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Alkylation of Cysteine 82 of p11 Abolishes the Complex Formation with the Tyrosine-Protein Kinase Substrate p36 (Annexin 2, Calpactin 1, Lipocortin 2)*

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p36 (annexin 2) is the major cytoplasmic target of the src tyrosine-kinase and forms *in vitro* and *in vivo* a stable tetrameric complex in which two p36 polypeptides interact with a dimer of a unique p11 polypeptide. p11 belongs into the superfamily of EF-hand proteins. Upon mild cysteine modification conditions, both cysteines (positions 61 and 82) of the free p11 become substituted, and the ability to form the p36·p11 complex is lost. Under the same conditions, the 2 cysteines of p11 incorporated into the complex display differential reactivity. Here, cysteine 61 is fully substituted while cysteine-82 is protected. p11 derivatives substituted only on cysteine 61 retain binding activity for p36 unless cysteine 82 is substituted by a second cycle of modification of the isolated p11. Thus, the C-terminal extension protruding from the second EF-hand of the p11 molecule (residues 77–96) is important for the interaction with p36. As a consequence of our analysis, we report a new separation of p36 and p11 from the p36·p11 complex. This is based on a reversible cysteine modification and thus is an alternative to the denaturation and renaturation cycle used previously.

p36 (annexin 2), a major cytoplasmic substrate of the src tyrosine-specific protein kinase, is a member of the annexins, a newly defined family of Ca^{2+}/lipid-binding proteins (see Klee, 1988, for review and references). Depending on their molecular weight, annexins contain either 4 or 8 homologous segments arranged in tandem. These segments, which are around 75 residues in length, together form the large core domain, which is protease-resistant and displays both Ca^{2+} and lipid binding activities. A short N-terminal tail domain, which is highly protease-sensitive, contains all currently known phosphorylation sites (De *et al.*, 1986; Glenney and Tack, 1985, Gould *et al.*, 1986; Johnsson *et al.*, 1986a; Schlaepfer and Haigler, 1988; Weber *et al.*, 1987). It differs strongly in length and sequence among the different annexins. Within the annexin family, p36 displays a unique property. It forms a tight complex with a dimer of a unique p11 molecule (Erikson *et al.*, 1984; Gerke and Weber, 1984). p11 is a member of the EF-hand type Ca^{2+}-binding proteins and shows particularly high sequence similarity to the S100 proteins (Gerke and Weber, 1985b; Glenney and Tack, 1985). In the heterotetrameric complex, p11 modulates some properties exerted by p36: *e.g.* it lowers the Ca^{2+} requirement for phospholipid binding of the complexed p36 in comparison to the monomeric form (Drust and Creutz, 1988; Powell and Glenney, 1987). Due to a deletion in the first loop and some amino acid substitutions in the second loop, the two EF-hands in p11 are functionally inactive (Gerke and Weber, 1985b; Glenney, 1986). Thus p11, unlike other EF-hand proteins, is not regulated by Ca^{2+}, and its interaction with its p36 target reflects a permanent "on" situation.

Studies with proteolytic fragments map the p11 binding site to the N-terminal tail domain of p36. These data are confirmed and extended by the analysis of natural and synthetic peptides of the p36 tail (Glenney *et al.*, 1986; Johnsson *et al.*, 1986b, 1988). Current evidence shows that residues 1 to 14 of p36, probably acting as an amphiphatic helix, form the p11 binding site, which includes the N-terminal acetyl group (Johnsson *et al.*, 1988).

Here we locate part of the p36 binding site on the C-terminal sequence of the p11 molecule. We show that under mild alkylating conditions both cysteines of the free p11 are substituted, while in the p36·p11 complex cysteine 82 of p11 is protected by p36 unless more drastic conditions are used. Alkylation of cysteine 82 in p11 is paralleled by the loss of p36 binding activity. Dissociation of the p36·p11 complex by reversible cysteine modification can be used to separate p11 from p36.

EXPERIMENTAL PROCEDURES

Materials

Fresh pig intestines were obtained from a local abattoir and processed within 1 h of slaughter. Superose S-12 column and Sephadex S-200 were from Pharmacia LKB GmbH (Freiburg, FRG). EGTA,[1] urea, and DTT were from Serva (Heidelberg, FRG). VP was from Janssen Chemicals (Beerse, Belgium). DTP, iodoacetamide, dimethylsuberimidate, amino acid standards, and protein standards for SDS polyacrylamide gel electrophoresis were from Sigma Chemie GmbH (Deisenhofen, FRG). CNBr and phenylisothiocyanate were from Pierce (Rockford, IL). Reagents for protein sequencing were from Applied Biosystems (Foster City, CA). The Shandon C18-Hypersil HPLC column was from Knauer (Berlin). Phosphatidylserine was kindly provided by Dr. H.-J. Eibl.

Methods

Protein Purification—The p36·p11 complex was purified from pig intestines following the methods of Gerke and Weber (1985a), with the minor modifications described in Johnsson *et al.* (1988). For the separation of the subunits, between 5 and 12 mg of lyophilized protein was dissolved in 9 M urea, 100 mM NaCl, 2 mM NaN_3, 1 mM EGTA, 5 mM DTT, 20 mM Tris-HCl, pH 7.5 (buffer A) and incubated for 1 h at 35 °C under nitrogen. Separation was achieved by gel filtration

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[1] The abbreviations used are: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; VP, 4-vinylpyridine; DTP, 2,2′-dithiopyridine; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography.

on a Sephadex S-200 column equilibrated in buffer A. Renaturation of the subunits was achieved by extensive dialysis against 20 mM imidazole-HCl, 100 mM NaCl, 2 mM NaN_3, 1 mM EGTA, 0.5 mM DTT, pH 7.5 (buffer B) at 6 °C.

Cysteine Alkylation—For alkylation with VP, the protein was transferred to buffer B by PD10 gel filtration (Pharmacia LKB Biotechnology Inc.) and subsequently concentrated by the Centricon 10 microconcentrator (Amicon, Danvers, MA) to 2 mg/ml of the p36·p11 complex and to 0.5 mg/ml of the p11 subunit.

In Experiments 1, 2, and 3, VP in a small volume of buffer B was added to the p36·p11 complex and incubated under nitrogen. In Experiment 1, 1.4-fold excess of reagent over the total SH, 2 h, room temperature. In Experiment 2, 7-fold excess of VP, 8 h, 35 °C. In Experiment 3, 1.4-fold excess of VP, 2 h, room temperature. The reaction was stopped by the addition of DTT to 50 mM, and a small aliquot (12 μl) was analyzed by gel filtration on Superose S-12, equilibrated in buffer B. The remaining protein solution was dialyzed against 0.1 M NH_4HCO_3, lyophilized, and dissolved in 1 ml of 9 M urea (buffer A). The subunits were separated and renatured as described above. After dialysis against 0.1 M NH_4HCO_3, a small portion of the two subunits was tested for their ability to bind to the unmodified binding partner; the rest was used for amino acid analysis and CNBr cleavage (200-fold excess of reagent over the methionines in a small volume of 70% formic acid; 22 h at 23 °C). About 1 nmol of the peptide mixture was directly applied on a gas phase sequenator. In Experiment 3, the separated p11 was again treated with VP in buffer B (1.4-fold excess of reagent over total SH, 2 h, room temperature). The reaction was stopped with DTT and p11 was analyzed as above. Alkylation with iodoacetamide was done in 50 mM Tris-HCl, 80 mM NaCl, 1 mM EGTA, 2 mM NaN_3, 1 mM DTT, pH 8.2, using a 2-fold excess of reagent over the total SH. After 30 min at 35 °C, the reaction was stopped with DTT. Modification of p11 (0.5 mg/ml) or dissociation of the p36·p11 complex with DTP was performed in 20 mM Tris, 0.5 mM EGTA, pH 7.8 (buffer C), on ice. The reagent was added in a small volume of dimethyl sulfoxide (less than 5%) to a final concentration of 0.2 mM and 0.5 mM, respectively.

Sequence Analysis—Sequence analysis was performed on a gas phase Sequenator (Applied Biosystems, Model 470 A) using the standard 03RPTH program supplied by the manufacturer. Phenylthiohydantoin-*S*-ethylpyridine-cysteine eluted between phenylthiohydantoin-valine and DPTU from the on-line connected HPLC column. The integration of the peaks was performed automatically by a Merck/Hitachi D-2000 chromatointegrator.

Miscellaneous Techniques—Binding assays were performed in buffer B, and the samples were adjusted to 5 mM DTT. The degree of complex formation after an incubation time of 30 min at room temperature was monitored by gel filtration on Superose S-12, equilibrated in buffer B without DTT. CD measurements were made at 20 °C on a Jobin Mark V, interfaced to a microcomputer. Cuvettes of 1 mm path length were used. Spectra were taken in steps of 0.2 nm. Protein samples were in 20 mM sodium phosphate buffer, pH 7.2. Samples for amino acid analysis were hydrolyzed in propionic acid/HCl at 150 °C for 150 min. The analysis was performed on an HPLC column after pre-column derivatization of the amino acids with phenylisothiocyanate (Bidlingmeyer *et al.*, 1984). *S*-Ethylpyridine-cysteine was eluted with acetonitrile between the derivatives of methionine and isoleucine. The phosphatidylserine liposome matrix was made according to Uchida and Filburn (1984). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

RESULTS

Cysteine Alkylation of p11 Inactivates p36 Binding Activity—When p11 in buffer B was treated with a 1.4-fold molar excess of 4-vinyl pyridine over the total SH concentration, it gradually lost its ability to form the heterotetrameric p36·p11 complex when mixed with stoichiometric amounts of p36. The Superose S-12 column profiles of Fig. 1 show that a treatment for 2 h at 20 °C fully destroyed the binding activity of p11. Similar results were obtained when p11 was treated with other cysteine-specific reagents such as iodoacetic acid, iodoacetamide, or 2,2′-dithiopyridine (data not shown). Amino acid analysis of p11, which had been treated by VP or iodoacetamide, showed that the loss of binding activity is accompanied by the alkylation of both cysteine residues of the protein (see Table I for vinylpyridine).

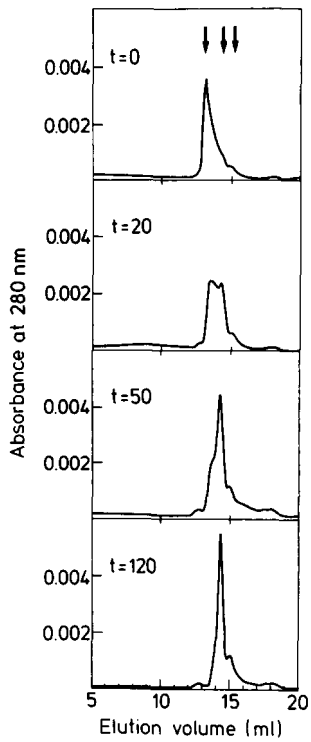

FIG. 1. **Porcine p11 in 20 mM imidazole HCl, 100 mM NaCl, 0.5 mM EGTA, 0.2 mM DTT, pH 7.5, was treated at 22 °C with a 1.4-fold molar excess of VP over total SH.** The reaction was stopped after different times with an excess of DTT. p36 was added in stoichiometric amounts, and the formation of the resulting p36·p11 complex was monitored by fast protein liquid chromatography on Superose S-12. The *arrows* at increasing elution volumes mark the position of the complex, p36 monomer, and p11 dimer, respectively. Since p36 contains 1 tryptophan and 18 tyrosines and p11 only 2 tyrosines (Gerke and Weber, 1985b), the absorbance of p11 is relatively small in comparison to that of free p36. Note the progressive loss of p36 binding activity upon alkylation of p11. The reaction seems complete after 2 h, when amino acid analysis shows that both cysteines of p11 are recovered as *S*-ethylpyridine-cysteine.

TABLE I

p11 derivatives with 1 or 2 residues of S-ethylpyridine-cysteine in complex formation with p36

Experiment	Treatment	No. of modified cysteines	Complex stability	Interaction with p36
	p11; mild alkylation	2		–
1, 3A	p11 in p36·p11; mild alkylation	1 (Cys-61)	+	+
3B	p11 from Experiment 3A plus second mild alkylation	2		–
2	p11 in p36·p11; strong alkylation	2	–	–

The p11 binding region is confined to the N-terminal 14 amino acids of p36. We have previously described a fluorescence assay to measure the binding of p11 to p36 or its N-terminal peptides labeled at cysteine 8 by the fluorescent dye Prodan (Johnsson *et al.*, 1988). As expected, p11, alkylated by iodoacetamide, showed no binding to a synthetic peptide covering the 18 N-terminal residues of p36 in this highly sensitive assay (data not shown).

For the protein chemical studies described below, we preferred alkylation by VP since *S*-ethylpyridine-cysteine is easily quantified by amino acid analysis and by automated gas phase sequencing using the standard program of the Applied Biosystems Sequenator.

Alkylation of Cysteine 82 Is Reduced in the p36·p11 Complex and Causes the Dissociation of the Complex—To decide whether the reactivity of the 2 cysteines of p11 is influenced by p36, we treated the p36·p11 complex with VP under mild (1.4-fold molar excess of reagent over the total SH for 2 h at 22 °C; Experiment 1) and strong alkylating conditions (7-fold molar excess for 8 h at 36 °C; Experiment 2). After mild alkylation, the p36·p11 complex remained intact (see the gel filtration profile in Fig. 2*A*). Under the same conditions, p11 loses its ability to bind p36 (see above). After strong alkylation, more than 80% of the p36·p11 complex dissociated into its subunits (Fig. 2*D*). The p11 subunits from both experiments were separated from p36 and from the reagent by gel filtration on a Sephadex S-200 column in 9 M urea. Amino acid analysis of p11 obtained from Experiment 1 revealed a single residue of *S*-ethylpyridine-cysteine (see Table I). After renaturation by dialysis against physiological buffer, p11 from Experiment 1 formed the p36·p11 complex when mixed with untreated p36 (Fig. 2*C*). The p11 obtained from Experiment 2 revealed close to 2 residues of modified cysteine. Less than 15% of this preparation was able to reform the complex when mixed with free p36 (Fig. 2*F*). The p36 subunits obtained from both experiments were readily renatured from 9 M urea and retained their ability to bind unmodified p11 (see profiles in Fig. 2, *B* and *E*). Nonetheless, we detected significant modification at cysteine 8 of p36, even in samples subjected to mild alkylating conditions, when the peptides of the tail piece of p36 were sequenced (not shown). This confirms our previous observation that modification of cysteine 8 of p36 does not impair the binding to p11 (Johnsson *et al.*, 1988).

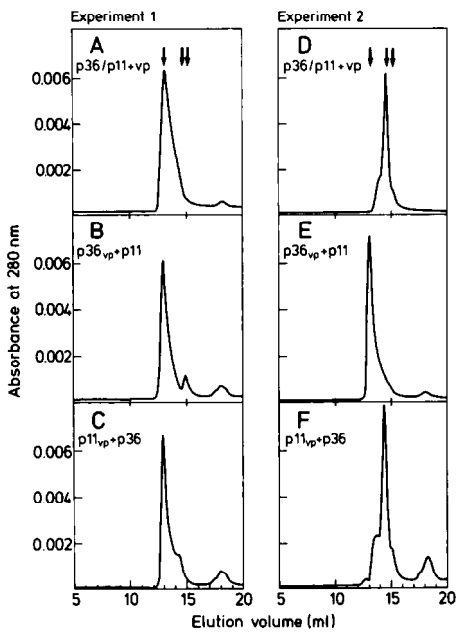

FIG. 2. **The p36·p11 complex in buffer B was treated either for 2 h at 22 °C with a 1.4-fold molar excess of VP over the total SH (Experiment 1; *panels A* to *C*) or for 8 h at 36 °C with a 7-fold excess of the reagent (Experiment 2; *panels D* to *F*).** Alkylation was stopped by DTT. Superose profiles of corresponding aliquots show that the complex remains stable under the mild alkylation conditions (*panel A*) but dissociates after the stronger alkylation conditions (*panel D*). The *arrows* at the increasing elution volumes mark the positions of the complex, monomeric p36, and p11 dimer, respectively. The bulk of the material from the two reactions was used to separate p11 and p36 by gel filtration on Sephadex S-200 in 9 M urea. Subunits were renatured and assayed by Superose gel filtration for complex formation by the addition of the complementary subunit. *Panels B* and *E* show that p36 from both alkylation reactions is fully competent to form the complex when mixed with untreated p11. *Panels C* and *F* document a differential alkylation of p11. When obtained from the mildly treated complex (*Experiment 1, panel A*), p11 readily forms the complex with normal p36 (*panel C*), while p11 obtained from the strong alkylation reaction (*Experiment 2, panel D*) fails to bind p36 (*panel F*). Amino acid analysis shows modification of only 1 cysteine of p11 in Experiment 1, while both cysteines are modified in Experiment 2.

The combined experiments suggest that, under mild alkylating conditions, 1 of the 2 cysteines in p11 is protected in the p36·p11 complex by the p36 subunit. Alkylation of the unprotected cysteine does not interfere with the binding ability of p11, whereas the modification of the second cysteine causes dissociation of the complex and consequently destroys the ability of p11 to bind to p36.

In an independent series of experiments, the complex was again mildly alkylated (see Experiment 1) and the subunits separated in 9 M urea (Experiment 3A). After renaturation, p36 and p11 were separately treated again with VP using the mild alkylating conditions (Experiment 3B). Gel filtration analysis on a Superose S-12 column showed that the p36 from both experiments retained its ability to bind unmodified p11 (not shown). In contrast, the binding activity of p11 was only retained after the first alkylation (Experiment 3A, Fig. 3*A*) but was nearly completely abolished by the second alkylation (Experiment 3B, Fig. 3*B*). Amino acid analysis showed that the level of *S*-ethylpyridine-cysteine in p11 was about 1 residue after the first and 2 residues after the second alkylation reaction.

We next tried to identify which of the two cysteines of p11 (Gerke and Weber, 1985b) is protected against mild alkylation in the p36·p11 complex. The isolated p11 derivatives of Experiments 1, 2, 3A, and 3B were treated with CNBr and directly analyzed by automated gas phase sequencing. The 2 cysteines of porcine p11, located at positions 61 and 82, lie in the same CNBR fragment which covers residues 56 to 90 (Gerke and Weber, 1985). The direct sequencing approach

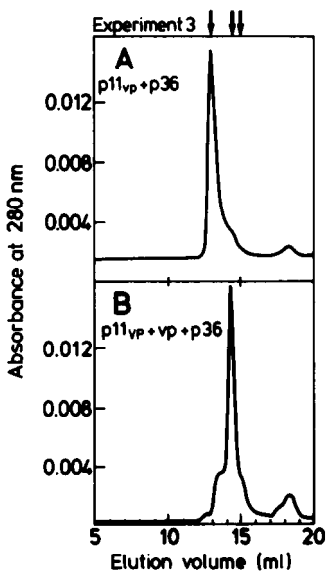

FIG. 3. **p11 with a single modified cysteine loses p36 binding ability upon further alkylation.** The p11 derivative was obtained by treatment of the p36·p11 complex using the mild alkylation conditions given for Experiment 1. It was isolated by gel filtration in 9 M urea and renatured. *Panel A* documents that upon addition of normal p36 the p11 derivative is fully incorporated into the resulting p36·p11 complex (*Experiment 3A*). This ability is lost when the p11 derivative is subjected to a second mild alkylation (*Experiment 3B*) prior to addition of p36 (*panel B*). Amino acid analysis shows the presence of 1 residue of *S*-ethylpyridine-cysteine in the p11 of Experiment 3A and of two substituted cysteines in the p11 of Experiment 3B.

without a preceding separation by HPLC avoids the problems of multiple peaks due to differential substitution of the 2 cysteines and to variations in the homoserine and homoserine-lactone content of the CNBR fragments. Inspection of the p11 sequence reveals that the *S*-ethylpyridine derivative can be expected at either cycle 6 (cysteine 61) and/or cycle 27 (cysteine 82) and that the extent of the modification can be estimated from the yield of glutamine and alanine in the preceding cycles 5 and 26, respectively. No other CNBr fragment contributes these amino acids at these cycle numbers. Thus, for calculation, the integrated areas of these amino acid derivatives were set to 1. Using this approach, we found for p11 from Experiments 1 and 3A that cysteine 61 was recovered to 80–90% as the *S*-ethylpyridine-cysteine derivative, while cysteine 82 showed a modification of less than 10%. This level also increased to about 80% when p11 from the strong alkylation reaction was analyzed (Experiment 2) or when p11 was subjected to a second modification cycle (Experiment 3B). We conclude that cysteine residue 61 of p11 is readily alkylated without loss of binding affinity for p36. In contrast, cysteine 82 is protected against alkylation when the complex is subjected to mild alkylation. However, under stronger modification conditions, cysteine 82 is alkylated in either free p11 or in the complexed p11, in which case, dissociation occurs upon modification. Thus, cysteine 82 is located within or very near an important p11·p36 contact region.

p11 Modified at Both Cysteines Remains a Dimer—When the p11 dimer was modified with VP, iodoacetamide, or DTP, the resulting derivatives eluted from a Superose S-12 column at 14.9 ml, the position characteristic of the unmodified p11 dimer. Modification of the cysteines does not reduce the portion of the p11 dimer detected by gel electrophoresis after dimethylsuberimidate cross-linking (not shown). To further confirm that the alkylation of the cysteines does not disturb the structure of p11, we compared the CD spectra of the alkylated protein with the unmodified p11. As seen in Fig. 4, the CD spectra of both preparations are nearly identical and are dominated by α-helix. We conclude that the alkylation of both cysteines does not affect the secondary structure of the molecule.

The p36·p11 Complex Can Be Reversibly Dissociated with DTP—Using DTP, the p36·p11 complex was dissociated under very mild conditions. The p36·p11 complex is nearly completely dissociated into its subunits when treated with a 4-fold molar excess of the reagent for 30 min on ice. Thus, dissociation of the p36·p11 complex with DTP required much less severe conditions than those needed for VP. When the dissociated complex was treated with an excess of DTT, the p36·p11 complex was reformed (not shown).

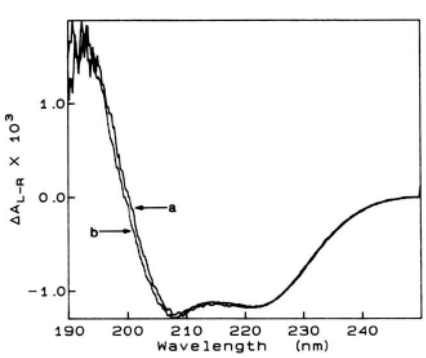

FIG. 4. **CD spectroscopy of p11 (*a*) and p11, alkylated with iodoacetamide at both cysteines (*b*).** For the direct comparison, the spectrum of the alkylated protein was multiplied by a factor of 1.21 to compensate for the lower protein concentration. In this experiment, p11 and the alkylated p11 had an optical density at 280 nm of 0.125 and 0.103, respectively.

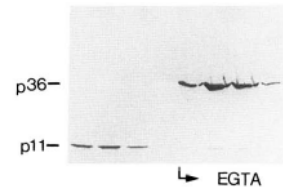

FIG. 5. **Separation of p11 and p36 under nondenaturating conditions.** The p36·p11 complex (1 mg/ml) in buffer C was treated with 5 mM DTP for 30 min on ice and then applied to a small affinity column of immobilized phosphatidylserine after addition of Ca^{2+} to 1 mM. The column was washed with the buffer and finally developed with buffer C containing 8 mM EGTA. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis. Pure p11 is recovered in the flow through fractions. The EGTA step elutes p36 and a small but variable amount of p11 resulting from some remaining p36·p11 complex.

Using the reversible thiol-specific reagent DTP, it is now possible to separate p11 from p36 under nondenaturating conditions. In a first set of experiments, we combined this approach with the Ca^{2+}-dependent binding of p36 to phosphatidylserine liposomes (Glenney, 1985). After the p36·p11 complex was treated with excess DTP for 30 min on ice, the solution was made 1 mM in Ca^{2+} and applied to a column of immobilized phosphatidylserine. Since p11 does not bind to lipids (Glenney, 1986; Johnsson *et al.*, 1986a), the modified p11 is not retarded and is found in the breakthrough fractions (Fig. 5). p36 and a trace of remaining complex is bound by the column and can be eluted by EGTA-containing buffer.

DISCUSSION

p11 is a dimer, as are the highly homologous S-100 proteins. In contrast to other EF-hand proteins, changes in the two loop sequences have made p11 a modulator no longer controlled by Ca^{2+}. p11 contains 2 cysteines per polypeptide chain. Both occur in the C-terminal half of the molecule. Cysteine 62 lies in the loop of the second EF-hand, while cysteine 82 locates to the C-terminal extension, which follows the last helix of the second EF-hand (Gerke and Weber, 1985b; Saris *et al.*, 1987). We have now shown that, in the free p11 dimer, both cysteines are modified under relatively mild conditions. The resulting derivative can no longer form the heterotetrameric $(p36 \cdot p11)_2$ complex. When the same modification conditions are used on p36·p11, the complex remains stable, and only 1 cysteine per p11 polypeptide is modified. Sequence analysis documents that under these conditions cysteine-61 is modified, while cysteine 82 is protected from alkylation by the p36 subunits in the complex. p11 modified only at cysteine 61 can again form the p36·p11 complex. This property is lost once the p11 derivative is subjected to a second cycle of mild alkylation, which leads to substitution of cysteine 82. We conclude that cysteine 82 is at, or very close to, the contact region between p36 and p11. However, our analysis cannot discriminate between two possible explanations. Thus, alkylation of cysteine 82 might either directly inhibit an important contact between the cysteine and a corresponding residue on p36 or, alternatively, cause a conformational alteration in p11 which is incompatible with the binding of p36. However, the alkylated p11 is still a dimer and displays no alterations in its CD spectrum.

Our results provide the first mapping of the p36 binding site on the p11 molecule and have obvious implications for p11-related molecules, which also have a C-terminal extension past the second EF-hand. Four of them, S100α and -β, S100L, and 18A2 have a cysteine at the position corresponding to the cysteine 82 of p11 (Glenney *et al.*, 1989; Isobe and Okuyama,

1978; 1981; Jackson-Grusby *et al.*, 1987). In contrast, MRP14, MRP8, and calcyclin display instead of the cysteine a serine, an alanine, and a tyrosine, respectively (Calabretta *et al.*, 1986; Odink *et al.*, 1987). If some of the p11-related molecules exert their function as Ca^{2+}-controlled regulators, one can speculate that their C-terminal extensions might be involved in the binding to their specific targets, which are not yet identified. The lack of a C-terminal extension in the sequence of the intestinal vitamin D-dependent calcium-binding protein would therefore emphasize its proposed function as a Ca^{2+} buffer (Wasserman and Fullmer, 1982). Interestingly, the C-terminal extension of the S100 proteins is more exposed to the solvent in the presence of Ca^{2+}. This property was demonstrated by spectroscopical studies of the single tryptophan of S100α (position 90) and by the increased chemical accessibility of the cysteines in positions 84 and 85 of S100α and -β, respectively (Baudier and Gerard, 1986; Mani and Kay, 1983). One can therefore speculate that Ca^{2+} transforms the S100 proteins into their active conformation, which also involves a conformational change in the C-terminal extension. p11 does not bind Ca^{2+}. Thus, it has to be in a permanent on state in regard to the binding of its ligand, p36. Consequently, cysteine 82 of the free p11, in contrast to the corresponding cysteine of the S100 proteins, is accessible to alkylating agents even in the absence of Ca^{2+}.

For the various members of the S100 family, a functional assay is only available for p11 and the glial-specific S100β protein. Kligman and Marshak reported that a possibly extracellular form of S100β displays neurite extension stimulating activity (Kligman and Marshak, 1985). Recent experiments point to a critical role of both cysteines of S100β (positions 68 and 84) in this stimulatory activity. Genetically engineered S100β proteins missing either both or only one of the cysteines fail to stimulate the outgrowth of neurones. The authors propose that the formation of either one or two disulfide bridges is important for the extracellular function of the S100β protein (Winningham-Major *et al.*, 1989). Consequently, the reduced S100β protein, which should reflect the intracellular form of the protein, is inactive when tested in the neurite extension assay (Kligman and Marshak, 1985). The reverse seems to be true for the ligand binding activity of p11. Here a fully reduced molecule is a prerequisite for the binding to p36. Incubation of p11 under nonreducing conditions reduces the ability of p11 to bind to p36. This loss in activity can be restored by treatment with DTT.[2] This behavior may be related to the localization of the p36·p11 complex, which is thought to be exclusively intracellular (Zokas and Glenney, 1987; Osborn *et al.*, 1988). We assume that in the absence of reducing agent an inter- or intramolecular disulfide bridge involving cysteine 82 is formed.

The p36·p11 complex is very stable, and titration experiments indicate that the dissociation constant is less than 30 nM (Johnsson *et al.*, 1988). All previous attempts to separate the two subunits have relied on denaturation by 9 M urea or 6 M guanidinium HCl followed by gel filtration in these solvents (Gerke and Weber, 1985a; Glenney, 1986). The separated subunits are then individually renatured and, when mixed, readily form the p36·p11 complex. We have made use of our observation that the p36·p11 complex can be dissociated by reversible cysteine-specific reagents such as DTP without denaturation. After this treatment, the p36 subunit is retained on a phospholipid affinity matrix due to its known Ca^{2+}-dependent interaction with the phospholipid, while p11 is found in the breakthrough. Since subsequent reduction leads to a reversal of the cysteine modification, the individual subunits prepared by this method can be used for further biochemical studies.

[2] N. Johnsson and K. Weber, unpublished observations.

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