Phosphorylation of the IQ Domain Regulates the Interaction between Ca\(^{2+}\)-vector Protein and Its Target in Amphioxus*

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Calcium vector protein target (CaVPT), a 26-kDa endogenous target of calcium vector protein from Amphioxus (CaVP), contains three distinct regions: a N-terminal Pro-Ala-Lys-rich motif, segment 36–50 displaying sequence similarity to the calmodulin-binding site in neuromodulin and neurogranin where they are designated as the IQ domain; and two immunoglobulin-like folds. The phosphorylation by protein kinase C of Ser-43 in the IQ domain drastically decreases the affinity of CaVPT for CaVP and CaVP protects CaVPT from phosphorylation. Phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase has a similar effect, but in addition to Ser-43 four other phosphorylated sites were identified. Removal of the Pro-Ala-Lys-rich region and the IQ domain in CaVPT by trypsin leads to the loss of binding to CaVP, whereas the chymotryptic fragment, containing these regions and first immunoglobulin-like domain, retained the ability to interact with CaVP. A synthetic IQ domain alone interacts strongly with calmodulin, but not with CaVP. Two main conclusions can be drawn from this study: 1) the regulation of interaction between CaVP and CaVPT is very similar to the mechanism observed in the complex between neuromodulin or neurogranin and calmodulin; 2) in spite of this similarity the entire CaVP-binding site is not restricted to the IQ domain; in addition the Pro-Ala-Lys-rich motif may be necessary for high affinity binding to CaVP.

Calcium vector protein (CaVP)1 and its endogenous target (CaVPT) are present in muscle of Amphioxus at unusually high concentration, namely 50 to 100 \(\mu\)M in terms of intracellular water, comparable to that of the abundant muscle component troponin C (Cox, 1986; Head and Perry, 1974). In spite of about 30% of sequence similarity to troponin C and CaM, CaVP does not substitute for the latter proteins in functional assays and bona fide CaM and troponin C are present in Amphioxus (Takagi et al., 1994), suggesting that CaVP assumes a particular function in the muscle of this higher invertebrate. Moreover, the only known endogenous target of CaVP is CaVPT, a unique 26-kDa protein displaying the following domain structure: 1) segment 1–23, a Pro-Ala-Lys-rich repetitive motif at the N terminus; 2) segment 36–50 displaying sequence similarity to CaM-binding sites in neuromodulin and neurogranin (Alexander et al., 1988; Apel et al., 1990; Baudier et al., 1991) where they are designated as the IQ domains; 3) segment 63–243 composed of two Ig-folds, structural motifs also found in myosin light chain kinase (Olson et al., 1990), telokin (Ito et al., 1989), C-protein (Fürst et al., 1992), twitchin (Benian et al., 1989), and titin (Labeit et al., 1990), proteins believed to be involved in homotypic interactions as well as in the interaction with myosin. At present the role of CaVPT is not well established, although recent data indicate that it interacts with a paramyosin-like protein and thus may participate in regulation of thick filament dynamics (Petrova et al., 1995b).

Our previous study indicated that while the complex CaVP-CaVPT possesses a rather rigid structure and does not display pronounced interactive properties, CaVP and CaVPT have characteristics of highly dynamic proteins (Petrova et al., 1995a). This allowed us to hypothesize that the complex represents a non-interactive end product and that CaVPT itself is the relay protein toward the unknown cellular response element. Under physiological conditions the affinity of CaVP for CaVPT is 80-fold higher in the presence of Ca\(^{2+}\), but Ca\(^{2+}\) removal does not lead to dissociation. In search of a physiological mechanism of CaVP-CaVPT dissociation we have studied the effect of phosphorylation by PKA and PKC on the interactive properties of CaVPT, in analogy with the dissociation mechanism proposed for neuromodulin/neurogranin and CaM (Apel et al., 1990; Baudier et al., 1991). We have also investigated by means of controlled proteolysis the domain structure of CaVPT and the interactive properties of the individual domains. Finally, we studied the interaction of CaVP with a synthetic peptide IQ comprising the IQ domain of CaVPT, in order to evaluate whether this sequence represents the CaVP-binding site, as it was suggested earlier (Takagi and Cox, 1991).

EXPERIMENTAL PROCEDURES

Materials—CaVPT/CaVP and bovine brain CaM have been purified as described by Petrova et al. (1995a) and Gopalakrishna and Anderson (1983), respectively. PKC from rat brain and catalytic subunit of PKA from porcine heart were purchased from Boehringer Mannheim and Sigma. Protein concentrations were determined spectrophotometrically using the extinction coefficients of 13,700 and 26,600 M\(^{-1}\) cm\(^{-1}\) for CaVP and CaVPT, respectively. The IQ\(^{-}\) peptide (NH\(_2\)-VISAATRIQAS-FRMHKNRMALKERKIPKF-COOH) was synthesized on a Labotech SP650 peptide synthesizer in combination with a Tecan RSP 5052 laboratory robot using 9-fluorenylmethoxycarbonyl-protected amino acids and a Wang resin (Bachem). CaVP (0.8–1 mg/ml of resin) was immobilized on vinylsulfone-activated agarose (Sigma) according to
Phosphorylation of CaVPT—Phosphorylation of CaVPT (0.3 mg/ml) by PKC was performed in 20 mM Tris-HCl, pH 7.4, 2 mM CaCl₂, 5 mM MgCl₂, 0.32 μg/ml phosphatidylserine, 7.5 μM [γ-³²P]ATP (5 μCi/100 μl) of the reaction mixture. Phosphorylation of CaVPT by the catalytic subunit of PKA was carried out in 20 mM MES, pH 6.5, 5 mM magnesium acetate, 1 mM DTT, and 50 μM ATP. Phosphorylation of the complex was performed in the presence of 1 mM CaCl₂ or 1 mM EGTA. The reaction was initiated by the addition of PKC or PKA. At the end of the reaction ATP was removed either by gel-filtration in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, or by precipitation of the protein in the presence of 5% trichloroacetic acid.

The phosphorylation was routinely monitored by autoradiography after SDS-PAGE or by liquid scintillation counting. To determine the degree of phosphorylation the band of CaVPT was excised from the gel, dissolved in 1 ml of Soluene 350 (Packard), and subjected to liquid scintillation counting. The incorporation of phosphate was calculated from the known quantity of the protein on the gel and the specific activity of ATP. The molecular weights of phosphorylated and non-phosphorylated CaVPT were determined by electron spray ionization-mass spectroscopy.

Interaction of Phosphorylated CaVPT with Immobilized and Native CaVP—The mixture of phosphorylated and non-phosphorylated CaVPT (100 μg/ml) was electrophoretically separated into 1 ml of CaVP-agarose resin was mixed with the digest, dialyzed for 12 h against 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA and subjected to SDS-PAGE. The gel was stained with Coomassie to estimate the relative protein concentration in the different fractions. The phosphorylated protein was determined by autoradiography of the same gel.

The interaction of phosphorylated CaVPT with native (non-immobilized) CaVP was studied by gel-filtration. A 3-fold molar excess of CaVP was added to the mixture of phosphorylated and non-phosphorylated CaVPT (100 μg/ml) in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM CaCl₂. Proteins were incubated for 1 h in the presence of 1 mM CaCl₂ or 1 mM EGTA, then applied to a TSK-125 gel-filtration column (7.8 × 300 mm), equilibrated in the same buffer and connected to a HPLC system (LKB). Migration of proteins was monitored by UV absorption and by SDS-PAGE, elution of phosphorylated CaVPT was monitored by liquid scintillation counting. The column was calibrated using the Bio-Rad standards for gel filtration.

Controlled Proteolysis of CaVPT and the Complex CaVP—CaVPT—The controlled digestion of CaVPT (0.4–0.8 mg/ml) and of the complex (0.8 mg/ml) by trypsin or N-tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (Sigma) was carried out in buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT (trypsin) or in 20 mM MES, pH 6.5, 1 mM DTT (chymotrypsin). When indicated 1 mM CaCl₂ or 1 mM EDTA were added. The protease/protein ratio was 1:500 (trypsin) or 1:100 (chymotrypsin) by weight. The digestion was stopped by the addition of a 2-fold excess of soybean trypsin inhibitor (Sigma), followed by heating at 100 °C, or by the addition of 1 mM phenylmethylsulfonyl fluoride.

Molecular weight of chymotryptic fragment of CaVPT was determined by electron spray ionization-mass spectrometry. Mass spectra were obtained in positive mode on a Trio 2000 instrument (Fisons Analitica) as described by Rose (1994). Prior to the analysis, the fragment was separated from non-digested CaVPT, CaVP, and small peptides by reverse-phase HPLC on a Nucleosil 300 A-5 μm C8 column (4 × 250 mm) with a linear gradient of acetonitrile from 0 to 70% in the presence of 0.1% trifluoroacetic acid.

Interaction of CaVPT Fragments with Immobilized CaVP—To identify the fragments of CaVPT capable of binding CaVP, 500 μg of the complex was digested with chymotrypsin in the presence of 1 mM CaCl₂. After 4 min the reaction was stopped and 2 μl urea and 1 mM EDTA were added in order to achieve the complete dissociation of the complex. 0.8 ml of CaVP-agarose resin was mixed with the digest, dialyzed for 12 h against 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM CaCl₂, 1 mM DTT, and transferred into a column. Unbound proteins were washed out with 10 volumes of the same buffer, then bound material was eluted by the buffer containing 1 mM EDTA instead of CaCl₂. Void and eluted fractions were analyzed by SDS-PAGE and Western blotting. The interaction of CaVPT tryptic fragments with immobilized CaVP was monitored in a similar way, but without the addition of urea, since free CaVPT was used in the last experiment. 200 μg of free CaVPT were subjected to the trypsinolysis.

Interaction of IQ Peptide with CaM and CaVP—Complex formation of IQ peptide with CaM or CaVP was followed by alkaline PAGE on 12.5% rod gels in the presence of either 1 mM CaCl₂ or 1 mM EDTA, as described previously (Duruisse et al., 1993). Before loading on the gels the samples of CaM or CaVP (1-2 mg/ml) were incubated for 15 min with 1 mM CaCl₂ or 1 mM EDTA, pH 7.5, 50 mM NaCl, 1 mM CaCl₂, 1 mM EDTA and 1 mM CaCl₂, or 1 mM EDTA. The concentrations of IQ peptide were chosen so as to obtain the peptide/protein ratio indicated in Fig. 8.

Electrophoresis and Western Blotting—12.5% or 15% SDS-PAGE was performed according to Laemmli (1970). The proteins were visualized by staining with Coomassie Brilliant Blue G. For immunoblotting after separation by SDS-PAGE (15% gels) the proteins were electroblotted in 25 mM Tris, 200 mM glycine to nitrocellulose membranes. The efficiency of the transfer was controlled with the pre-stained protein standards (Bio-Rad). Membranes were blocked for 1 h in 10 mM sodium phosphate, pH 7.2, 150 mM NaCl containing 0.4% casein and 0.07% polyvinylpyrrolidone, then probed for 30 min with immunopurified antibodies against CaVP (40 ng/ml), CaVP (85 ng/ml), or IQ peptide (60 ng/ml). Bound antibodies were detected with the chemiluminescent immunosass Tropix (Bedford, MA) as described earlier (Bronstein et al., 1991). Alkaline phosphatase-conjugated goat antibody against rabbit immunoglobulin G (Sigma) was used as secondary antibody.

Identification of the N-terminal Sequences of Proteolytic Fragments of CaVPT—After digestion and separation by SDS-PAGE the protein fragments were transferred onto a TSK-125 gel-filtration column (7.8 × 300 mm) and eluted with 40% methanol. The sequences of the peptides were determined with an automated sequencer (Applied BioSystem Model 477A on-line with a Model 120A PTH-analyzer).

Identification of Phosphorylation Sites—To identify the phosphorylation sites, 500 μg of CaVPT were phosphorylated by PKC with non-radioactive ATP as described above, dialyzed against 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM CaCl₂ and passed through the column of CaVP-agarose. The phosphorylated protein, which is not retained by the column, was precipitated by the addition of 5% trichloroacetic acid. The pellet was washed three times with methyl ether in order to remove traces of trichloroacetic acid, dried, then reduced with 2 mM DTT and alkylated with 4-vinylpyridine in the presence of 6 μg guanidine-HCl, 0.5 mM Tris-HCl, pH 8.5, 10 mM EDTA. After dialysis against water the sample was lyophilized and redissolved in 10 μl of 8 M urea. After the addition of 90 μl of 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, the sample was digested at 37 °C with 3 μl of 1 mg/ml N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin ( Worthington). The peptides were separated on a Toso ODS-80 column (4.6 × 300 mm) with a linear gradient of acetonitrile from 0 to 45% in the presence of 0.1% of trifluoroacetic acid. A control sample of non-phosphorylated CaVPT was prepared in the same way and the two elution profiles were compared. A new protein peak present in the phosphorylated sample but not in the non-phosphorylated one was sequenced as described above.

To determine the PKA phosphorylation sites, 200 μg of CaVPT were phosphorylated by the catalytic subunit of PKA in the presence of [γ-³²P]ATP as described earlier. After 30 min the reaction was stopped by the addition of 5% trichloroacetic acid. The pellet was collected after centrifugation, washed twice with cold 5% trichloroacetic acid in order to remove traces of ATP, and dissolved in 300 μl of 100 mM (NH₄)₂CO₃, pH 8.0. 10 μl of 1 mg/ml of N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin were added, and the sample was left overnight at room temperature. Peptides were separated on a μBondapak C18 column (3.9 × 300 mm) in the presence of 0.1% trifluoroacetic acid with a gradient of acetonitrile (10–60%). Fractions corresponding to radioactive peaks were dried and redissolved in 10 μl of 8 M urea. After the addition of 90 μl of 0.1 M Tris-HCl, pH 8.0, peptides were digested at 37 °C with 3 μl of 0.1 mg/ml endoprotease Asp-N (sequencing grade, Boehringer Mannheim). After digestion, peptides were separated on the same column with a gradient of acetonitrile from 5 to 60% and the radioactive peptides were sequenced as described above.

RESULTS

Phosphorylation of CaVPT by PKC and the Catalytic Subunit of PKA—Fig. 1 shows that CaVPT can be phosphorylated by PKC in vitro. Similar results were obtained for the catalytic subunit of PKA (data not shown). The incorporation of the
phosphate represents 0.3 mol/mol of the protein in both cases. The addition of an equimolar amount of CaVP under conditions which favor reconstitution of the complex (Petrova et al., 1995a) inhibits the phosphorylation by 80% and phosphorylation is totally suppressed when the amount of CaVP is increased to a 3:1 ratio (Fig. 1, data for PKA are not shown).

Identification of PKC Phosphorylation Site—Non-phosphorylated and phosphorylated CaVPT show molecular masses of 26621.6 ± 1.5 Da and 26698.7 ± 2.3 Da, respectively, as determined by electron spray ionization-mass spectrometry. The difference corresponds to the incorporation of a single phosphate group per CaVPT. Moreover only one radioactive peptide was eluted at 15% acetonitrile during separation of the tryptic digest of 32P-phosphorylated CaVPT by reverse phase HPLC (Fig. 2a). In order to identify this phosphorylation site in CaVPT, we compared the elution profiles of the tryptic digests of phosphorylated and non-phosphorylated samples. The two profiles were almost identical, except for one peptide which was eluted at 16% acetonitrile in the non-phosphorylated sample and shifted to 15% acetonitrile in the phosphorylated sample.

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The sequences of the peptide in both samples were the same (IQASFR), which identifies Ser-43 as a phosphorylation site for PKC in CaVPT (Fig. 2a, table). The fact that the phosphorylated peptide is eluted at a lower percentage of acetonitrile than the non-phosphorylated one is in agreement with its decreased hydrophobicity.

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Effect of Phosphorylation on the Interaction of CaVPT with Immobilized and Native CaVP—To evaluate the effect of phosphorylation on the interactive properties of CaVPT we have used two approaches: 1) binding of phosphorylated CaVPT to immobilized CaVP, and 2) binding of phosphorylated CaVPT to native CaVP monitored by gel filtration. The immobilization of CaVP on the agarose resin weakens the interaction with CaVPT to such an extent that the decrease of the affinity in the absence of Ca2+ (Petrova et al., 1995a) becomes sufficient to allow the dissociation of CaVPT. When the mixture of phosphorylated and non-phosphorylated CaVPT was passed through the column of CaVP-agarose in the presence of Ca2+, almost all phosphorylated protein was found in the void volume (Fig. 3a), whereas the non-phosphorylated CaVPT was retained and eluted by 1 mM EGTA (Fig. 3b). This indicates that immobilized CaVP is unable to sequester phosphorylated CaVPT even in the presence of Ca2+. The inability of phosphorylated CaVPT to form a complex with CaVP both in the presence and absence of
Ca\(^{2+}\) was further confirmed by the second approach, i.e. gel filtration. All phosphorylated CaVP eluted with an apparent molecular mass of 40 kDa corresponding to that of free CaVP. The complex and the excess of free CaVP migrated at 60 and 26 kDa, respectively (Fig. 4), as expected from previous work (Petrova et al., 1995). Thus, the phosphorylation of CaVP by PKC at Ser-43 efficiently abolishes its ability to form a complex with CaVP. Phosphorylation by PKA influences the binding of CaVP to immobilized CaVP in a very similar manner (data not shown). However, since this enzyme phosphorylates CaVP at multiple sites all over the protein, including Ser-43 of the IQ motif, it is difficult to evaluate if and how the phosphorylation of the additional sites contributes to the modification of the interactive properties of CaVP.

Controlled Proteolysis of CaVP—Intact CaVP migrates in the SDS-PAGE system with an apparent molecular mass of 31 kDa. Trypsinolysis of CaVP yields one major fragment with apparent molecular mass of 25 kDa which revealed to be very stable and was not positive to anti-IQ peptide. Immunoactivity to anti-IQ peptide was detected only in the intact protein (Fig. 5a). Automated Edman degradation of the 25-kDa fragment revealed the following N-terminal sequence MALKEKSIPK, corresponding to the residues 51–60 of CaVP. Thus, the 25-kDa peptide represents segment 51–243 of CaVP and will be called further T\(_{51-243}\), where T stands for “tryptic.” Interestingly, the trypsin digestion pattern of the complex was Ca\(^{2+}\)-dependent. In the presence of Ca\(^{2+}\) CaVP protects the IQ domain from trypsinolysis, whereas in the presence of EDTA both CaVP and CaVP are degraded by the enzyme (Fig. 5b).

Digestion of CaVP by chymotrypsin yields a large number of fragments, underlining the high susceptibility of free CaVP to this protease (data not shown). On the contrary the complex CaVP-CaVP produces one major fragment with an apparent molecular mass of 25 kDa, either in the presence of Ca\(^{2+}\) or EGTA (Fig. 6, lane 1). Interestingly, CaVP is very resistant to chymotrypsin and no proteolysis occurs even after prolonged incubation. The 25-kDa fragment reacts with anti-IQ (Fig. 6, lane 2) and has an N terminus PKPPAEAK which corresponds to that of the intact protein. The molecular mass of the 25-kDa fragment is 20315.36 ± 0.07 Da as determined by electron spray ionization-mass spectrometry. Taken together, these data allow to identify the fragment as C\(_{1-186}\) of CaVP with a calculated molecular mass of 20312.29 Da. The proteolytic cut thus occurs after Trp-186 in the beginning of the second Ig-fold which is in agreement with the specificity of chymotrypsin.

Interaction of T\(_{51-243}\) and C\(_{1-186}\) with CaVP-agarose—After trypsin digestion of CaVP, the mixture of the fragments and non-digested protein was chromatographed on CaVP-agarose in the presence of Ca\(^{2+}\). T\(_{51-243}\) fragment was found in the void fractions, whereas non-digested CaVP was retained by the column and eluted in the presence of 1 mM EDTA (Fig. 7a). Thus, T\(_{51-243}\), corresponding to the tandem Ig-folds of CaVP, is not able to interact with CaVP.

After treatment with chymotrypsin in the complex was dissociated in the presence of urea and EDTA and renatured in the presence of CaVP-agarose and Ca\(^{2+}\). Both CaVP and C\(_{1-186}\) bind to immobilized CaVP and can be eluted in the presence of 1 mM EDTA (Fig. 7b, lane 2). However, a significant part of CaVP and the fragment remains in the void fractions, likely because of the presence of non-proteolyzed CaVP during renaturation (Fig. 7b, lane 1). As estimated from the band intensity on the gel, the relative amounts of CaVP and C\(_{1-186}\) are the same in the eluate and in the void fractions, suggesting that the affinities of C\(_{1-186}\) and CaVP for CaVP are comparable. Therefore, it can be concluded that C\(_{1-186}\), comprising the Pro-Ala-Lys-rich region, the IQ domain and one complete Ig-fold, fully retains the ability to interact in a Ca\(^{2+}\)-dependent manner with immobilized CaVP.

Fig. 3. Effect of phosphorylation of CaVP by PKC on its interaction with immobilized CaVP. A mixture of non-phosphorylated and phosphorylated CaVP was passed through a CaVP-agarose column in the presence of 1 mM CaCl\(_2\), then retained protein was eluted by 1 mM EDTA. Distribution of phosphorylated CaVP and total protein in the void fractions and the eluate are shown after 12.5% SDS-PAGE and autoradiography (a) or after Coomassie staining (b), respectively.

Fig. 4. Interaction of phosphorylated and non-phosphorylated CaVP with native CaVP. A mixture of PKC-phosphorylated CaVP, non-phosphorylated CaVP, and a 3-fold excess of CaVP was incubated in the presence of Ca\(^{2+}\) or EDTA, then the components were separated on a gel-filtration column. The dashed line shows the elution profile of phosphorylated CaVP as determined by radioactivity; the solid line corresponds to optical density at 214 nm (OD\(_{214\text{nm}}\)). Inset, the column was calibrated using gel-filtration standards (44, 17, and 1.35 kDa, Bio-Rad, filled squares). Open circles correspond to the peaks 1, 2, and 3 on the main figure, \(t_0\) and \(t_e\) correspond to the void and the elution time.
Interaction of IQ Peptide with CaM and CaVP—Complex formation between IQ peptide and CaVP or CaM was monitored by a gel mobility shift assay in the presence of either 1 mM Ca$^{2+}$ or EDTA. Whereas CaM forms a 1:1 complex with IQ peptide in the presence of Ca$^{2+}$ (Fig. 8a), no interaction was observed in the presence of 1 mM EDTA, even at a 3-fold excess of the peptide (data not shown). The interaction of IQ peptide with CaVP was revealed to be quite different: upon electrophoresis of a mixture of CaVP and increasing amounts of the peptide in the presence of Ca$^{2+}$, the band of CaVP tends to disappear, but no new protein band is observed. Instead a broadband above CaVP shows Coomassie staining, suggesting that a not well established complex is formed. It should be noted that even a 6-fold excess of IQ peptide did not lead to the complete disappearance of CaVP (Fig. 8b). Under identical experimental conditions intact CaVP forms a well defined 1:1 Ca$^{2+}$-dependent complex with CaVP but binds CaM rather weakly (Cox, 1986), thus indicating a basic difference in the interactive properties of IQ peptide and CaVPT.

**DISCUSSION**

The Ig-fold is a common structural motif found in many intracellular muscle proteins, such as the giant protein titin (Labeit and Kolmerer, 1995), myosin light chain kinase (Olson et al., 1990), C-protein (Fürt et al., 1992), M-protein (Noguchi et al., 1992), myomesin (Obermann et al., 1995), H-protein (Vaughan et al., 1993) etc. It is composed of about 100 amino acids that form a $\beta$-barrel consisting of 8 $\beta$ strands (Holden et al., 1992; Pfuhl et al., 1995). Although the number of Ig-folds per protein varies from 112 copies in titin (Labeit and Kolmerer, 1995) to only 1 in the gizzard smooth muscle component telokin (Ito et al., 1989), all intracellular Ig-fold containing proteins are thought to be implicated in the regulation of myosin activity and assembly of thick filaments. CaVPT from muscle of Amphioxus is one of the smallest members of this family: it contains only two Ig-folds that account for 80% of its sequence (Fig. 9a). Residues 36–50 preceding the Ig-folds, display a high degree of sequence similarity to the CaM-binding domains of neuromodulin and neurogranin (Fig. 9b), as it was pointed by Cheney et al. (1991). Takagi and Cox (1991) have demonstrated that CaVPT can bind to CaM but in vivo it rather forms a strong complex with CaVP, a particular Ca$^{2+}$-binding protein in Amphioxus muscle.
for the PKC-regulated interaction with Ca\(^{2+}\)-binding proteins in otherwise very different protein systems. The biological consequence of this phosphorylation likely varies from one protein to another as they show very little sequence homology except for the IQ domain. It was proposed that the phosphorylation of neurenomodulin and neurogranin leads to an increase of the “local” concentration of CaM and to the activation of CaM-regulated processes (Alexander et al., 1987). However, in the case of the CaVP/CaVPT system, we rather suggest that the phosphorylation is necessary to maintain CaVPT in its free state. Indeed, free CaVPT but not the complex was found to interact with the 106-kDa protein from Amphioxus muscle (Petrova et al., 1995b).

With PROSITE, a dictionary of post-translational modification sites (Geneva University Hospital and University of Geneva, Geneva, Switzerland), no consensus sequence for PKA can be detected in CaVPT. It is known, however, that many well established substrates of PKA do not possess a canonical phosphorylation site RXXS (Walsh and Van Patten, 1994). Our data clearly show that CaVPT can be phosphorylated by the catalytic subunit of PKA at multiple sites, including Ser-43, and that the phosphorylation is prevented by CaVP. Moreover, these results provide an interesting example of allosteric propagation of a conformational change in CaVPT induced by CaVP: four out of the five phosphorylation sites are located in the second Ig-fold (Fig. 9a), i.e., far away from the suggested CaVP-binding site, yet in the complex CaVP-CaVPT these sites cannot be phosphorylated. Likewise we previously observed a strong decrease in the reactivity of two thios located in the second Ig-fold of CaVPT upon formation of the complex with CaVP (Petrova et al., 1995a). We suggest, therefore, that the binding of CaVPT provokes an allosteric re-arrangement in the structure of CaVPT which propagates from the N to the C terminus of the protein. Interestingly, a similar model of ligand-induced conformational changes may be proposed for C-CAM1, an epithelial cell adhesion molecule of the immunoglobulin superfamily. Binding of CaM to the cytoplasmic domain of C-CAM1, situated at the C terminus of the molecule, causes a down-regulation of the homophilic self-association of C-CAM1 which is thought to mediate cell-cell adhesion (Edlund et al., 1996).

**Domain Structure of CaVPT and CaVP-binding Site**—The phosphorylation of Ser-43 within the IQ motif is prevented by CaVP suggesting that the phosphorylation occurs in the CaVP-binding domain. Moreover, the phosphorylation of Ser-43 efficiently inhibits the interaction between CaVPT and CaVP. The question arises whether the IQ domain alone represents an entire CaVP-binding site. The synthetic IQ peptide, which comprises residues 36–50 of CaVP, revealed to be a good target for CaM but, surprisingly, interacted only weakly with CaVP. In the case of the entire protein the situation is inverse: the affinity of CaVP for intact CaVPT exceeds significantly that of CaVP alone and is associated with a Ca\(^{2+}\)-binding component.

Neurenomodulin and neurogranin constitute a separate group within the family of CaM-binding proteins due to their ability to fix CaM with almost equal and relatively low affinity in the absence as in the presence of Ca\(^{2+}\) (Alexander et al., 1987; Baudier et al., 1991). The regulation of their interaction with CaM is achieved through the phosphorylation by PKC of a Pro-Ala-Lys-rich region (Fig. 9a) (Baudier et al., 1991). Similar IQ homologues have also been identified in many unconventional myosins where they serve to anchor CaM to the cytoplasmic domain of the molecule, causing a down-regulation of the homophilic self-association of CaM (Espreafico et al., 1995). Sequences of bovine neurenomodulin and neurogranin were retrieved from the SwissProt data bank (accession numbers P06836 and P35722).

**Phosphorylation of CaVPT**—Of four potential phosphorylation sites for PKC in CaVPT, namely Ser-43, Ser-145, Thr-164, and Thr-239, only Ser-43 is situated in the IQ domain can be phosphorylated in vitro. The phosphorylation of Ser-43 drastically decreases the affinity of CaVPT for CaVP and a 3-fold excess of CaVP protects CaVPT from phosphorylation. These data underline the universality of the IQ domain as a module for the PKC-regulated interaction with Ca\(^{2+}\)-binding proteins in otherwise very different protein systems.
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