus the Ca^{2+}-induced change in the emission spectrum reflects the Ca^{2+}-dependent binding of S100C to the annexin I peptide and indicates that the local environment of the peptide tryptophan becomes less aqueous upon S100C binding. In line with the ligand-blotting data (Figure 4), the D91stop mutant of S100C fails to elicit the Ca^{2+}-induced change in Ac1–18 fluorescence emission (Figure 5, right panel). Taken together these data show that the binding site for the N-

terminal domain of annexin I resides in the C-terminal extension of S100C and specifically involves one or more residues within the sequence spanning positions 91–94.

The S100C sequence essential for annexin I binding (D91–I94) contains a hydrophobic amino acid, Phe-93, found in a position which in a linear sequence comparison is occupied by a hydrophobic residue in other S100 proteins as well. In a recent structural analysis of calcyclin (S100A6) the equivalent hydrophobic residue was implicated in dimer formation and it was suggested that perturbation of the monomer–dimer equilibrium of an S100 protein could affect target protein binding [32]. To test the possibility that the sequence spanning residues 91–94 of S100C, in particular Phe-93, is involved in dimer formation and that interference with dimerization in the D91stop mutant leads to the loss of annexin I binding, we performed a series of chemical cross-linking and analytical gel-filtration experiments with the different S100C deletion mutants. Figure 6(A) shows that covalent stabilization of the S100C dimer by the cross-linker EGS is not affected in the K95stop and the D91stop mutants. This result is corroborated by analytical gel filtration of the purified truncation mutants. Both K95stop– and D91stop–S100C show an elution profile basically indistinguishable from that of wt–S100C, with the protein peak migrating at a position corresponding to a globular protein of approximately 20 kDa (Figure 6B). Thus dimer formation is not affected in the D91stop mutant which has lost the ability to interact with annexin I.

DISCUSSION

The annexin I–S100C interaction represents the third example of an annexin–S100 pair. In all cases the specific complex-formation is mediated through the N-terminal domain of the annexin, thus supporting the hypothesis that the unique N-terminal domain carries the functional specificity for individual annexins ([12,17,18]; this study). The annexin-binding sites in the S100 proteins also reside in unique regions of the molecules, i.e. the N- and C-terminal extensions, which show the lowest level of sequence conservation among the S100 family members. However, whereas the annexin XI-binding site in calcyclin appears to be restricted to the N-terminal extension, particularly residues 4–7 [19], annexin II binding to p11 critically depends on hydrophobic residues in the C-terminal extension [14]. We show here that, in the case of annexin I–S100C, the interaction is also mediated through the C-terminal domain of the S100 molecule.

The S100-binding site on both annexin I and II is restricted to the N-terminal 13 or 14 residues respectively. In annexin II this sequence has been shown to form an amphipathic α-helix with the hydrophobic amino acids Val-3, Ile-6, Leu-7 and Leu-10, which lie on one face of the helix, representing the major contact sites for p11 [12,13]. In annexin I, the N-terminal sequence is also predicted to have a high probability of forming such a helix, with Met-2, Val-3, Phe-6 and Leu-7 potentially providing contacts with S100C [17]. Interestingly, acetylation of the N-terminal residue of both annexins is also required for S100 binding. Whereas this has been shown directly for annexin II using a series of peptides modified differently at their N-termini [13], our comparison of S100C binding to authentic and bacterially expressed annexin I (Figure 4) clearly emphasizes the importance of a processed N-terminus in this case as well. Moreover, the acetylated annexin I peptide, Ac1–18, is capable of competing with the annexin I–S100C interaction (Figure 2A) whereas the non-acetylated form of the peptide fails to show this effect (not shown). Nevertheless, it remains to be seen whether other forms of N-terminal modifications of annexin I are also tolerated in complex-formation. While this study was under way, Mailliard et al. [17] showed that a fusion protein of glutathione S-transferase linked to the N-terminal domain of annexin I (residues 1–46) is able to bind S100C. In this construct the glutathione S-transferase fusion part might substitute for the *N*-acetyl group, and might induce within the N-terminal annexin I residues a conformation required for S100C binding.

With the hydrophobic face of an amphipathic α-helix probably representing an important S100-binding site in both annexin I and II, it is tempting to speculate that hydrophobic side chains in S100C and p11 respectively provide contact sites for the hydrophobic surfaces of the annexin helices. Such residues have been identified in p11 as Tyr-85 and Phe-86 [14]. Interestingly, two hydrophobic amino acids are also present among the four residues of S100C identified here as being indispensable for annexin I binding (Glu-91 to Ile-94; Figure 3). One of these residues, Phe-93, is in fact found in a position which in a linear sequence alignment is equivalent to Phe-86 of p11 (see [32] for a sequence alignment). In a recent NMR study of the calcyclin (S100A6) dimer the corresponding residue, Leu-88, has been implicated in the homodimer formation. It has been speculated that perturbations of the monomer–dimer equilibrium would result from mutations reducing significantly the hydrophobic nature of this side chain [32]. As a consequence of such perturbations, a reduced affinity for target proteins (annexin II in the case of p11) might be expected [32]. However, our direct analysis of the D91stop mutant of S100C, in which the corresponding residue, Phe-83, has been removed, reveals that dimer formation is not affected by this truncation (Figure 6). Thus, at least in S100C, the conserved hydrophobic residue, Phe-93, is not essential for dimerization but appears to be involved in annexin I binding.

A major difference between annexin II–p11 and annexin I–S100C complex-formation lies in the Ca^{2+}-dependence of the interaction. Whereas p11 binding to annexin II occurs in the absence of Ca^{2+} [33], the presence of Ca^{2+} is a prerequisite for annexin I–S100C interaction. Although both annexin I and S100C are Ca^{2+}-binding proteins, the Ca^{2+} regulation of complex-formation only occurs through S100C. This is clearly seen in the direct binding studies carried out with the N-terminal annexin I peptides. Since the peptides are not capable of binding Ca^{2+}, such regulation has to be provided through Ca^{2+} binding to S100C.

The physiological consequences of the annexin I–S100C interaction are not yet known. By analogy with annexin II–p11, it seems likely that annexin I binding by a S100C dimer leads to the formation of a heterotetrameric complex, i.e. that two annexin I-binding sites are present per S100C dimer. It remains to be seen whether this annexin I–S100C complex has altered biochemical properties and/or different intracellular distribution compared with the individual subunits. Since annexin I appears to be found in different intracellular locations, i.e. as a soluble protein in the cytoplasm, at or close to the plasma membrane, and on multivesicular endosomes [9,21,34], it is tempting to speculate that S100C binding influences one or more of the subcellular locations. This is in fact observed in the structurally related annexin II–p11 pair where formation of the annexin II_2p11_2 heterotetramer is required for anchoring of annexin II in the cortical cytoskeleton [9,10].

The S100C-binding site in the N-terminal domain of annexin I is in close spatial proximity to Tyr-20, which is phosphorylated by the EGF receptor/kinase, and Thr-23, Ser-26 and Ser-27, which are sites of phosphorylation by protein kinase C [20,35]. *In vitro*, S100C has an inhibitory effect on the phosphorylation of annexin I by protein kinase C [16]. The protein kinase C site(s) affected have, however, not been mapped. Future studies need to

reveal whether there is a mutual influence of S100C binding and phosphorylation on the biochemical and functional properties of annexin I.

We thank Lars von Olleschik and M. Alexander Schmidt for peptide spot synthesis and Anja Rosengarth for help with fluorescence spectroscopy. J.S. is supported by a predoctoral fellowship from the Max Planck Society.

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Received 2 May 1996; accepted 30 May 1996