Identification of Effector Binding Sites on S100β: Studies with Guanylate Cyclase and p80, a Retinal Phosphoprotein[†]

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ABSTRACT: S100β is a calcium-binding protein, which regulates the activities of several enzymes and inhibits the phosphorylation of a variety of protein kinase C substrates in a calcium-dependent manner. The protein was recently found to activate a retinal membrane guanylate cyclase, and in this paper, we report that it inhibits the phosphorylation of an 80 kDa retinal protein (p80). Structurally, S100β consists of two EF-hands connected by a hinge region. In view of its small size, wide distribution in a variety of tissues, and regulation of many different proteins, it is of interest to identify the sites on the protein that interact with the effectors, and to determine if the same sites are responsible for interaction with different effectors. We addressed these questions with the use of synthetic peptides with sequences corresponding to different regions of S100β and testing their effects on the protein's activation of guanylate cyclase, and inhibition of p80 phosphorylation. Peptides with sequences corresponding to effector interaction sites were anticipated to either block or simulate the effects of S100β. The results show that two regions of S100β interact with effectors: the C-terminal region of Thr81−Glu91 and the hinge region of Leu32−Leu40. The synthetic peptide containing the latter sequence blocked the S100β activation of guanylate cyclase and inhibition of p80 phosphorylation, while the peptide containing the former sequence blocked cyclase activation and simulated S100β in inhibiting p80 phosphorylation. By determining the effects of including or excluding dithiothreitol in the assays, we observed that the cysteine residue in the C-terminal region of S100β (Cys84) participates in the regulation of guanylate cyclase but not of p80 phosphorylation. We conclude from these results that the C-terminal and hinge regions of S100β are important in the regulation of effector proteins and that Cys84 is essential for interaction with only specific effectors.

S100β is a short, acidic, calcium-binding protein first discovered in brain and subsequently found at lower concentration in many other tissues (*1*−*5*). The dimer of the protein is referred to as S100b. It regulates a variety of intracellular effector proteins in a calcium-dependent fashion: activating fructose-1,6-bisphosphate aldolase, phosphoglucomutase, and adenylate cyclase (*6*, *7*) and inhibiting the phosphorylation of several protein kinase C substrates, including microtubule-associated τ protein, MARCKS (myristoylated alanine-rich C kinase substrate, p80 or 80 kDa protein), neuromodulin, and neurogranin (*8*−*10*). S100β was also shown to activate the retinal rod outer segment guanylate cyclase (ROS-GC) in a calcium-dependent manner (*11*). A retinal protein purified and characterized as calcium-dependent guanylate cyclase activator protein (CD-GCAP) has recently been found to be a conformational isoform of S100β (*12*, *13*). In addition to intracellular regulation of proteins and enzymes, S100β promotes neurite extension and glial cell proliferation (*14*, *15*) when it is added to cell cultures.

Since S100β is only 91 amino acids long and it regulates the activities of several effector proteins, identifying the effector interaction epitopes of the protein is of interest. S100β is a member of the family of S100 proteins, which are characterized by two EF-hand (helix−loop−helix) calcium-binding motifs (*4*). The calcium-coordinating loop of the N-terminal hand consists of 14 amino acids and that of the C-terminal hand 12 amino acids. A comparison of the amino acid sequences of several S100 proteins revealed that the C-terminal region and the hinge region between the two EF-hands are less conserved, leading to the suggestion that these regions may form the sites of interaction with effector proteins (*4*). It is also thought that binding of calcium to regulatory EF-hand-containing proteins such as calmodulin and S100β exposes hydrophobic areas, which then interact with effector proteins (*16*−*19*). On the basis of chemical shift changes upon calcium binding, such exposed regions in S100β were shown to be in helix I, helix II, the linker region, helix IV, and the C-terminal loop (*20*−*24*).

This study was undertaken to identify the regions of S100β which mediate activation of ROS-GC. Our approach was to synthesize peptides whose sequences correspond to specific regions of S100β and determine their ability to simulate or block the activation of the enzyme by S100β. While the study was in progress, we found that the phosphorylation of an 80 kDa retinal protein (p80) is inhibited by S100β and calmodulin in a calcium-dependent manner. It appears to be similar to the MARCKS protein (*25*, *26*) since it could be immunoprecipitated by anti-MARCKS antibodies. This protein gave us the opportunity

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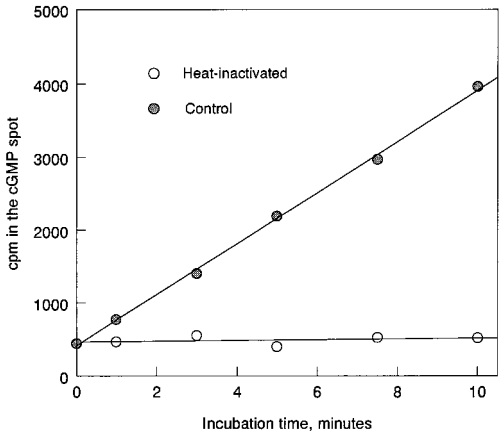

FIGURE 1: Time course of cGMP production in the guanylate cyclase assay as determined by the TLC method. Control and heat-inactivated enzyme preparations were assayed.

to measure the influence of the synthetic peptides simultaneously on one effector activated by S100β, and on another whose phosphorylation is inhibited by it. The results demonstrate that two S100β regions, one comprising helix II and the loop in the hinge region and the other consisting of helix IV and loop 4 in the C-terminal region, are involved in regulating both effectors.

MATERIALS AND METHODS

Guanylate Cyclase Preparation. Retinal rod outer segments isolated from fresh dark-adapted bovine eyes were washed to remove soluble and peripheral proteins (*13*). Guanylate cyclase activity in washed membranes was unaffected by calcium in that it was not influenced by 1 mM $CaCl_2$ or 2 mM EGTA which was added to the assays. The membranes were suspended in 5 mM isobutylmethylxanthin (IBMX) with or without 5 mM dithiothreitol (DTT) as required, aliquoted, and stored in the dark at −80 °C.

Guanylate Cyclase Assay. The membranes were assayed for guanylate cyclase activity under infrared light. All assays were carried out in triplicate. The 40 μL assay mixture contained 20 μg of membrane protein, 40 mM Hepes (pH 7.4), 1.0 mM GTP, 6 μCi of [α-^{32}P]GTP, 2 mM cyclic GMP, 0.2 μCi of cyclic [^{3}H]GMP, 15 mM $MgCl_2$, 1.25 mM IBMX, and 1 mM $CaCl_2$. Unless specified otherwise, the assays also contained 10 mM DTT. The reactions were carried out at 37 °C for 10 min and terminated by the addition of 20 μL of 150 mM EDTA containing GTP, cGMP, and GMP, all at 2 mM. After 10 min at 4 °C, the reaction mixtures were clarified by centrifugation and the cyclic GMP formed was measured by thin layer chromatography essentially as described earlier (*27*). Briefly, 5 μL of the clarified reaction mixture was spotted on a PEI-F cellulose TLC plate, dried, and developed for 1 h in 0.15 M LiCl. The plate was blow-dried, and the spots of the nucleotides were detected under UV light. The radioactive cyclic GMP spot was identified by reference to a simultaneously run standard, excised from the plate, extracted in 1 mL of 2 M KCl, and counted in 10 mL of scintillation fluid.

Figure 1 shows the time course of guanylate cyclase activity of a typical preparation of washed rod outer segment membranes. There was a linear time-dependent increase in radioactivity appearing in the cGMP spot when control membranes were assayed, and no increase in the heat-inactivated. The radioactivity in the cGMP spot, when heat-inactivated membranes were assayed, was between 400 and 550 cpm (total radioactivity in the assay mixture was 11 × 10^6 cpm). Recovery of [^{3}H]cGMP added to the assay mixtures was about 98 ± 2%. The specific activity of cyclase calculated from this experiment was 2.95 nmol of cGMP formed min^{-1} mg of protein^{-1} and represented the "basal" activity. The basal activity in the various membrane preparations used in this study was between 1.5 and 3.0 nmol min^{-1} mg of protein^{-1}.

Preparation of Retinal Subcellular Fractions. Retinas were extracted from dark- or light-adapted bovine eyes and homogenized in the homogenization medium (HM) [0.32 M sucrose, 20 mM Tris (pH 7.5), 2 mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL trypsin inhibitor, 50 μg/mL benzamidine, and 10 mM DTT]. The suspension was filtered on a 200 μm nylon mesh, and the supernatant was subjected to differential centrifugation to isolate nuclear, mitochondrial, and microsomal fractions sedimenting respectively at 700*g* (10 min), 10000*g* (10 min), and 100000*g* (1 h). Each fraction was washed once in the HM, suspended in the same medium, aliquoted, and frozen at −80 °C. The postmicrosomal supernatant was centrifuged a second time to remove any residual membranes, and the supernatant (cytosol) was frozen in small aliquots.

Phosphorylation Assay. The assay mixtures (40 μL) contained about 80 μg of protein, 130−160 mM sucrose, 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10−50 μM ATP, and 6 μCi of [γ-^{32}P]ATP. Unless specified otherwise, the assays also contained 10 mM DTT and 1 mM $CaCl_2$. The reactions were initiated with the addition of ATP. After incubation for 10 min at 30 °C, the reactions were terminated by the addition of 20 μL of a 3-fold-concentrated electrophoresis sample buffer containing SDS. The samples were electrophoresed in either a 10 to 20% gradient polyacrylamide gel (*28*) or a Tris-tricine 16.5% polyacrylamide gel (*29*). The gel was stained with Coomassie Blue, destained, photographed, air-dried, and exposed to X-ray film to detect radioactive protein bands. Following autoradiography, radioactive bands were excised from the gel and their activities were measured in 3 mL of scintillation fluid.

Immunoprecipitation of the MARCKS Protein. Retinal membranes were phosphorylated (180 μg of protein in 90 μL) as described above either in the absence (control) or in the presence of 5 μM S100β. Reactions were terminated with the addition of 30 μL of 4× hot lysis buffer [1× = 1.0% SDS and 10 mM Tris (pH 7.4)]. Samples were heated for 5 min at 95 °C and diluted to 500 μL with water. An equal volume of 2× immunoprecipitation buffer [1× = 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, and 0.5% NP-40] was added to each sample followed by overnight incubation at 4 °C with 10 μL of the anti-MARCKS monoclonal antibody. The mixture was incubated for 1 h with 10 μL of horse anti-mouse IgG. Twenty microliters of a 50% protein A−agarose suspension was added to each sample, and the mixture was agitated for 1 h at 4 °C. The immunoprecipitate was collected by centrifugation and washed six times with immunoprecipitation buffer. Proteins in the pellet were dissolved in 25 μL of 3× electrophoresis sample buffer, heated for 5 min at 95

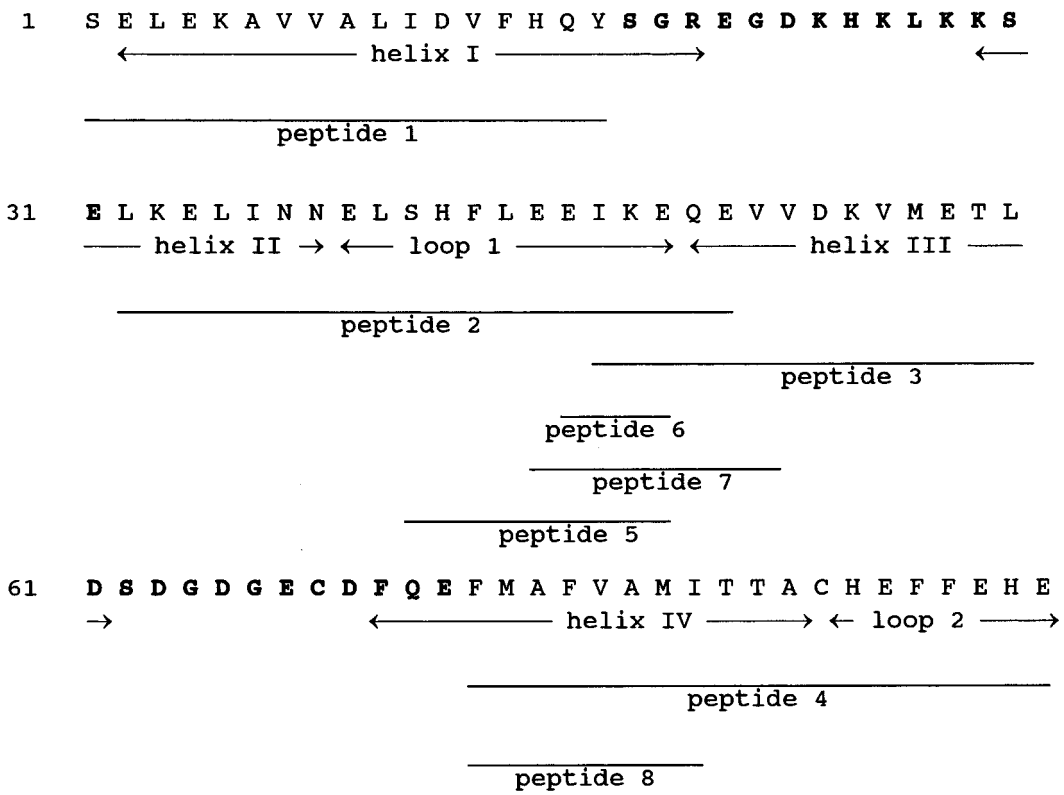

FIGURE 2: Synthetic peptides used in this study. The amino acid sequence of S100β is shown with calcium-coordinating loops shown in bold. Helices and loops are numbered. Underlines mark the sequences for which corresponding synthetic peptides were made.

°C, and separated by SDS−PAGE. Gels were dried and autoradiographed.

Materials. Bovine eyes were purchased from Wolverine Packing Co. (Detroit, MI). Peptides corresponding to the sequences of different regions of S100β were synthesized and purified by Quality Controlled Biochemicals (Hopkinton, MA). [α-^{32}P]GTP and [γ-^{32}P]ATP were purchased from NEN (Boston, MA). S100β, S100α, and calmodulin, all purified from bovine brain, were purchased from Sigma Chemical Co. (St. Louis, MO), as were phorbol 12,13-dibutyrate (PDB) and 8-bromo analogues of cyclic AMP and cyclic GMP. Anti-MARCKS protein monoclonal antibody was a generous gift from Dr. P. J. Blackshear (National Institute of Environmental Health Sciences, Research Triangle Park, NC).

RESULTS

Choice of Sequences of the Synthetic Peptides Used in the Study

Eight peptides with sequences corresponding to different regions of S100β were synthesized. These are shown in Figure 2. When the peptides were chosen, the sequences belonging to calcium-coordinating loops (Ser18−Glu31 and Asp61−Glu72) were excluded assuming that these regions, being calcium-bound, would not be available for effector binding. Peptide 1 (Ser1−Tyr17) approximately corresponded to helix I preceding the N-terminal calcium loop. Peptide 2 (Leu32−Glu51) included the sequences of helix II and loop 1. Peptide 3 (Iso47−Leu60) overlapped a portion of peptide 2 and included the sequence of helix III. And peptide 4 (Phe73−Glu91) included the sequences of helix IV and loop 2 (helices are numbered as in refs *24* and *30*; when the loops were being numbered, calcium-coordinating regions were not considered, just for convenience).

Ser41−Glu49 was reported to be a loop region by Amburgey et al. (*30*), while Kilby et al. (*31*) observed that residues Ser41−Glu45 are in a helical arrangement, leaving Glu46−Glu49 to form the loop. To test the influence of this loop region on the effector proteins, we designed peptides to match both possibilities: peptide 5 corresponded to Ser41−Glu49 and peptide 6 to Glu46−Glu49.

Peptides 7 (Glu45−Val52) and 8 (Phe73−Ile80) were chosen to represent S100β sequences less conserved in other S100 proteins (*4*). Peptide 8 also corresponded to a highly hydrophobic patch in the S100β protein.

Influence of Peptides on ROS-GC

We have shown earlier that ROS-GC is stimulated about 8-fold by S100β in the presence of calcium (*11*). In this series of experiments, the influence of the above peptides was tested on both the basal and S100β-stimulated cyclase activity.

Basal ROS-GC Activity. All the peptides were tested at 10−300 μM for their influence on basal cyclase activity. None of them inhibited the activity or simulated the effect of S100β. Cyclase activity in the presence of any one of the tested peptides at 300 μM was within 90−105% of the basal activity. The presence of 1 mM EGTA or 1 mM calcium chloride in the assay, which did not affect the basal cyclase activity, also did not have any influence on the inactivity of the peptides.

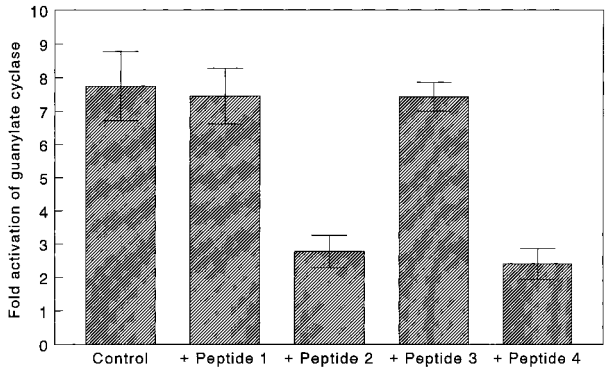

FIGURE 3: Influence of peptides 1−4 on S100β-stimulated guanylate cyclase activity. The peptides were tested at 300 μM, at which they had no influence on basal cyclase activity. The concentration of S100β in the assays was 6 μM. The data from four experiments are shown as the mean ± SD.

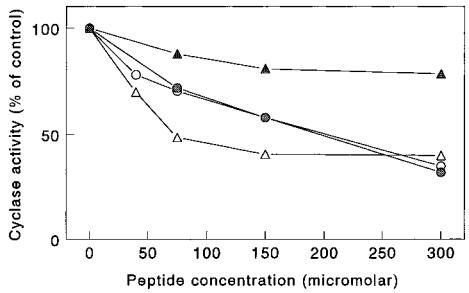

FIGURE 4: Dependence of the inhibition of S100β-stimulated cyclase activity on the concentrations of peptides 2 and 4 in the presence and absence of DTT: peptide 2 without (●) and with (○) DTT and peptide 4 without (▲) and with (△) DTT. Guanylate cyclase was stimulated by 6 μM S100β. The concentration of DTT in the assay, when it was present, was 10 μM.

S100β-Stimulated ROS-GC Activity. When the peptides were screened, all at 300 μM, for effects on S100β-activated ROS-GC, we showed that peptides 1 and 3 did not interfere with the activation (Figure 3). Peptides 5−8 were also without influence (data not shown). However, peptides 2 and 4 reduced the stimulation of cyclase from about 8-fold to about 2−3-fold (Figure 3).

Dose−response curves for peptides 2 and 4 are shown in Figure 4. At 300 μM, peptide 2 (open circles) and peptide 4 (open triangles) inhibited the activation of cyclase by about 60%. Half-maximal inhibition by peptide 2 was achieved at about 90 μM and by peptide 4 at about 40 μM. The inhibition by peptide 4 reached a plateau, while that by peptide 2 appeared to progress. However, increasing the concentration of peptide 2 to 600 μM inhibited the basal cyclase activity, besides further reducing the S100β-stimulated activity.

Peptide 2 does not have a cysteine, while peptide 4 contains one cysteine corresponding to Cys84. We have therefore investigated the influence of the removal of DTT from the assays on the effects of peptides 2 and 4. The membranes used in these assays as a source of ROS-GC activity were also prepared in the absence of DTT. The activation of cyclase by S100β was slightly higher (695%) in the presence of DTT than in its absence (530%), but the activated level was shown normalized to 100% for the sake of comparison. As is evident from Figure 4, the absence of DTT had no influence on the effect of peptide 2 but reduced the effectiveness of peptide 4 by nearly 70%.

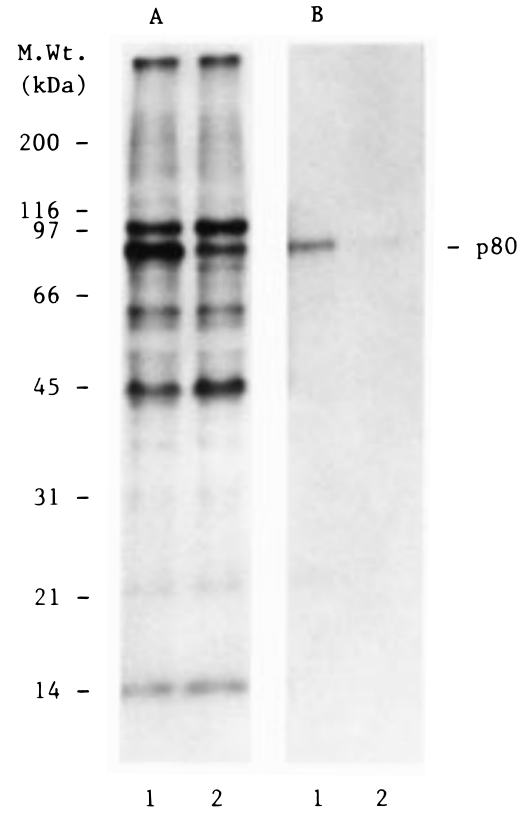

FIGURE 5: Phosphorylation of an 80 kDa protein and its immunoprecipitation with anti-MARCKS antibody. Membrane proteins were phosphorylated by incubation with [^{32}P]ATP: lane 1, control; and lane 2, with 6 μM S100β. Phosphorylated proteins were separated by SDS−PAGE and identified by autoradiography (A). A second set of phosphorylated membranes were solubilized and immunoprecipitated using an anti-MARCKS monoclonal antibody, and the precipitated proteins were separated by SDS−PAGE and detected by autoradiography (B). The antibodies precipitated a single protein of 80 kDa.

Characteristics of the Phosphorylation of an 80 kDa Retinal Protein

In our attempts to identify other effectors of S100β among the retinal proteins, we determined its influence on protein phosphorylation. When retinal subcellular fractions were incubated with [^{32}P]ATP in the presence and absence of S100β, and the phosphorylated proteins were detected by SDS−PAGE and autoradiography, an 80 kDa protein (p80) was found to be phosphorylated in all subcellular fractions and its phosphorylation was inhibited by S100β. Further studies were conducted mostly on the mitochondrial fraction in which fewer proteins were phosphorylated, and p80 was the most prominent of them (Figure 5). Calcium was required for S100β inhibition of p80 phosphorylation; when it was excluded from the assay, S100β did not inhibit phosphorylation (data not shown). p80 phosphorylation was also inhibited by calmodulin and S100α in a calcium-dependent manner (data not shown). As seen in Figure 5, S100β caused prominent reduction only in the phosphorylation of p80, not a nonspecific reduction in the phosphorylation of every protein.

Earlier reports indicated that S100β inhibits phosphorylation of specific proteins, including an 80 kDa protein kinase C substrate, the MARCKS protein. The presence of the MARCKS protein in bovine retina was reported (*32*). To

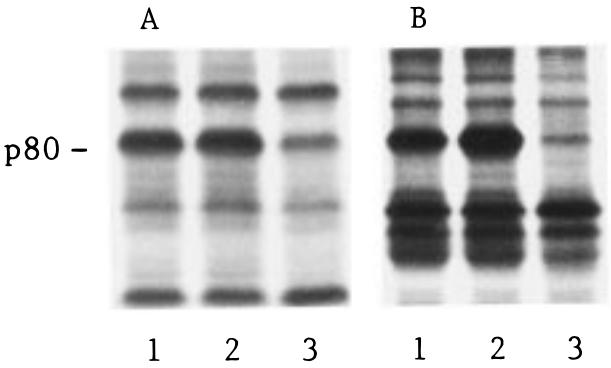

FIGURE 6: Effect of PDB on the phosphorylation of p80. Membrane (A) or cytosolic (B) fractions were phosphorylated as described in Materials and Methods: lane 1, control; lane 2, with 300 nM PDB; and lane 3, with 6 μM S100β.

determine if the p80 in our study is the MARCKS protein, retinal proteins labeled by incubation with [^{32}P]ATP were immunoprecipitated using an anti-MARCKS monoclonal antibody. If the protein is a MARCKS protein, there should be more radioactivity precipitated in the control than in the preparation labeled in the presence of S100β. Figure 5 shows that a single 80 kDa radioactive protein was immunoprecipitated by the antibody, and that there was less radioactivity immunoprecipitated from the preparation labeled in the presence of S100β. Little is known about the physiological role of p80 in retina. On the basis of studies in other tissues, it is suggested that the MARCKS protein cross-links actin in the membrane fraction and that phosphorylation releases it into the cytosol. Interaction with calcium-binding proteins (calmodulin and S100β) disrupts the ability of the MARCKS protein to cross-link actin and prevents its phosphorylation. We therefore anticipated that the phosphorylation of p80 would be more favored in the membrane fraction than in the cytosol. However, as shown in Figure 6, PDB stimulated the phosphorylation of p80 in the cytosol, but not in the membranes. The radioactivity in the presence of PDB (lane 2) was 250% of the control value (lane 1). 8-Bromo analogues of cyclic AMP and cyclic GMP had no influence on p80 phosphorylation in either fraction (data not shown). On the basis of these observations, it appears that p80 is the MARCKS protein, but its functional role in retina remains to be determined.

We then tested the possibility that S100β inhibits p80 phosphorylation by competing with it as a protein kinase substrate. Figure 7 shows a Tris-tricine gel in which the lower-molecular mass proteins are better separated. As is evident, S100β was not phosphorylated when it was added to the phosphorylation assay mixture. Inhibition of the p80 phosphorylation by S100β was therefore not due to competition between two substrates.

Dose-Dependent Inhibition of p80 Phosphorylation by S100β

Figure 8 shows that inhibition of p80 phosphorylation by S100β is dose-dependent. Since S100β is shown to exist as a dimer (*33*, *34*), even at micromolar concentrations (*33*), the molecular mass of the protein was treated as 21 000 Da in calculating its concentration. Ninety percent of the phosphorylation of p80 was inhibited at 20 μM S100β. Half-maximal inhibition was observed at about 3 μM. It might

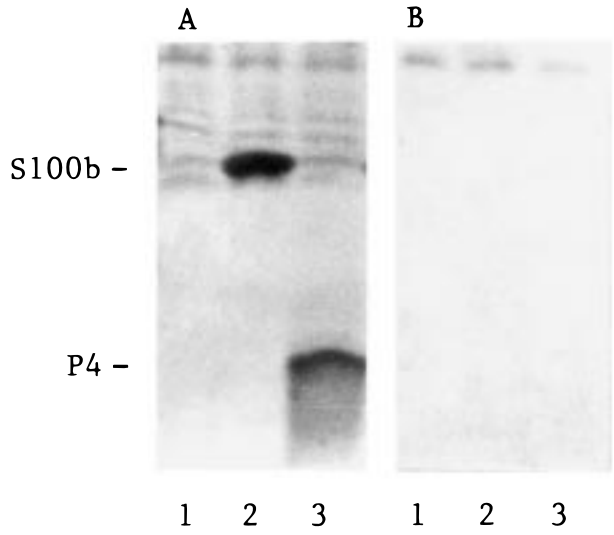

FIGURE 7: S100β and peptide 4 were not labeled in the phosphorylation assays. Membrane proteins were phosphorylated with no additions (lane 1) or in the presence of 6 μM S100β (lane 2) or 300 μM peptide 4 (lane 3). The phosphorylated proteins were separated by SDS−PAGE as in Figure 5 with the exception that a Tris-tricine gel was used so that lower-molecular mass proteins could be better resolved. The portion of the gel with proteins of less than 15 000 Da is shown. Panel A shows the proteins stained with Coomassie Blue; panel B shows the corresponding autoradiogram. S100β and peptide 4 were not phosphorylated. Phosphorylation of higher-molecular mass proteins is shown in Figure 5.

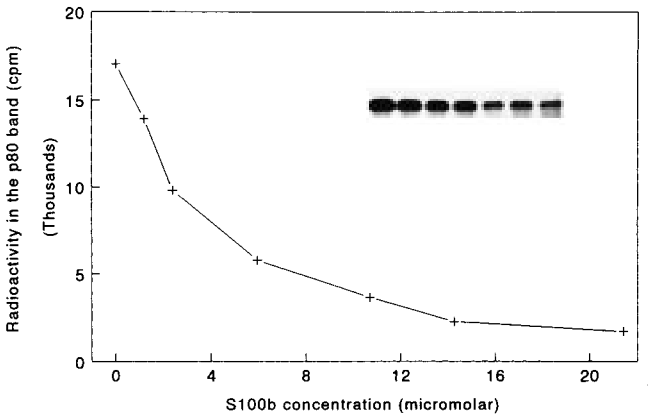

FIGURE 8: Dose-dependent inhibition of p80 phosphorylation by S100β. After identification of the p80 by autoradiography (inset), the bands were excized from the gel and the radioactivity was measured. The data points in the graph correspond to the different lanes in the inset.

be noted here that activation of ROS-GC by S100β was also maximal at about 20 μM and half-maximal at about 2 μM (*11*). These concentrations are in the physiological range since S100 is known to be present in bovine brain at about 12 μM (*54*). The concentration in retina remains to be determined.

Effect of Synthetic Peptides on p80 Phosphorylation

As in the case of ROS-GC, synthetic peptides were tested for their influence on the phosphorylation of p80 and its inhibition by S100β. As shown in Figure 9A, at 300 μM, peptides 1−3 did not affect the phosphorylation, but peptide 4 inhibited it by about 70%, a level of inhibition observed with 10 μM S100β. Peptide 4 itself was not phosphorylated in the incubations (Figure 7), and therefore, inhibition of p80

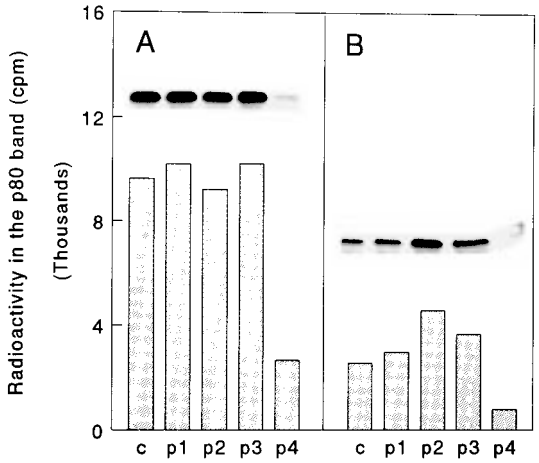

FIGURE 9: Influence of peptides 1−4 on p80 phosphorylation (A) and its inhibition by S100β (B). All the peptides were tested at 300 μM. The concentration of S100β in the assays, when it was present, was 6 μM. c in panel A represents control; c in panel B represents control and S100β. p1−4 are peptides 1−4.

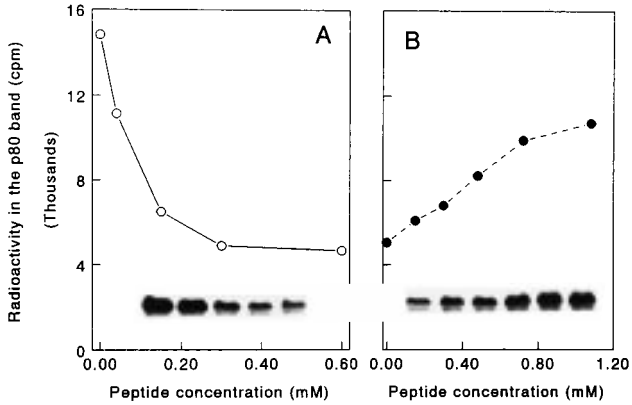

FIGURE 10: Inhibition of p80 phosphorylation at different concentrations of peptide 4 (A) and the reversal of S100β inhibition by different concentrations of peptide 2 (B). The concentration of S100β in assays, when it was present, was 6 μM. p80 was identified by autoradiography (insets). The protein bands were excized from the gel, and the radioactivity was measured.

phosphorylation could not have been due to competition between two substrates.

When added at a final concentration of 300 μM to assays in which the phosphorylation was inhibited by 75% by S100β, peptide 2 relieved 30% of the inhibition, while peptide 4 enhanced the inhibition to a level of >90% (Figure 9B). Peptide 1 had no effect, while peptide 3 decreased the inhibition marginally (as shown in Figure 9B) but not consistently. Peptides 5−8 had no influence on the phosphorylation of p80, or on its inhibition by S100β (data not shown).

Figure 10 shows the concentration dependence of peptide 4 in inhibiting the phosphorylation of p80 and that of peptide 2 in relieving the inhibition caused by S100β. Nearly 70% of the phosphorylation was inhibited by peptide 4, but the inhibition reached a plateau at about 300 μM peptide. Half-maximal inhibition was observed at about 70 μM. Peptide 2 was tested in the presence of 10 μM S100β which normally reduced the phosphorylation by about 70%. As seen in Figure 10, the inhibition was reversed by peptide 2 in a concentration-dependent manner. Nearly 60% of the inhibition was reversed at 1.08 mM peptide. Higher concentrations of peptide were not tested, but it appears that further reduction in the inhibition could be achieved at higher concentrations. Figure 10 also suggests that peptide 2 was less efficient in blocking the inhibition than peptide 4 was in inhibiting it.

As shown above, peptide 4 was most effective in blocking the S100β stimulation of ROS-GC under reducing conditions (in the presence of 10 mM DTT). To test whether reducing conditions were essential for the inhibition of p80 phosphorylation, assays were carried out in the absence and presence of 10 mM DTT. Neither the level of phosphorylation of p80 nor its inhibition by S100β or peptide 4 was influenced by DTT (data not shown).

DISCUSSION

The main goals of this investigation were identifying the site of S100β involved in stimulating the retinal guanylate cyclase, ROS-GC, and determining if the same site interacts with another effector protein from the same tissue, p80, whose phosphorylation is inhibited by S100β. The results indicate that there are two regions in S100β that interact with the effectors investigated. Though the same regions interact with both proteins, there are differences, particularly in the involvement of Cys84.

Binding Sites Involved in Guanylate Cyclase Activation. The investigation was initiated with the anticipation that the synthetic peptides would either mimic or interfere with the effects of S100β. Of the four peptides (1−4) which covered the entire sequence of S100β outside the calcium-binding loops, only peptides 2 and 4 blocked the activation of ROS-GC by S100β. Since peptide 2 contains the sequences of peptides 5 and 6, and nearly the entire sequence of peptide 7, and these peptides had no effect on ROS-GC activation, we conclude that the effectiveness of peptide 2 is not due to sequences covered by these peptides, i.e., the loop region of the hinge. The rest of peptide 2 has the sequence of helix II, and it therefore appears that helix II is involved in the stimulation of guanylate cyclase. Peptide 4 (Phe73−Glu91), which covers the sequences of both helix IV and loop 2, also blocked the activation, but peptide 8 (Phe73−Iso80), whose sequence is entirely covered by peptide 4, was ineffective. This suggests that the effectiveness of peptide 4 is likely due to the residues in the Thr81−Glu91 part of the peptide. It may therefore be concluded that two sites on S100β appear to be involved in the activation of guanylate cyclase, and they are in the regions Leu32−Leu40 and Thr81−Glu91.

Of the two effective peptides, only peptide 4 has a cysteine residue (corresponding to Cys84), and it blocked activation of cyclase more effectively under reducing conditions. This suggests that reduced S100β is a better activator of cyclase, a conclusion supported by our earlier observation (*13*). S100b is a dimer ($\beta\beta$) at the micromolar concentrations used in this study (*33*). The dimer could exist in either an oxidized or reduced state (*33*). Apparently, reducing conditions favor activation of ROS-GC by $\beta\beta$ and its block by the synthetic peptide.

Binding Sites Involved in Inhibition of p80 Phosphorylation. Peptides 1, 3, and 5−8 had no influence either on the phosphorylation of p80 or on its inhibition by S100β. Peptide 2 did not affect phosphorylation but partially reversed

the inhibition by S100β. Peptide 4, however, simulated the effect of S100β in inhibiting the phosphorylation. These results strongly suggest that the regions of S100β corresponding to peptides 2 and 4 are the epitopes involved in regulating the phosphorylation of p80. As in the case of guanylate cyclase, the lack of effect by peptides 5−8 suggests that the effect of peptide 2 could be due to the helix II region and that of peptide 4 due to the Thr81−Glu91 region.

Unlike its effect on guanylate cyclase, DTT had no effect on the phosphorylation of p80, or its inhibition by S100β and peptide 4, suggesting a lack of involvement of Cys84. Baudier et al. (*9*) showed that S100β inhibits the phosphorylation of τ protein and that Cys84, though involved in forming a covalent bond with the protein, is not essential for the inhibition. Recently, Landar et al. (*35*) found that mutation of Cys84 to Ser eliminated S100β's ability to inhibit the phosphorylation of the τ protein. The mutation however did not prevent S100β from activating another effector, phosphoglucomutase, though the calcium dependency of the activation was lost. It also had no influence on S100β activation of aldolase. On the basis of our results and those of Landar et al. (*35*), we conclude that Cys84 is involved in interactions with only specific effector proteins.

While this paper was being written, Kilby et al. (*21*) reported the binding sites on S100β involved in interaction with another effector, the CapZ protein. On the basis of NMR data, these authors suggested that residues Val8, Iso11, and Asp12 in the N-terminal region and residues Ala78, Thr81, Thr82, and Cys84 in the C-terminal region are likely to be involved in the interaction. These residues are found in peptides 1 and 4 of our study. Since peptide 1 had no effect in blocking the activation of cyclase or inhibiting the phosphorylation of p80, the involvement of residues in this region appears to be effector-specific. Also, the consensus S100β-binding sequence in CapZ proteins, TRTKIDWNKILS (*36*), does not have a corresponding homologous sequence in either ROS-GC or the MARCKS protein (which we presume the p80 is), indicating that S100β-binding sequences vary in different effector proteins.

Rustandi et al. (*37*) recently investigated the sites on rat S100β that interact with a peptide derived from p53. p53 is another protein kinase C substrate whose phosphorylation is inhibited by S100β (*38*). The p53 peptide serves as a protein kinase C substrate, just as the parent molecule does, and its phosphorylation is inhibited by S100β (*39*). On the basis of the chemical shifts observed for various residues in the calcium-bound S100β, and the calcium-bound S100β interacting with the p53 peptide, Rustandi et al. suggested that residues Ser41, Leu44, Glu45, Glu46, and Iso47 in the hinge region and Ala83−Phe88 in the C-terminal loop are likely to be involved in effector binding. It is noteworthy that the sequence of the C-terminal region, which we found to be effective in interacting with guanylate cyclase and p80, includes residues Ala83−Phe88. However, it may be recalled that peptide 5, which contained the hinge region residues Leu44−Iso47, was ineffective in blocking the effects of S100β on cyclase or p80.

Our results with guanylate cyclase and p80, the reported observations on CapZ (*21*) and p53 (*37*, *39*) peptides, and other NMR studies (*21*−*24*) together suggest that there are at least two sites on S100β that interact with the effector proteins. Of these, one is in the C-terminal portion of the protein, and it appears to be common to interactions with most effector proteins. The other site could be effector-specific, and located N-terminal with respect to the first EF-hand calcium-binding loop or in some part of the hinge region between the two EF-hand calcium-binding loops. The role of Cys84 also appears to be effector-specific, as it does not appear to be involved in interactions with all effectors.

Significance of S100 Interaction with Guanylate Cyclase. The reported interaction of the S100 protein with calponin (*40*), caldesmon (*41*), capZ (*21*, *36*), and MARCKS proteins (*8*) suggests a possible physiological role for its regulation of ROS-GC. All four of the above are actin-binding proteins. It may be recalled that ROS-GC was thought to be an axoneme-attached protein, very difficult to solubilize with detergent alone (*42*, *43*). Following the discovery that a combination of a nonionic detergent and a molar salt concentration could solubilize the protein (*44*), the enzyme was purified and cloned (*45*−*47*), but the significance of its attachment to cytoskeleton was not further investigated. Recently, Hallett et al. (*48*) provided definitive evidence that ROS-GC is an actin-binding protein. ROS-GC is therefore similar to caldesmon, calponin, capZ, and MARCKS proteins in several properties: (I) its actin-binding ability, (II) its interaction with S100 proteins, and (III) the calcium dependence of the S100−effector interaction. In the case of the other four proteins, calcium-dependent interaction with S100 protein detaches them from actin. The similarities suggest that the S100 protein could also detach ROS-GC from actin. Whether this occurs in rod outer segments or the synaptic layers of retina, where the immunoreactivity of ROS-GC was demonstrated (*49*), remains to be determined. In addition, ROS-GC is a dually regulated enzyme: activated by GCAPs at nanomolar calcium and by S100 proteins at micromolar calcium (*11*, *12*, *50*−*53*). Studies on whether actin binding is influenced by all the calcium-binding proteins that interact with ROS-GC are likely to reveal the physiological significance of such interactions in rod outer segments and the retinal synaptic layers.

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