High-Affinity Binding and Localization of the Cyclic GMP-Dependent Protein Kinase with the Intermediate Filament Protein Vimentin†

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ABSTRACT: The major receptor protein for cyclic GMP (cGMP) in smooth muscle is the cGMP-dependent protein kinase (cGMP kinase). The more abundant Iα isoform (subunit $M_r \simeq 78\,000$) of this enzyme mediates the effects of cGMP to relax contracted vascular smooth muscle preparations. In this study, we have addressed the hypothesis that the cGMP kinase is anchored to intracellular proteins which might serve to target cGMP kinase to protein substrates. Using a gel overlay technique, immunoprecipitation, and a fluorescence binding assay for cGMP kinase, we have identified vimentin as a high-affinity and specific binding protein for cGMP kinase. Binding of cGMP kinase to vimentin is reversible and stoichiometric (one cGMP kinase dimer/vimentin dimer) with a K_D of approximately 49 nM. The site of high-affinity binding between cGMP kinase and vimentin did not appear to be localized to the catalytic domain of the kinase since vimentin phosphorylated by cGMP kinase and peptide substrates for cGMP kinase did not compete for high-affinity binding. Neither the proteolytically-derived 69-kDa catalytic fragment nor the 8-kDa N-terminal fragment bound vimentin with high affinity, suggesting that the cGMP kinase dimer was necessary for the interaction. Vimentin was readily phosphorylated *in vitro* with the dimer, but not the monomeric 69-kDa catalytic fragment even though the monomeric 69-kDa fragment was catalytically active toward other substrates such as histone F2b and peptides. This suggests that the high-affinity interaction between cGMP kinase and vimentin occurs at the N-terminal region, thus allowing the interaction between the phosphorylation site of vimentin and the catalytic site of cGMP kinase to occur. Finally, immunocytochemical colocalization of cGMP kinase and vimentin was observed in intact vascular smooth muscle cells in culture. A model is proposed to describe the targeting of cGMP kinase to the substrate vimentin and the physiological consequences of this interaction.

The major receptor protein for cGMP[1] in vascular smooth muscle and other tissues is the cyclic GMP-dependent protein kinase (cGMP kinase). This enzyme is a serine/threonine protein kinase which mediates the actions of cGMP to relax contracted vascular smooth muscle in part by decreasing intracellular Ca^{2+} levels (Cornwell & Lincoln, 1989). Several potential substrate proteins or sites of action for cGMP kinase have been identified, including the phosphorylation of the inositol 1,4,5-tris(phosphate) (IP_3) receptor (Jin et al., 1993; Komalavilas & Lincoln, 1994), Ca^{2+} ATPase activation (Rashatwar et al., 1987; Vrolix et al., 1988; Yoshida et al., 1991), activation of Ca^{2+}-activated K^+ channels (White et al., 1993; Robertson et al., 1993), phosphorylation of cytoskeletal actin binding proteins such as VASP (Reinhard et al., 1992), and inhibition of phosphoinositide turnover (Rapoport, 1986; Hirata et al., 1990). The list is not exhaustive.

There are two forms of cGMP kinase: a dimeric, soluble type I form with an approximate subunit M_r of 78 000 (Lincoln et al., 1977; Monken & Gill, 1980) and a particulate type II form with an $M_r = 86\,000$ (de Jonge, 1981; Uhler, 1993). The type I enzyme exists as two isoforms (α and β) which differ only in the N-terminal 106 amino acids forming the dimerization region and the pseudosubstrate domain (Lincoln et al., 1988; Wolfe et al., 1989a). The expression of the two type I isoforms appears to be the result of alternate mRNA splicing (Wernet et al., 1989; Francis et al., 1988). As mentioned above, cGMP kinase has been implicated in the control of intracellular Ca^{2+} levels, and the more abundant Iα form has been shown to mediate relaxation of contracted smooth muscle strips (Sekhar et al., 1992). However, few specific (i.e., unique) substrates for the enzyme have been identified. Substrates for many serine/threonine protein kinases have been shown to contain relatively specific consensus phosphorylation motifs, such as RRXS/T for the cAMP kinase where X is a small noncharged residue (Kemp & Pearson, 1990). While selective phosphorylation motifs have been described for cGMP kinase (Glass & Krebs, 1982; Colbran et al., 1992), cAMP and cGMP kinases have many similar and overlapping consensus phosphorylation motifs (Lincoln & Corbin, 1977). In addition, the relatively low cytosolic concentration of cGMP kinase in most cells suggests that mechanisms other than recognition of specific consensus phosphorylation motifs may contribute to selective cGMP-mediated protein phosphorylation in cells. One such mechanism for selective cGMP-dependent protein phosphorylation is the localization of cGMP kinase with substrate proteins in the cell. Cornwell et al. (1991) reported that cGMP kinase was colocalized in the endoplasmic reticulum of rat aortic smooth muscle cells with the Ca^{2+}-ATPase regulatory protein and cGMP kinase substrate, phospholamban. Another protein substrate whose colocal-

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[1] Abbreviations: cGMP kinase, cyclic GMP-dependent protein kinase; cAMP kinase, cyclic AMP-dependent protein kinase; SE-HPLC, size exclusion high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; BSA, bovine serum albumin; VSMC, vascular smooth muscle cells; IP_3, inositol 1,4,5-tris(phosphate).

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ization with cGMP kinase is important is the intermediate filament protein, vimentin. Wyatt et al. (1991) suggested that cGMP kinase must be transiently colocalized with vimentin in activated neutrophils in order to catalyze phosphorylation of this protein. Evidence for other proteins being localized in the cell with substrates has been presented for protein kinase C (Mochly-Rosen et al., 1991) and cAMP kinase (Scott et al., 1990).

Cytoskeletal proteins are involved with many diverse cellular functions such as mitosis, locomotion, and organization of cell architecture. In addition, many cytoskeletal proteins are readily phosphorylated by numerous protein kinases, thereby regulating their function. It is believed that the cytoskeleton, especially intermediate filaments, may also serve to localize organelles near regulatory proteins within the cytoplasm of cells (Goldman et al., 1986). In this report, we have examined the possibility that cGMP kinase Iα binds to vimentin with high affinity, and that this interaction may regulate the phosphorylation of the protein by cGMP kinase.

EXPERIMENTAL PROCEDURES

Materials. Bovine lens vimentin was purchased from Fluka Chemicals (Ronkonkoma, NY) or Sigma (St. Louis, MO). The monoclonal vimentin antibody was purchased from Dako Laboratories (Carpinteria, CA), and the mouse horseradish peroxidase linked secondary antibody was purchased from Amersham Inc. (Arlington Heights, IL). The IP_3 receptor peptide, GRRESLTSFG, was synthesized by Research Genetics (Huntsville, AL). Fluorescamine and α-chymotrypsin were purchased from Sigma. Iodination of cGMP kinase Iα was performed using the Indogen method at the Iodination Laboratory in the Cancer Center at the University of Alabama at Birmingham. The SE-HPLC column (G3000SW, 7.5-mm i.d. × 30 cm) was purchased from Perkin Elmer.

Cytoskeletal Protein Extract Preparation. Rat aortic smooth muscle cells were isolated using the procedure of Smith and Brock (1983) with modifications as previously described (Cornwell & Lincoln, 1989). Cells were grown to confluence in 100-mm dishes and used within the first two passages. Cytoskeletal extracts were prepared from these cells following the method of Tsuda et al. (1988). Cells were washed with phosphate-buffered saline (PBS) and then incubated at 4 °C in extraction buffer [20 mM Tris, pH 7.4, 0.1–1% Triton X-100, 50 mM NaCl, 4 mM EDTA, and 0.5 mM phenylmethanesulfonyl fluoride (PMSF)] for 5 min. The supernatant was removed, and the adherent cells were washed two times with extraction buffer in the absence of Triton X-100. Next, the cells were incubated at 4 °C for 10 min in extraction buffer containing 1% Triton X-100 and 1.5 M KCl. The supernatant was removed and the insoluble fraction washed two times with extraction buffer containing no Triton X-100. Insoluble proteins (cytoskeletal proteins) were isolated by scraping the plates using a plastic cell scraper. The extract was resuspended in extraction buffer without Triton X-100.

Immunoprecipitation. To determine whether cGMP kinase Iα binding proteins were bound to the enzyme in the intact cell, VSMC were homogenized in extraction buffer containing 1% Triton X-100 as described above, except that 1.5 M KCl was replaced with 200 mM NaCl. The homogenate was centrifuged for 20 min at 14000*g* to yield a Triton-soluble fraction and a Triton-insoluble pellet. The pellet was resuspended in extraction buffer without Triton X-100 and sonicated. Both the Triton-soluble and -insoluble fractions were subjected to immunoprecipitation analysis as follows: protein A–sepharose (1:1 slurry, Sigma) was washed with extraction buffer and cleared by incubating with 5 mg of nonimmune rabbit IgG for 30 min before use. Next, samples were precleared by being incubated with preabsorbed protein A–sepharose for 30 min. The precleared samples ($\simeq$0.4 mL) were incubated at 4 °C in a neutator with 5 μL of either anti-cGMP kinase or nonimmune rabbit IgG for 24 h. Immune complexes were precipitated using 30 μL of protein A–sepharose for 45 min. The suspension was centrifuged at 14000*g* for 1 min, and the supernatant was discarded. The immobilized immune complex was washed three times in extraction buffer and once in PBS. The pellet was resuspended in 50 μL of electrophoresis loading buffer and heated to 100 °C for 5 min. Proteins were separated on a 7.5% SDS–acrylamide gel according to the procedure of Laemmli (1970). Western blot analysis was then performed using a monoclonal anti-vimentin antibody as described below.

Western Blot Analysis. Following protein separation by SDS–PAGE, proteins were electrophoretically transferred to nitrocellulose at 100 V for 1 h, and the nitrocellulose membranes were incubated in a 2.5% bovine serum albumin (BSA) solution for 1 h at room temperature to block nonspecific protein binding. For detection of vimentin in extracts, blots were incubated with mouse monoclonal anti-vimentin antibody (1:4000) overnight at 4 °C. Following three washes in Tris-buffered saline (TBS), two 7-min washes in buffer B (TBS containing 0.05% NP40, 0.125% sodium deoxycholate, and 0.05% SDS), and three additional washes in TBS, the blots were incubated in TBS/Tween (0.1%) containing 1% BSA with an anti-mouse horseradish peroxidase (HRP) linked secondary antibody (1:8000) for 45 min at room temperature. The blots were quickly washed three times in TBS followed by five 5-min washes in buffer B and three washes in TBS. Bands were detected using the enhanced chemiluminescent assay (ECL) method (Amersham, Inc.).

Fluorescence Spectroscopy. Fluorescence spectroscopy was used to study cGMP kinase Iα binding to vimentin as follows: Bovine lung cGMP kinase Iα (8–20 nM) was labeled with fluorescamine in a 100-μL reaction volume containing 50 mM borate buffer, pH 9.1. Two 5-μL aliquots of fluorescamine (5 mg/mL in acetone) were quickly added to the protein solution while a vortex was maintained. This solution was diluted 20-fold into a buffer containing 100 mM potassium phosphate, pH 7.4, 150 mM NaCl, and 1 mM dithiothreitol (DTT) and placed in a cuvette. Prescans were performed to determine maximal excitation and emission wavelengths for fluorescamine-labeled cGMP kinase Iα (excitation at 399 nm and an emission at 482 nm). Unlabeled proteins had no fluorescence at 482 nm, and fluorescamine-treated H_2O also showed no fluorescence, since unreacted fluorescamine hydrolyzes to a nonfluorescent species. A standard curve of fluorescamine-labeled tyrosine based on its absorbance at 399 nm was done to determine how many fluorescamine molecules were incorporated into cGMP kinase Iα. The results indicated that approximately 16 fluorescamine molecules per monomer of cGMP kinase Iα were covalently attached. No further increase in protein labeling was seen with successive additions of fluorescamine. Unlabeled tyrosine, hydrolyzed fluorescamine, or fluorescamine-labeled H_2O had virtually no absorbance at 399 nm. For studying the interaction between cGMP kinase Iα and potential binding proteins, small volumes of putative binding protein were added directly to fluorescamine-labeled cGMP kinase Iα and continuously stirred at 25 °C. After 30 s the change in fluorescence was integrated for 4 s. The increase in fluorescence is consistent with a binding

interaction which shielded the hydrophobic fluorescamine molecules from the polar environment. The final fluorescence value (F_0) was divided by the initial fluorescence (F_i) to yield a ratio of relative fluorescence (F_0/F_i). The data were fitted using the MINSQ Program (Micromath, Inc., Salt Lake City, UT), which is a nonlinear regression program utilizing a user-derived single-site binding equation (see below).

Single-Site Binding Equation. Data were analyzed assuming one type of binding site, and the dissociation constant (K_D) was calculated by fitting experimental data to eq 5. As described below, E_f is free receptor, S_f is free ligand, and ES is the receptor–ligand complex. E_t is total receptor and S_t is total ligand.

$$K_d = [E_f][S_f]/[ES] \quad (1)$$

Since

$$[E_f] = [E_t] - [ES] \quad \text{and} \quad [S_f] = [S_t] - [ES] \quad (2)$$

then

$$K_d = ([E_t] - [ES])([S_t] - [ES])/[ES] \quad (3)$$

so that

$$[ES]^2 - ([E_t] + [S_t] + K_d)[ES] + [E_t][S_t] = 0 \quad (4)$$

This quadratic equation can be solved for [ES] as shown in eq 5.

$$[ES] = \frac{([E_t] + [S_t] + K_d) - ([E_t] + [S_t] + K_d)^2 - 4[E_t][S_t])^{1/2}}{2} \quad (5)$$

The fluorescence change is related to formation of ES by eq 6.

$$F = 1 + (B/A - 1)[ES]/[E_t] \quad (6)$$

where A is the fluorescence associated with E_t (E_{total} or totally "unbound" E) and B is the fluorescence associated with ES. Thus, F is the fluorescence of a mixture of E_f and ES relative to that of E_t only, at any given ligand concentration ($[S_t]$). Note that at [ES] = 0 (i.e., in the absence of ligand), $F = 1$, and at [ES] = E_t (i.e., at saturating ligand), $F = B/A$. $[E_t]$, K_d, and B/A are parameters which are allowed to vary in order to produce the best fit to the experimental data (measured F as a function of added S_t) using eq 6 where [ES] is calculated using eq 5.

The linear transformation of fluorescence data, based on Scatchard analysis, is essentially as described by Gutfreund (1971). This equation was used to determine the stoichiometry of cGMP kinase Iα binding to vimentin:

$$R = \frac{1 - (\text{fluorescence}_{obs})}{1 - (\text{maximal relative fluorescence})}$$

where R = fractional saturation and $\text{fluorescence}_{obs}$ is the relative fluorescence observed at any ligand concentration.

Phosphorylation of Vimentin Using cGMP Kinase. Vimentin (880 nM) was phosphorylated using 80 nM of pure cGMP kinase Iα for 3–60 min as follows: Kinase and vimentin were preincubated in a 100-μL buffer containing 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 0.1 mM cGMP. The reaction was initiated by addition of 0.1 mM [^{32}P]ATP (2.2×10^{-4} cpm/pmol) and was terminated by addition of SDS-electrophoresis stop mix and heating at 100 °C for 5 min. Proteins were separated by SDS–PAGE, and the gels were stained with coomassie blue, destained, dried, and subjected to autoradiography at −70 °C. For use in the fluorescence binding assay vimentin was maximally phosphorylated for 1 h as described above with unlabeled ATP and small aliquots were added to the fluorescamine-containing binding buffer. The components of the phosphorylation reaction were shown not to interefere with fluorescence binding.

Limited Digestion of cGMP Kinase Iα with α-Chymotrypsin. The catalytic and dimerization domains of cGMP kinase Iα were proteolytically isolated as described previously (Monken & Gill, 1980) with minor revisions. Small molecular weight components were separated from cGMP kinase Iα by Millipore ultrafiltration concentrators (MWCO = 10 000) against 10 mM potassium phosphate, pH 6.8. α-Chymotrypsin was prepared as 2.5 mg/mL stocks, freshly diluted to 0.25 mg/mL prior to use, and used at a ratio of 1:200 (chymotrypsin/cGMP kinase). Limited proteolysis was performed in 10 mM potassium phosphate, pH 6.8, for 5 min at 30 °C, and the reaction terminated by separation of the fragments at room temperature using size-exclusion HPLC (SE-HPLC). A G3000SW (7.5-mm i.d. × 30 cm) column was used to resolve the dimerization and catalytic domain. The mobile phase consisted of 20 mM potassium phosphate, pH 6.8, with a flow rate of 1 mL/min. Limited digestion revealed two major bands on SDS–PAGE: a 69-kDa constitutively active fragment and an 8-kDa amino-terminal fragment. Prior to separation on SE-HPLC, the digested solution was labeled with fluorescamine as described above. This allowed for collection of fluorescamine-labeled peaks based on the absorbance at 365 nm. Separate fragments were then used in typical fluorescence titration experiments as described above.

Immunocytofluorescence Localization of Vimentin and cGMP Kinase. Rat aortic vascular smooth muscle cells were prepared for fluorescence immunocytochemistry as described by Cornwell et al. (1991). Cells in passage 1 were plated on 12-mm glass coverslips in the presence of Dulbecco's minimal essential medium plus 10% fetal bovine serum for 24 h before treatment. Monolayers were fixed in 3% formaldehyde at room temperature for 10 min, followed by permeabilization with 0.5% Triton X-100 in PBS for 3 min. The cells were washed three times with PBS after formaldehyde and Triton and were incubated for 1 h at room temperature with 1% BSA in PBS. Finaly the cells were incubated for 1 h with rabbit anti-bovine cGMP kinase (Cornwell & Lincoln, 1989) or with the monoclonal vimentin antibody. Cells were washed in PBS and stained with FITC-goat anti-rabbit IgG and rhodamine-rabbit anti-mouse IgG for 45 min at room temperature. Control cells were incubated with preimmune serum controls, and no fluorescence was observed in the control-treated cells. The cells were then mounted in poly(vinyl alcohol) and viewed with a Zeiss fluorescence microscope where images were recorded.

Other Methods. Bovine lung cGMP kinase was purified to apparent homogeneity by affinity chromatography on 8-hexylamine cAMP agarose using a modified method of that previously described (Lincoln et al., 1977). The molecular weights for dimer cGMP kinase Iα (156 kDa) and dimer vimentin (114 kDa) were used for calculating molarity and stoichiometry of binding. Protein determinations were done by the method of Bradford (1976).

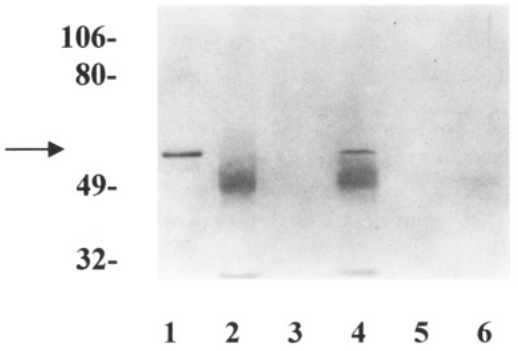

FIGURE 1: Co-immunoprecipitation of cGMP kinase and vimentin from VSMC extracts. Western blot analysis using a monoclonal antibody to vimentin was used to detect the protein following immunoprecipitation using the cGMP kinase polyclonal antibody. Triton-soluble VSMC extracts (lanes 2 and 5) and Triton-insoluble VSMC extracts (lanes 4 and 6) were incubated with the cGMP kinase antibody (lanes 2 and 4) or a nonimmune rabbit IgG (lanes 5 and 6). The complexes were resolved on 7.5% polyacrylamide gels as described in Experimental Procedures and transferred to nitrocellulose. Western blotting was then performed using the vimentin monoclonal antibody. The arrow represents vimentin migration. It was noted that the vimentin antibody recognized the cGMP kinase polyclonal antibody (lanes 2 and 4) but not the nonimmune antibody (lanes 5 and 6). Lane 1 is a vimentin standard, lane 3 is blank, and molecular weights in kilodaltons (kDa) include phosphorylase B (106), bovine serum albumin (80), ovalbumin (49), and carbonic anhydrase (32).

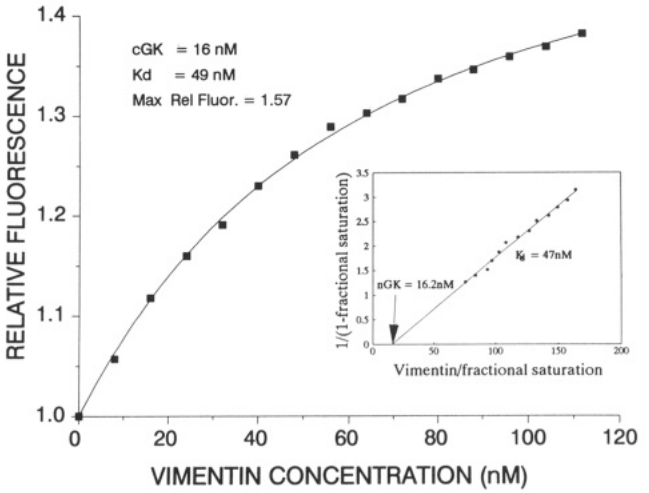

FIGURE 2: Fluorescence spectroscopy illustrating cGMP kinase binding to vimentin. cGMP kinase (16 nM) was labeled with fluorescamine and titrated with increasing concentrations of vimentin dimer (0–110 nM). Excitation was at 399 nm and emission at 482 nm. The inset represents the linear transformation of the data (Gutfreund, 1971; see Experimental Procedures), which indicated that binding was one to one (dimer cGMP kinase to dimer vimentin), $n = 6$.

RESULTS

Identification of cGMP Kinase Iα Binding Proteins. Initial attempts to identify binding proteins for the cGMP kinase Iα isoform were performed using a solid-phase overlay method similar to that used for the cAMP kinase (Lohmann et al., 1984). Triton-soluble (predominantly membrane-bound proteins) and Triton-insoluble proteins (predominantly intermediate filaments and other cytoskeletal proteins) from cultured rat aortic vascular smooth muscle cells (VSMC) were analyzed. A large number of coomassie blue bands (at least 19 major bands on a 7.5% SDS gel) were observed in the cytoskeleton fraction; however, [^{125}I]cGMP kinase Iα only labeled one relatively minor protein from the Triton-insoluble fraction having M_r = 57 kDa. The 57-kDa protein had an apparent molecular weight similar to that of the intermediate filament protein, vimentin. Confirmation that the 57-kDa Triton-insoluble binding protein was vimentin and not another protein with similar mobility on SDS–PAGE was obtained by western blot analysis using a monoclonal antibody to vimentin (data not shown).

The overlay method for detecting and characterizing binding proteins has been used extensively and has allowed us to identify vimentin as a cGMP kinase Iα binding protein. Another method, based on co-immunoprecipitation of cGMP kinase and vimentin, was used to study the interaction of these two proteins in the intact cell. As shown in Figure 1 a polyclonal antibody to cGMP kinase (Cornwell & Lincoln, 1989) immunoprecipitated vimentin from cultured VSMC as detected using a monoclonal antibody to vimentin in the western blot analysis. The cGMP kinase antibody did not immunoprecipitate purified vimentin, however, indicating the necessity for cGMP kinase to be present for vimentin immunoprecipitation (not shown). Nonimmune (or preimmune) IgG was not able to immunoprecipitate either vimentin or cGMP kinase (compare lane 4 to lane 6 in the figure). Similar to the blot-overlay technique mentioned above, only Triton-insoluble vimentin appeared to be bound to cGMP kinase. Cyclic GMP kinase from VSMC was also detected in the immunoprecipitate by western blot analysis using the polyclonal antibody to cGMP kinase (data not shown). As seen in Figure 1, the monoclonal anti-vimentin also appeared to recognize the IgG heavy chain of the polyclonal anti-cGMP kinase antibody, but not that to nonimmune rabbit IgG. Thus, several control experiments were performed to validate that the immunoprecipitation was specific for cGMP kinase. As mentioned above, the antibody toward cGMP kinase did not recognize vimentin, nor did the monoclonal antibody toward vimentin recognize cGMP kinase. Likewise A–sepharose did not absorb either protein in the absence of primary antibody. It appeared, therefore, that the vimentin antibody did indeed recognize an epitope on the cGMP kinase antibody. One possible explanation for this unusual antibody reactivity is that the epitope on vimentin recognized by the vimentin antibody is complementary to a sequence on the cGMP kinase antibody. This type of phenomenon might occur if the binding site on vimentin for cGMP kinase is similar to the epitope that the vimentin antibody recognizes on vimentin. In addition, the cGMP kinase antibody would require one epitope corresponding to the vimentin binding region on cGMP kinase, a likely occurrence in that the antibody for cGMP kinase is of polyclonal origin. Consistent with this explanation, co-immunoprecipitation using the monoclonal vimentin antibody to bind cGMP kinase was unsuccessful due to the epitope on vimentin being blocked by the bound cGMP kinase. These data and those described below indicate that vimentin and cGMP kinase form a tight association in the cell that can be isolated using immunoprecipitation techniques.

Fluorescence Spectroscopy Assay for cGMP Kinase Binding. In order to study the more quantitative properties of cGMP kinase Iα binding, a fluorescence spectroscopy assay was developed. Fluorescamine-labeled cGMP kinase Iα binding was monitored at 482 nm while exciting at 399 nm. No unlabeled cGMP kinase was detected following the labeling procedure using HPLC analysis, and intrinsic protein fluorescence due to vimentin or any other protein was negligible at this wavelength. As shown in Figure 2, cGMP kinase Iα binding to vimentin was saturable and occurred with a high affinity (K_D = 47 ± 1.03 nM, n = 6). The stoichiometry of binding based on the linear transformation of the data using

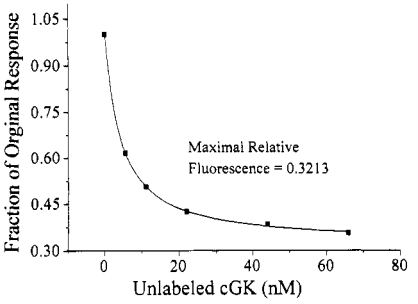

FIGURE 3: Effects of unlabeled cGMP kinase on binding of fluorescamine-labeled cGMP kinase to vimentin. Unlabeled cGMP kinase was added at different concentrations (0–66 nM) to a constant amount of fluorescamine-labeled cGMP kinase (11 nM) and vimentin (4 nM). As shown on the ordinate, increasing amounts of unlabeled cGMP kinase decreased the fraction of the original response, $n = 3$.

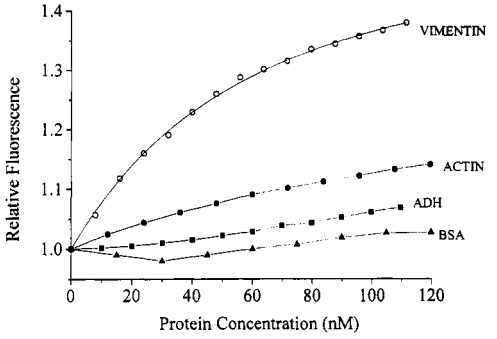

FIGURE 4: Specificity of binding of cGMP kinase to vimentin. Using fluorescence spectroscopy as described in Figure 3, actin, alcohol dehydrogenase (ADH), and bovine serum albumin (BSA) were examined for their capacity to bind fluorescamine-labeled cGMP kinase with high affinity or specificity. Note that the data points from ADH and BSA were unable to fit the equation, while actin fit a low-affinity ($K_D > 1$ mM) binding and failed to approach a saturable limit. These curves are compared to the titration curve seen with native vimentin, $n = 3$.

an equation first described by Scatchard (inset of Figure 2) indicated that the dimer cGMP kinase Iα was bound to the dimer of vimentin (Gutfreund, 1971).

To examine the reversibility of binding, unlabeled cGMP kinase Iα was added at different concentrations to the fluorescamine-labeled cGMP kinase Iα and vimentin. As shown in Figure 3, unlabeled cGMP kinase Iα competed with labeled cGMP kinase Iα, yielding a half-maximal inhibition at approximately 11 nM and equivalent to the amount of fluorescamine-labeled cGMP kinase Iα present. The capacity of unlabeled cGMP kinase Iα to bind equally well if not slightly better to vimentin ($K_D \simeq 11$ nM, $n = 3$) indicated that the binding was specific for the cGMP kinase *per se*, and not due to binding of the immobilized fluorescamine molecule itself.

To further validate the use of the fluorescence spectroscopy assay, binding of the ligand cGMP and the substrate Mg-ATP to fluorescamine-labeled cGMP kinase Iα revealed K_D's of 15 nM (high-affinity cGMP site), 74 μM (low-affinity site) and 2.7 μM (data not shown). These values were similar to those expected for the interaction of these ligands with native cGMP kinase on the basis of other procedures. This indicates that the fluorescamine-labeled cGMP kinase retains much of its native structure.

Because it is possible that other proteins may bind nonspecifically with fluorescamine-labeled cGMP kinase, several proteins were analyzed for their potential interactions with cGMP kinase Iα. As shown in Figure 4, bovine serum albumin (BSA), alcohol dehydrogenase (ADH), and actin all failed to bind labeled cGMP kinase Iα in a specific manner

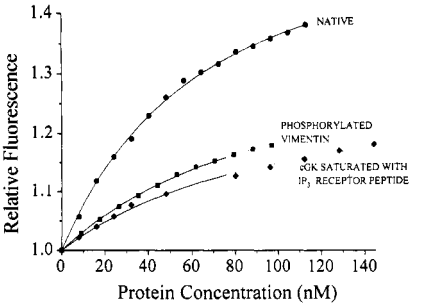

FIGURE 5: Effects of phosphovimentin and the IP_3 receptor substrate peptide on cGMP kinase binding to vimentin. Using fluorescence spectroscopy the effect of phosphorylated vimentin binding to cGMP kinase and the effect of native vimentin binding to cGMP kinase presaturated with IP_3 receptor substrate peptide (GRRESLTSFG) were examined. Vimentin (880 nM) was phosphorylated in a separate reaction using cGMP kinase (80 nM) and unlabeled ATP as described in Experimental Procedures and used to titrate fluorescamine-labeled cGMP kinase (middle curve). Fluorescamine-labeled cGMP kinase was saturated with IP_3 receptor substrate peptide (1.5 μM) and titrated with native vimentin (lower curve). In both instances, binding of vimentin was preserved with a slight shift in affinity. Both approaches are compared to the titration curve seen with native vimentin (top curve), $n = 3$.

like vimentin (K_D's of greater than 1 mM). In the presence of these nonspecific proteins, vimentin still bound cGMP kinase Iα with high affinity thus confirming the specificity (data not shown).

Identification of the Binding Domain of cGMP Kinase Iα. Many protein kinases including cGMP kinase Iα catalyze the phosphorylation of vimentin (Wyatt et al., 1991). Therefore, it was important to determine if the vimentin binding domain on cGMP kinase Iα was merely the substrate binding site on the catalytic domain of the kinase or a separate interaction site altogether. To determine if the substrate binding site interacted with vimentin with high affinity, the ability of substrates themselves to compete with vimentin for high-affinity binding was examined. We used a relatively high affinity peptide substrate for cGMP kinase Iα to saturate the catalytic site and to determine if this peptide affected cGMP kinase Iα binding to vimentin. The peptide substrate corresponding to the IP_3 receptor phosphorylation site has a relatively high affinity for cGMP kinase: 117 nM (Komalavilas & Lincoln, 1994). As shown in Figure 5, saturation of labeled cGMP kinase Iα with the IP_3 receptor peptide had little effect on the binding affinity of cGMP kinase Iα for vimentin (K_D changed from 49 to 88.3 ± 1.52 nM, $n = 3$). Although relative fluorescence decreased, this effect was most likely due to effects of the peptide alone (possibly the phenylalanyl residue in the peptide) on cGMP kinase Iα fluorescence, which had little effect on the high-affinity interaction between vimentin and cGMP kinase Iα. Thus, saturation of the phosphorylation site on cGMP kinase Iα did not prevent the high-affinity interaction between cGMP kinase Iα and vimentin.

Because of the effect of the peptide on fluorescence, another approach was used to address whethe cGMP kinase Iα binds vimentin at the substrate binding site. The effect of phosphorylation of vimentin itself by cGMP kinase Iα on cGMP kinase Iα binding was examined. The rationale was that the phosphorylated protein would dissociate rapidly from the catalytic domain of cGMP kinase Iα. As shown in Figure 5, phosphorylated vimentin bound to fluorescamine-labeled cGMP kinase Iα with only a slight shift in apparent affinity (K_d increased from 49 to 120.3 ± 1.53 nM, $n = 3$), suggesting that cGMP kinase Iα binds to vimentin at a site distinct from

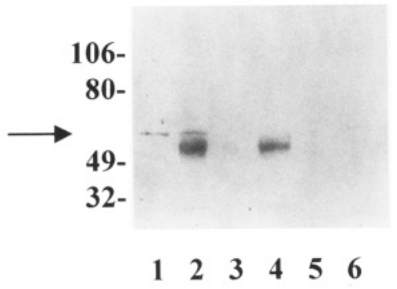

FIGURE 6: Co-immunoprecipitation of purified cGMP kinase or the catalytically-active monomer of cGMP kinase with purified vimentin. Western blot analysis using a monoclonal antibody to vimentin was used to detect the protein following immunoprecipitation using the cGMP kinase antibody. The monomer was prepared as described in Experimental Procedures. Lanes 2 and 5 represent native cGMP kinase, while lanes 4 and 6 represent the proteolytically-generated monomer of cGMP kinase. Lanes 2 and 4 are immunoprecipitates using the polyclonal cGMP kinase antibody while lanes 5 and 6 are nonimmune IgG controls. The arrow indicates vimentin migration. Lane 1 is the vimentin standard and lane 3 is blank, and molecular weights are as described in Figure 1.

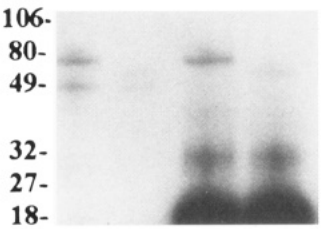

FIGURE 7: Phosphorylation of vimentin by holoenzyme cGMP kinase Iα and the catalytic 69-kDa monomeric kinase. Phosphorylation of vimentin (880 nM) with native cGMP kinase (80 nM) or chymotrypsin-cleaved monomeric cGMP kinase (80 nM) (lanes 1 and 2, respectively) was conducted as described in Experimental Procedures. Lanes 3 and 4 show phosphorylation of histone F2b (3 μM) with native (80 nM) or monoeric cGMP kinase (80 nM). All reactions were carried out in an identical manner.

the catalytic site and that catalytic site binding contributes little to the overall affinity. Total fluorescence change was less using the phosphorylated protein compared with the nonphosphorylated protein, but this was possibly due to a change in vimentin conformation whereby not as many fluorescamine molecules are shielded upon cGMP kinase Iα binding. Finally, binding of cGMP kinase Iα to phosphorylated and nonphosphorylated vimentin in the gel overlay assay demonstrated that cGMP kinase Iα bound equally well to both forms of vimentin (data not shown). Thus it appeared unlikely that cGMP kinase Iα bound vimentin with high affinity at the catalytic site of the kinase.

In an attempt to more specifically define the binding region on cGMP kinase Iα for vimentin, co-immunoprecipitation experiments using purified proteins were performed on the native holoenzyme and enzyme isolated as a proteolytically-generated and catalytically-active monomer. The monomeric form of the enzyme (M_r = 69 kDa) containing the cGMP binding domain and the catalytic domain was isolated from the N-terminal autoinhibitory and dimerization domain (M_r = 8 kDa) using limited digestion with α-chymotrypsin (Lincoln et al., 1978; Monken & Gill, 1980; Heil et al., 1987). As shown in Figure 6, co-immunoprecipitation demonstrated that only purified native cGMP kinase was capable of binding to purified vimentin while the chymotrypsin-digested cGMP kinase was incapable of binding to vimentin (compare lane 2 with lane 4). Because the N-terminal domain contains the dimerization site for cGMP kinase Iα, these data suggest that this region of the enzyme contributes in part at least to binding to vimentin.

In other experiments, the isolated fragments were labeled with fluorescamine and separated by HPLC. The fluorescent peaks corresponding to the constitutively active monomer and the dimeric amino terminus were collected, and each fragment was analyzed for the capacity to bind vimentin using an HPLC gel shift technique. Neither fragment bound vimentin, suggesting that neither the amino terminus dimeric polypeptide nor the catalytically-active 69-kDa monomer contained the entire high-affinity vimentin binding site (data not shown).

Phosphorylation of Vimentin by cGMP Kinase. Vimentin is phosphorylated by cGMP kinase both *in vitro* and *in vivo* (Wyatt et al., 1991). We were interested in whether there was a difference in the capacity of the holoenzyme to catalyze phosphorylation of vimentin with that of the catalytic fragment (which did not bind vimentin). As shown in Figure 7, the degree of phosphorylation of vimentin by the monomeric fragment was dramatically reduced compared to that catalyzed by the holoenzyme. However, the monomeric fragment catalyzed the phosphorylation of histone F2b with equal effectiveness as the holoenzyme, thus confirming the results of many other studies (Lincoln et al., 1978; Monken & Gill, 1980; Heil et al., 1987). In addition, peptide substrates such as the Kemptide and the IP_3 receptor substrate peptide were phosphorylated equally well by both the holoenzyme and the catalytic monomer (unpublished data). These results suggest that cGMP kinase Iα interacts in a different manner with vimentin than it does with other protein and peptide substrates when catalyzing phosphorylation.

Fluorescence Immunocytochemical Localization of cGMP Kinase and Vimentin. Because vimentin was the only protein in the cytoskeletal fraction that bound cGMP kinase, and since the binding affinity for vimentin is of the order that could be physiologically relevant, it was possible that vimentin may be a binding protein for cGMP kinase Iα in the intact cell. To test this idea, cultured rat aortic smooth muscle cells in the first passage were fixed and stained with fluorescent antibodies toward cGMP kinase and vimentin. As shown in Figure 8, the staining pattern for the two proteins was remarkably similar. Both proteins stained perinuclear areas and filamental structures conspicuously. The possibility that cGMP kinase is bound to vimentin in the intact cell seems more likely inasmuch as the cultures were treated with Triton X-100 following the fixative step. Thus, any nonbound kinase would be expected to be removed with this procedure. In other experiments, myosin or actin staining did not colocalize with cGMP kinase, suggesting that the colocalization of vimentin and cGMP kinase was not due to a nonspecific cross-linking of the proteins during fixation for immunocytofluorescence (data not shown). Although staining was more intense for vimentin than for cGMP kinase, these results suggest that at least some cGMP kinase present in these cells would be bound to vimentin in the intermediate filament structures.

DISCUSSION

There is renewed interest in the role of cGMP in cell function following the elucidation of the natriuretic peptide and nitric oxide (NO) signaling pathways. Although the molecular mechanisms of NO synthesis are well-characterized, the signal transduction mechanisms following cGMP elevation are not

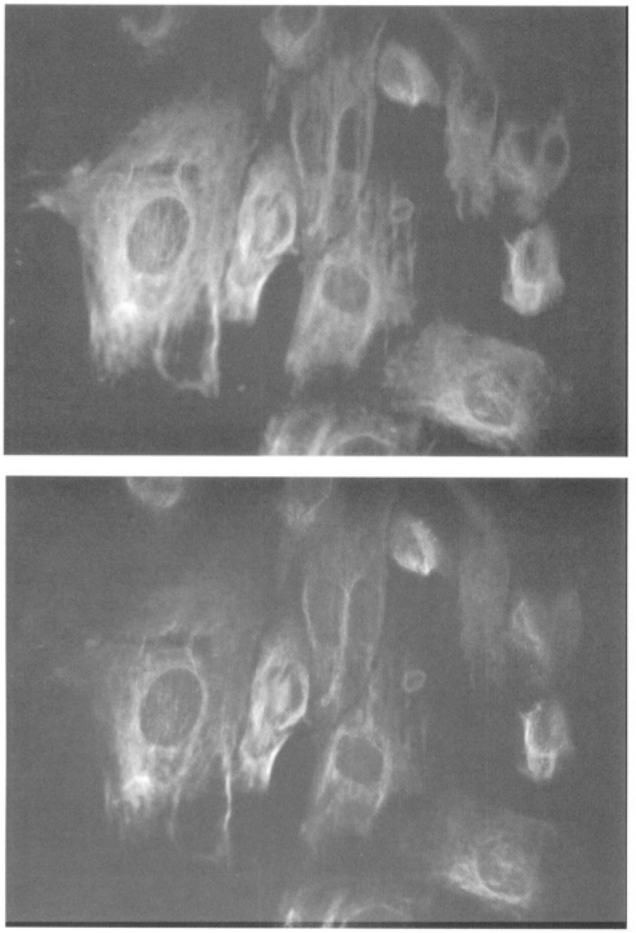

FIGURE 8: Fluorescence immunocytochemical localization of vimentin and cGMP kinase in cultured VSMC. Cells in passage 1 were plated and stained as described in Experimental Procedures. Top panel: vimentin. Bottom panel: cGMP kinase.

as well-defined. The major cGMP receptor in vascular smooth muscle is cGMP kinase, and thus it seems reasonable that cGMP kinase might play a role in NO signal transduction. One major gap in our knowledge is the elucidation of protein substrates for cGMP kinase. Because only a few substrates have been defined, and because localization of cGMP kinase with protein substrates may play an important role in cGMP action, we have been interested in potential cGMP kinase binding or targeting proteins in cells.

In order to study and characterize cGMP kinase binding proteins, we developed a fluorescence binding assay which has been used by other investigators to study binding interactions between proteins (Eftink & Ghiron, 1981; Eftink & Selvidge, 1982). This method was deemed suitable to study the interaction between cGMP kinase Iα and binding proteins due to its high sensitivity and the need to keep protein concentrations low both to accurately measure a high-affinity interaction and to conserve scarce proteins.

The results of this paper show that cGMP kinase Iα binds to one potential targeting protein, vimentin, in a reversible manner with high apparent affinity and specificity. Two basic assumptions were used in the data analysis: first, that the change in fluorescence indicates a conformational change (i.e., binding); and second, that binding is saturable. The equation used to fit the data for the cGMP kinase Iα and vimentin interaction is similar to a B_{MAX}/K_M curve, but accounts for the changes in free "receptor" as a function of added ligand (see Experimental Procedures). The maximal relative fluorescence value given for the titration is an extrapolated parameter based on the maximal conformational change that the labeled protein (i.e., cGMP kinase Iα) can undergo at infinite ligand concentration. Transformation of these data yields a "Scatchard like" plot which yields the affinity of the binding and the number of interaction sites on cGMP kinase Iα and vimentin (Gutfreund, 1971). As shown in the inset of Figure 2 this method agrees with the previous apparent affinity calculation and indicates that there is one vimentin dimer binding site per cGMP kinase Iα dimer. On the basis of these results, vimentin satisfies the criteria for a high-affinity cGMP kinase Iα binding protein.

Previous studies by Wyatt et al. (1991) indicated that cGMP kinase is transiently colocalized with vimentin in activated neutrophils. This finding complemented other findings demonstrating that much of the cGMP kinase is localized to the insoluble fraction in both smooth muscle cells (Cornwell et al., 1991) and neutrophils (Pryzwansky et al., 1990). Those studies and the findings reported in this paper indicate that vimentin could well serve as a targeting protein for cGMP kinase Iα in cells. Vimentin and other intermediate filament proteins are found abundantly in the perinuclear region of cells, including smooth muscle cells (Skalli & Goldman, 1991). The results shown in Figure 8 indicate that vimentin and cGMP kinase exist in similar if not identical regions in the smooth muscle cell. The perinuclear region of smooth muscle cells also contains substrates for cGMP kinase including phospholamban (Cornwell et al., 1991) and the IP_3 receptor (Komalavilas & Lincoln, 1994). Thus, localization of cGMP kinase with vimentin in the perinuclear cytoskeleton may provide a "docking site" for the kinase to recognize substrates.

The binding of cGMP kinase Iα to vimentin appears to occur at a noncatalytic site on the kinase. This is supported by several findings: (1) Peptide substrates do not decrease the affinity of cGMP kinase binding as would be expected from a "competitive inhibitor". (2) Vimentin that had been phosphorylated by cGMP kinase still binds to the enzyme with high affinity. (If vimentin were bound to cGMP kinase at the catalytic site, then phosphorylated vimentin would be expected to dissociate very rapidly from the enzyme as would any phosphorylated protein.) (3) The monomeric catalytic fragment of cGMP kinase does not bind vimentin. This larger chymotryptic fragment had been shown previously to be constitutively active and retain normal catalytic activity to a variety of protein substrates (Lincoln et al., 1978; Monken & Gill, 1980; Heil et al., 1987). Interestingly, however, the catalytically-active fragment was less efficient in catalyzing phosphorylation of vimentin than the holoenzyme (Figure 7), even though both forms of the kinase catalyze the phosphorylation of other substrates (i.e., histone F2b) equally well. This suggests that vimentin behaves differently from other substrates for cGMP kinase. The site(s) of phosphorylation on vimentin (for cGMP kinase) is (are) not known. However, previous studies have shown that serine-24 in the globular head region of vimentin is phosphorylated using cAMP kinase (Ando et al., 1989). This site is a potential cGMP kinase phosphorylation site in that there is an arginyl residue three residues N-terminal to the serine and an aromatic residue four residues C-terminal to the serine (Colbran et al., 1992). No other potential cGMP kinase phosphorylation sites are found in the protein. Nevertheless, the synthetic peptide corresponding to this site proved to be a poor substrate for cGMP kinase relative to other peptide substrates (unpublished data). Hence, these data together provide evidence for an unconventional type of cGMP kinase–substrate interaction which allows for cGMP-dependent phosphorylation. A model

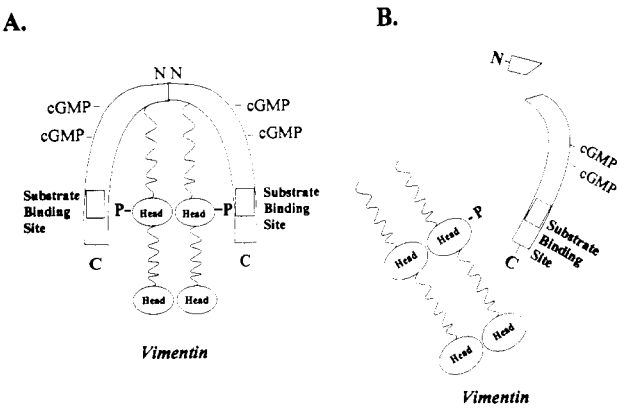

FIGURE 9: Model of possible interaction of cGMP kinase and vimentin. (A) Schematic of activated cGMP kinase dimer binding to vimentin via the tail portions of one vimentin dimer, thus aligning the head region near the catalytic domain of cGMP kinase. (B) Schematic of the trypsinized monomeric cGMP kinase failing to bind to vimentin due to the loss of the putative vimentin binding region located in the dimerization/hinge region of cGMP kinase.

for describing the interaction of cGMP kinase Iα with vimentin is shown in Figure 9. The dimeric cGMP kinase appears necessary for binding to vimentin. Binding of cGMP kinase is depicted to occur at the dimerization region and to adjacent regions in the regulatory domain. This would explain why neither the monomer nor the dimerization region (residues 1–77) alone is sufficient to bind vimentin. Although it is possible that proteolysis somehow affected a binding site distinct from the dimerization domain and thus prevented binding, this is unlikely inasmuch as both the binding sites for cGMP and the catalytic domain are known to be intact and fully functional in the monomeric fragment (Lincoln et al., 1978; Monken and Gill, 1980; Wolfe et al., 1989b). Thus, residues in the "hinge region" (residues 75–110) appear to be necessary for cGMP kinase binding to vimentin. The catalytic domain of cGMP kinase would be exposed to the site of phosphorylation on vimentin upon cGMP binding to the kinase. These results also demonstrate why there is a large difference in the capacity of the catalytic monomeric fragment to catalyze vimentin phosphorylation compared with the holoenzyme. Only the anchored kinase (i.e., holoenzyme) has the catalytic domain aligned with the sites of phosphorylation on vimentin. Because the phosphorylation site of vimentin (presumably serine-24) is not an ideal site of phosphorylation for cGMP kinase compared with other substrates such as histone F2b and the IP_3 receptor, alignment of the catalytic domain with the phosphorylation site seems to be required in the case of vimentin but perhaps not for other substrates. These results further provide an explanation for those of Wyatt et al. (1991), who observed that cGMP kinase only catalyzed vimentin phosphorylation in intact neutrophils when the kinase and the substrate were colocalized in the cells. Apparently cGMP kinase can only catalyze vimentin phosphorylation when it is bound to the substrate at a distinct high-affinity site.

Several other protein kinases, including cAMP kinase and protein kinase C, have been shown to interact with cytoskeletal proteins. Protein kinase C has been shown to interact with proteins known as RACKS (receptors for activated C kinase) that may target protein kinase C to specific cellular sites (Mochly-Rosen et al., 1991). The regulatory subunit (RIIβ) of the cAMP kinase has been shown to bind to proteins such as microtubule-associated proteins (MAP2). Conceivably, such binding could anchor the kinase to certain subcellular organelles containing substrates (Scott et al., 1990; Leiser et al., 1986; Bregman et al., 1989, 1991). Luo et al. (1990) and Scott et al. (1990) suggested that residues in the amino-terminal domain of RIIβ were critical for binding to MAPs and that dimerization of RIIβ was critical for the targeting process. Our results suggest that the dimeric cGMP kinase Iα interacts with vimentin at a site localized both at the dimerization site (AA 1–40) and at a region localized distal to this site (Figure 9). Such a model of binding of cGMP kinase Iα to vimentin is similar to that of RIIβ binding to MAP2 (Carr et al., 1991).

The functional consequence of cGMP kinase binding to vimentin is not known. As mentioned earlier, it is conceivable that this interaction anchors cGMP kinase Iα near substrate proteins. On the other hand, cGMP kinase Iα could regulate cell shape activity through the phosphorylation of the intermediate filament network. The precise molecular mechanism by which the vimentin-intermediate filament network forms is not known, but certain post-translational events such as phosphorylation can alter assembly of the network. The intermediate filament network disassembles during mitosis and during certain pathologic conditions (Skalli, 1992) such as Alzheimer's disease (Mayer et al., 1989; Shelanski, 1989). Vimentin is phosphorylated by several different kinases *in vitro* and *in vivo*, which alters its filamentous network. For example, $p34^{cdc2}$ kinase (which is the vimentin kinase in mitotic cells) is responsible for the collapse of the vimentin filaments during mitosis (Chou et al., 1990). Phosphorylation with cAMP kinase or the cGMP kinase may also promote a rearrangement of the filamentous vimentin network (Evans, 1989), while protein kinase C could cause a diassembly of the vimentin network. Further studies will be required to precisely map the binding region on cGMP kinase Iα, as well as the region of vimentin which is necessary for binding to cGMP kinase, and to understand the functional consequences of cGMP kinase vimentin phosphorylation.

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