Modulation of Protein 4.1 Binding to Inside-Out Membrane Vesicles by Phosphorylation†

Tsung-Shu Chao and Mariano Tao*

Department of Biochemistry, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612

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ABSTRACT: The effect of phosphorylation on the binding of protein 4.1 to erythrocyte inside-out vesicles was investigated. Protein 4.1 was phosphorylated with casein kinase A, protein kinase C, and cAMP-dependent protein kinase. An analysis of the phosphopeptides generated by α-chymotryptic and tryptic digestion indicates these kinases phosphorylate similar as well as distinct domains within protein 4.1. All three enzymes catalyze the phosphorylation to varying degrees of the 46-, 16-, and 8–10-kDa fragments derived from limited chymotryptic cleavage. In addition, casein kinase A phosphorylates a 24-kDa domain, whereas protein kinase C phosphorylates a 30-kDa domain. Protein 4.1 phosphorylated by casein kinase A and protein kinase C, but not cAMP-dependent protein kinase, exhibits a reduced binding to KI-extracted inside-out vesicles. On the other hand, phosphorylation of inside-out vesicles by casein kinase A does not affect their ability to bind protein 4.1. The inside-out vesicles, however, inhibit the phosphorylation of protein 4.1 by casein kinase A and protein kinase C, but not by cAMP-dependent protein kinase. These results suggest that casein kinase A and protein kinase C may modulate the binding of protein 4.1 to the membrane by phosphorylation of specific domains of the cytoskeletal protein. Since the 30-kDa domain has been suggested as a membrane-binding site, that phosphorylation by protein kinase C reduces the binding of protein 4.1 to inside-out vesicles is perhaps not surprising. On the other hand, the role of the casein kinase A substrate 24-kDa domain in membrane binding has not been established and needs to be examined. The results further suggest that the 16- and 8–10-kDa domains, which are phosphorylated by all three kinases, are probably not involved in the binding of protein 4.1 to membrane.

The cytoplasmic surface of the human erythrocyte membrane is underlaid with a layer of protein meshwork. This protein meshwork or membrane cytoskeleton is formed by the association of spectrin, actin, protein 4.1, and other minor peripheral membrane proteins including adducin and protein 4.9. The cytoskeleton is attached to the membrane in part by the interactions of protein 4.1 with the integral membrane proteins band 3 and glycophorins and by the linkage of spectrin to band 3 through an ankyrin bridge. The membrane cytoskeleton has been suggested to play an important role in maintaining the biconcave disk shape and rheological properties of the red cell (Chien & Sung, 1990).

There is increasing evidence to indicate that the assembly and dynamics of the membrane cytoskeletal structure may be subject to regulation by protein phosphorylation–dephosphorylation (Lu et al., 1985; Eder et al., 1986; Ling et al., 1988; Husain-Chishti et al., 1988; Danilov et al., 1990). Studies have shown that, with the exception of actin, all of the cytoskeletal proteins, including spectrin, ankyrin, protein 4.1, adducin, band 3, and band 4.9, are either phosphoproteins in nature or substrates for one or more protein kinases (Hosey & Tao, 1976, 1977; Ling et al., 1986, 1988; Boivin, 1988; Waseem & Palfrey, 1988; Husain-Chishti et al., 1988). That phosphorylation might regulate the interactions of the cytoskeletal proteins was first reported in studies with ankyrin (Lu et al., 1985). Phosphorylation of ankyrin by casein kinases isolated from human erythrocytes was found to reduce its affinity for either phosphorylated or unphosphorylated spectrin tetramers, but not spectrin dimers (Lu et al., 1985). In a similar study with protein 4.1, the affinity of this cytoskeletal protein for spectrin was likewise reduced about 5-fold by phosphorylation (Eder et al., 1986). Protein 4.1 is also a substrate for the cAMP-dependent protein kinase and protein kinase C. In vitro phosphorylation of 4.1 by these latter two kinases was found to reduce its ability to promote spectrin binding to actin (Ling et al., 1988). In addition, phosphorylation of protein 4.1 by protein kinase C resulted in a reduction of about 60–70% of the binding capacity of red cell inside-out vesicles for protein 4.1 (Danilov et al., 1990). These inside-out vesicles, which contain only band 3 and the full complement of glycophorins, are stripped of all peripheral membrane proteins by alkaline pH treatment. By contrast, the binding of protein 4.1 to inside-out vesicles was not affected by phosphorylation by the cAMP-dependent protein kinase (Danilov et al., 1990). Recently, Husain-Chishti et al. (1988, 1989) reported that the actin-bundling activity of protein 4.9 was abolished by phosphorylation by the cAMP-dependent protein kinase, but not by protein kinase C. Although spectrin constitutes the major protein of the cytoskeletal meshwork, the phosphorylation of spectrin, on the other hand, was found to be "silent" and exhibited no meaurable effect on its ability to interact with other cytoskeletal proteins (Lu et al., 1985; Eder et al., 1986). Taken together, these data suggest that phosphorylation may provide a mechanism for maintaining a relaxed, flexible cytoskeletal structure that is essential for the normal function of the red cell membrane.

Interestingly, several of the cytoskeletal proteins have been shown to be substrates for multiple kinases (Hosey & Tao, 1976, 1977; Ling et al., 1986, 1988; Husain-Chishti et al., 1988; Waseem & Palfrey, 1988; Chao & Tao, 1991). For example, ankyrin can be phosphorylated both by the cAMP-dependent protein kinase and by casein kinases (Hosey & Tao, 1977), protein 4.1 by protein kinase C, casein kinases, and cAMP-dependent protein kinase (Eder et al., 1986; Ling et

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al., 1988; Chao & Tao, 1991), protein 4.9 by protein kinase C and cAMP-dependent protein kinase (Husain-Chishti et al., 1988), and adducin by protein kinase C and cAMP-dependent protein kinase (Ling et al., 1986; Waseem & Palfrey, 1988). The role of phosphorylation by the various kinases in the regulation of cytoskeletal protein interactions is not fully understood. Since these kinases recognize different phosphorylation sequence motifs (Kemp & Pearson, 1990), they may or may not elicit the same effects on the cytoskeletal structure. In this study, we compare the phosphorylation of protein 4.1 by the red cell casein kinase, the catalytic subunit of bovine heart cAMP-dependent protein kinase, and the bovine brain protein kinase C. The effect of phosphorylation by these kinases on the binding of protein 4.1 to inside-out vesicles that are depleted of peripheral membrane proteins was also examined. Our results indicate that protein 4.1 contains multiple phosphorylation sites and that phosphorylation by the kinases elicits different effects on the binding of protein 4.1 to inside-out vesicles.

Experimental Procedures

Materials. Potassium iodide (KI), phosphatidylserine, 1,2-dioctanoyl-*sn*-glycerol, diisopropyl fluorophosphate (DFP),[1] Triton X-100, aprotinin, α-chymotrypsin (TLCK-treated), and the catalytic subunit of bovine heart cyclic AMP-dependent protein kinase were purchased from Sigma Chemical Co. TPCK-treated trypsin was from Cooper Biochemical Co. Sepacell (R-500A leukocyte removal recipient set) was purchased from Baxter Healthcare Corp. Bolton–Hunter reagent was from ICN Biomedicals, and [γ-^{32}P]ATP was from Amersham Corp. Pepstatin A and leupeptide were from Peptides International. The cytosolic casein kinase A was purified from human erythrocytes as described by Lu et al. (1985), and protein kinase C was prepared from bovine brain as described by Chao and Tao (1991). The cytoplasmic domain of band 3 was prepared as described previously (Soong et al., 1987). Fresh and outdated human red blood cells were obtained from the blood bank of the University of Illinois Hospital.

Preparation of Protein 4.1 and KI-Extracted Erythrocyte Inside-Out Vesicles. Human erythrocytes were filtered through Sepacell to remove leukocytes and other contaminating cell types (Gardner & Bennett, 1986). The erythrocytes were lysed, and the hemoglobin-free ghosts were prepared as described previously (Lu et al., 1985). Protein 4.1 was purified by a procedure modified from that described by Ohanian and Gratzer (1984). Briefly, 250 mL of the ghosts was solubilized by mixing with an equal volume of an ice-cold buffer containing 24 mM Tris-HCl, pH 7.5, 15% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.5 mM mercaptoethanol, and 0.4 mM DFP. Portions of 15 mL of this mixture were layered over a buffer containing 24 mM Tris-HCl, pH 7.5, 30% sucrose, 0.6 M KCl, 0.5 mM mercaptoethanol, 1 mM EDTA, and 1 mM EGTA and centrifuged at 20000 rpm for 60 min in a Beckman JA-20 rotor. The clear, gellike cytoskeleton pellets (composed primarily of spectrin, actin, and proteins 4.1 and 4.9) were dissolved in an equal volume of 2 M Tris-HCl, pH 7.5, and centrifuged at 45000 rpm in a Beckman 55.2 Ti rotor for 30 min to remove undissolved debris. The supernatant was dialyzed at 4 °C against a solution containing 1 mM EDTA (pH 7.5), 0.02% NaN_3, 0.5 mM mercaptoethanol, and 20 mM KCl in order to reduce the Tris-HCl concentration to about 20 mM. The dialyzed cytoskeleton solution was applied to a Whatman DE-52 column (3.2 × 12.5 cm) that had been equilibrated with 20 mM Tris-HCl, pH 7.5, 20 mM KCl, 1 mM EDTA, 0.02% NaN_3, 0.5 mM mercaptoethanol, and 0.4 mM DFP (buffer A). The column was eluted with a linear gradient of 20–400 mM KCl in buffer A. The fractions eluted from the column were analyzed by electrophoresis on an 8% SDS–polyacrylamide gel (Laemmli, 1970). The protein 4.1 eluted at KCl concentrations between 80 and 150 mM was about 98% pure and was used without further purification.

The KI-extracted inside-out vesicles (KI-IOV) were prepared essentially according to the method of Bennett (1983).

Membrane protein concentrations were estimated by the method of Lowry (1951) using bovine serum albumin as a standard.

Protein 4.1 Phosphorylation. The standard assays for the phosphorylation of protein 4.1 by protein kinase C, the catalytic subunit of cAMP-dependent protein kinase, and erythrocyte casein kinase A were conducted as described earlier (Chao & Tao, 1991). Briefly, the phosphorylation of protein 4.1 by protein kinase C was carried out at 30 °C for 60 min in 100 μL of a reaction mixture containing 20 mM Hepes–OH (pH 7.5), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.1 mM [γ-^{32}P]-ATP, 0.5–1 unit/mL of protein kinase C, freshly sonicated lipids (50 μg/mL phosphatidylserine and 5 μg/mL 1,2-dioctanoylglycerol), and protein 4.1. Phosphorylation by casein kinase A and the catalytic subunit of cAMP-dependent protein kinase was performed in a reaction mixture containing 50 mM Tris-HCl (pH 7.5 or 8.5), 5 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP, and 2 μg/mL each of the protease inhibitors, leupeptin, pepstatin A, and aprotinin, and protein 4.1.

The reactions were terminated by adding an electrophoresis sample buffer to the reaction mixtures to a final concentration of 2% SDS, 20 mg/mL dithiothreitol, 1 mM EDTA, 6% sucrose, and 0.01% bromophenol blue. The entire incubation mixture was subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). The ^{32}P-labeled protein 4.1 was identified by autoradiography and quantified by scintillation counting of the protein band excised from the gel.

Phosphorylation of Protein 4.1 in the Presence of KI-IOV. Purified protein 4.1 (4 μg) was preincubated at 24 °C for 3 h with 6.8, 13.6, and 20.4 μg of KI-IOV in isotonic KCl/NaCl "binding buffer" containing 10 mM Tris-HCl, pH 7.5, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM mercaptoethanol, 0.02% NaN_3, and protease inhibitors (0.4 mM DFP and 2 μg/mL each of leupeptin, pepstatin A, and aprotinin). For the casein kinase A catalyzed phosphorylation, the 130 mM KCl/20 mM NaCl in the binding buffer was replaced with 20 mM KCl. As reported earlier by Eder et al. (1986), the phosphorylation of protein 4.1 by casein kinase A was inhibited by high concentrations of salt. Phosphorylation was initiated by adding 10 μL of a "cocktail" containing protein kinase, Mg^{2+}, [γ-^{32}P]ATP, and activators (for protein kinase C) to 50 μL of preincubation mixtures. After incubation at 30 °C for 10 min, protein 4.1 phosphorylation was analyzed by subjecting the entire phosphorylation mixture to SDS–polyacrylamide gel electrophoresis on an 8% gel followed by autoradiography of the dried gels. The bands corresponding to band 4.1 were also excised from the gel and counted in a liquid scintillation counter in order to quantify the radioactivities incorporated.

Assay of ^{125}I-Labeled Protein 4.1 Binding to KI-IOV. Phosphorylated and unphosphorylated proteins 4.1 were radioiodinated on ice for 90 min in a reaction mixture containing

[1] Abbreviations: DFP, diisopropyl fluorophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; KI-IOV, potassium iodide extracted inside-out vesicles; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

0.5 mCi of ^{125}I-labeled Bolton–Hunter reagent, 0.16 M sodium borate, pH 8.5, 250 mM NaCl, and 0.5–1.0 mg of protein 4.1 in a final volume of 200 μL (Eder et al., 1986). The unreacted ^{125}I was removed by dialysis followed by repeated washings with isotonic binding buffer in an Amicon Centricon. The binding assay was conducted by incubating varying amounts of ^{125}I-labeled protein 4.1 with 16 μg of KI-IOV at 24 °C for 1 h in isotonic KCl/NaCl binding buffer. The entire (100 μL) incubation mixtures were layered over a cushion of 100 μL of 8% sucrose prepared in the binding buffer and centrifuged for 15 min at 12000 rpm in an Eppendorf microfuge. The amount of ^{125}I-labeled protein 4.1 bound to the pelleted vesicles was determined in a γ counter. Nonspecific binding to IOV was determined using heat-denatured (85 °C for 30 min) protein 4.1.

Limited Cleavage of Protein 4.1 by α-Chymotrypsin. Purified protein 4.1 (20 μg) was phosphorylated with [γ-^{32}P]ATP as described above except in the absence of protease inhibitors. The ^{32}P-labeled protein 4.1 was digested on ice for 30 min with 0.4 μg of TLCK-treated α-chymotrypsin, corresponding to an enzyme to substrate weight ratio of 1 to 50. The proteolysis was terminated by the addition of 1 mM DFP. As shown by Leto and Marchesi (1984), characteristic chymotryptic peptide fragments were generated under these limited digestion conditions. The peptide fragments were resolved by gel electrophoresis on a 15% SDS gel, and the phosphopeptides were localized by autoradiography.

Two-Dimensional Cellulose Peptide Mapping. Protein 4.1 (40 μg) was phosphorylated as described above. Following electrophoresis on an 8% SDS–polyacrylamide gel, the ^{32}P-labeled protein 4.1 band was excised from the gel, dried, and digested at 37 °C for 24 h with 0.5 mL of 50 μg/mL TPCK-treated trypsin in 10 mM Tris-HCl, pH 8.0. The supernatant of the digest was lyophilized, and the residue was dissolved in 50 μL of an electrophoresis solvent containing acetic acid/formic acid/water, 15:5:80 (v/v). Two-dimensional peptide mapping was carried out as described by Speicher et al. (1982). Briefly, aliquots of 2.5–5 μL of the dissolved digest containing (1–5) × 10^3 cpm were spotted on a cellulose-coated thin-layer sheet (20 × 20 cm; Kodak, 13255 cellulose, without fluorescent indicators) and electrophoresed in the above solvent at 4 °C on a Gelman flat-bed electrophoresis chamber at 900 V for 60 min. The sheet was dried and developed in the second dimension in butanol/pyridine/acetic acid/water, 32.5:25:5:20 (v/v), using a Kodak Chromagram developing apparatus. The autoradiogram was developed by exposing the cellulose thin-layer sheet to a Kodak X-Omat AR film in the presence of an intensifying screen at −80 °C.

Phosphoamino Acid Analysis. The ^{32}P-labeled protein 4.1 was digested with trypsin as described above. The tryptic digest was hydrolyzed in 0.5 mL of 6 N HCl at 105 °C for 1.5 h. The [^{32}P]phosphoamino acids were dissolved and separated by electrophoresis at pH 1.9 in formic acid/acetic acid/H_2O, 25:78:897 (v/v), as described earlier (Yan & Tao, 1982).

Results

Phosphorylation Domains of Protein 4.1. Since protein 4.1 is a multiphosphorylated protein and serves as a substrate for several red cell kinases, it is of interest to determine whether these kinases phosphorylate the same or different domains of protein 4.1 Protein 4.1 was phosphorylated with human erythrocyte casein kinase A, the catalytic subunit of bovine heart cAMP-dependent protein kinase, or bovine brain protein kinase C. The ^{32}P-labeled protein 4.1 was digested with proteolytic enzymes, and the phosphorylation domains were characterized either by SDS–polyacrylamide gel electrophoresis or by two-dimensional phosphopeptide mapping. The cleavage pattern of protein 4.1 by mild α-chymotryptic digestion has been analyzed by Leto and Marchesi (1984). Mild digestion with α-chymotrypsin cleaves protein 4.1 at three highly sensitive sites and generates a limited number of intermediate-sized peptide fragments consisting of 46, 30, 24, 16, and 10–8 kDa. The 30- and 16-kDa fragments are derived from the 46-kDa fragment. The order of alignment of these peptide fragments from amino to carboxyl terminal is 30, 16, 10, and 24 kDa (Leto et al., 1986).

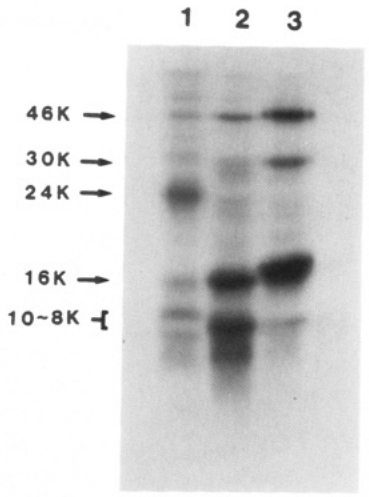

FIGURE 1: Limited proteolysis of ^{32}P-labeled protein 4.1 by α-chymotrypsin. Purified protein 4.1 (20 μg) was phosphorylated with casein kinase A (lane 1), cAMP-dependent protein kinase (lane 2), and protein kinase C (lane 3) as described under Experimental Procedures. The specific activity of [γ-^{32}P]ATP used was 250 cpm/pmol. The ^{32}P-labeled protein 4.1 was digested on ice for 30 min with α-chymotrypsin using an enzyme to substrate weight ratio of 1 to 50. The phosphopeptides were separated on an SDS–polyacrylamide gel, and the autoradiogram was developed from a Kodak X-OMAT film that had been exposed for 24 h to the dried gel with the aid of an intensifying screen.

An analysis of the labeling patterns of the chymotryptic peptides resolved by SDS–polyacrylamide gel electrophoresis indicates that there are significant differences in the phosphorylation of protein 4.1 by casein kinase A, cAMP-dependent protein kinase, and protein kinase C. Figure 1 (lane 1) shows that casein kinase A phosphorylates primarily a domain which migrates in the 24-kDa region with additional minor labeling seen in the 46-, 16-, and 10–8-kDa peptides. The catalytic subunit of the cAMP-dependent protein kinase phosphorylates sites within the 46-, 16-, and 10–8-kDa peptides with more intense labeling in the latter two regions (lane 2). This cAMP-dependent protein kinase phosphorylation pattern is in general agreement with that observed earlier by Horne et al. (1985). With protein kinase C, the primary ^{32}P-labeled peptide is the 16-kDa fragment with labels also found in the 46- and 30-kDa fragments (lane 3). The phosphorylation of the 30-kDa fragment was not observed in protein 4.1 isolated from erythrocytes treated with 12-*O*-tetradecanoyl phorbol 13-acetate (Horne et al., 1985).

The possibility that the three kinases might phosphorylate different domains of protein 4.1 was further examined by two-dimensional peptide mapping. The ^{32}P-labeled protein 4.1 was digested extensively with trypsin. The results presented in Figure 2 show that the phosphopeptide map of protein 4.1 phosphorylated by casein kinase A (Figure 2A) is significantly different from that phosphorylated by the cAMP-dependent protein kinase (Figure 2B) and protein kinase C (Figure 2C).

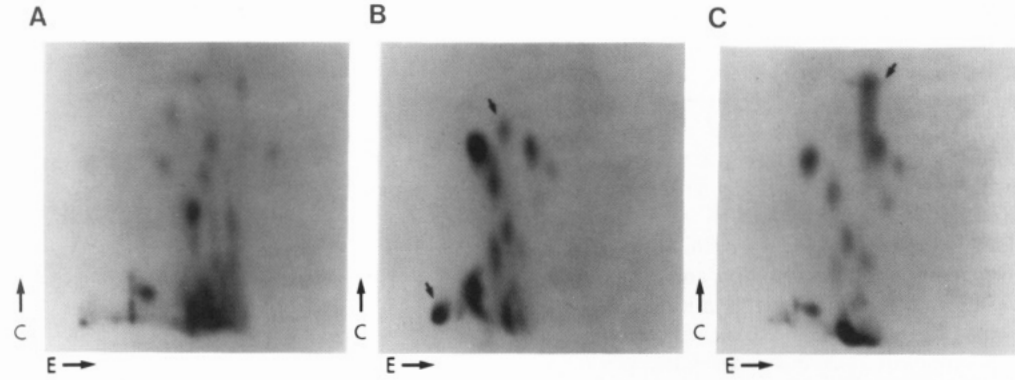

FIGURE 2: Autoradiograms of two-dimensional tryptic phosphopeptide maps of protein 4.1. Phosphorylation of protein 4.1 (40 μg) by casein kinase A (panel A), cAMP-dependent protein kinase (panel B), or protein kinase C (panel C), with [γ-^{32}P]ATP (300 cpm/pmol), digestion with trypsin, and two-dimensional phosphopeptide mapping were carried out as described under Experimental Procedures. Autoradiograms were developed for 24–36 h as described in the legend of Figure 1.

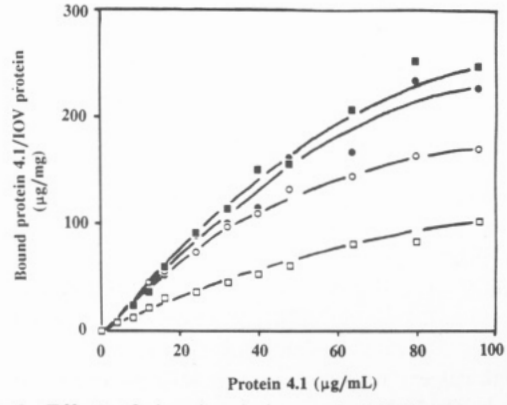

FIGURE 3: Effects of phosphorylation on the binding of protein 4.1 to KI-IOV. Unphosphorylated protein 4.1 (●) and protein 4.1 phosphorylated by casein kinase A (○), cAMP-dependent protein kinase (■), or protein kinase C (□) were radioiodinated with Bolton–Hunter reagent. The binding of ^{125}I-labeled protein 4.1 to KI-IOV was measured as described under Experimental Procedures.

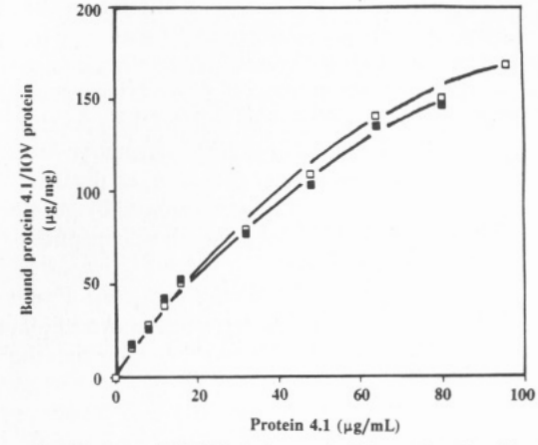

FIGURE 4: Binding of ^{125}I-labeled protein 4.1 to phosphorylated and unphosphorylated KI-IOV. Phosphorylation of KI-IOV by casein kinase A was conducted at 30 °C for 90 min in a reaction mixture (5 mL) containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM ATP, 2 units/mL kinase, and 2.4 mg of KI-IOV. The phosphorylated KI-IOV was pelleted by centrifugation, washed, and resuspended in 2 mL of the binding buffer. The binding of ^{125}I-labeled protein 4.1 to unphosphorylated control (■) or phosphorylated (□) KI-IOV was measured as described under Experimental Procedures.

On the other hand, the phosphopeptide map of protein 4.1 phosphorylated by the cAMP-dependent protein kinase exhibited only minor differences (indicated by arrows) from that phosphorylated by protein kinase C.

Phosphoamino acid analyses indicated that both cAMP-dependent protein kinase and protein kinase C phosphorylated primarily seryl residues of protein 4.1 whereas a significant incorporation into threonyl residues, in addition to seryl residues, was observed with casein kinase A (data not shown).

Effect of Phosphorylation on the Binding of Protein 4.1 to KI-IOV. Protein 4.1 has multiple functions in the cytoskeletal meshwork. In addition to promoting the tight association of spectrin to actin, it also serves to anchor the cytoskeleton to the membrane. This latter function of protein 4.1 is attributed to its ability to bind band 3 (Pasternack et al., 1985), glycophorins (Anderson & Lovrein, 1984; Mueller, 1984), and phospholipids (Sato & Ohnishi, 1983; Cohen et al., 1988). The possibility that the various kinases might regulate this anchoring activity of protein 4.1 was examined using KI-IOV. The KI-extracted inside-out vesicles are depleted of peripheral membrane proteins. The proteins that remain are band 3 and the full complement of sialoglycoproteins (Bennett, 1983). Figure 3 shows the binding of phosphorylated and unphosphorylated protein 4.1 to inside-out vesicles. Our results confirmed an earlier report by Danilov et al. (1990), who showed that protein 4.1 phosphorylated by protein kinase C exhibited a reduced binding to stripped inside-out vesicles, while that phosphorylated by the cAMP-dependent protein kinase exhibited the same binding capacity as the unphosphorylated control. As shown in Figure 3, the binding capacity of protein 4.1 phosphorylated by protein kinase C to IOV was reduced by about 60% as compared to the binding of unphosphorylated protein 4.1. In addition, we also observed a reduction of 25–30% in the apparent binding of protein 4.1 phosphorylated by the erythrocyte casein kinase A to stripped inside-out vesicles.

Since band 3 and glycophorins are substrates of casein kinase A (Simkowski & Tao, 1980), it is of interest to determine whether phosphorylation of these integral membrane glycoproteins affects the ability of IOV to bind ^{125}I-labeled protein 4.1. The results presented in Figure 4 show that phosphorylated and unphosphorylated IOV exhibit the same binding capacity for protein 4.1. Preliminary estimate indicates that the phosphorylation reaction results in the incorporation of about 0.5 mol of phosphate per mole of band 3 in IOV. Although the level of incorporation is significant, there remains the possibility that it may not be sufficient to elicit an effect on the binding of protein 4.1. Similar experiments were not conducted with the cAMP-dependent protein kinase and protein kinase C since both these kinases did not appear to phosphorylate band 3 and glycophorins.

In a separate study, we also examined the binding of phosphorylated and unphosphorylated ^{125}I-labeled protein 4.1 to phosphorylated IOV. The result obtained was essentially

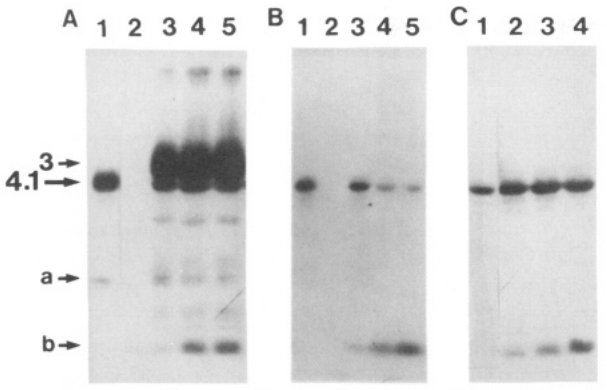

FIGURE 5: Autoradiograms showing the effects of KI-IOV on the phosphorylation of protein 4.1. The phosphorylation of protein 4.1 by casein kinase A (panel A), protein kinase C (panel B), and cAMP-dependent protein kinase (panel C) in the absence and presence of KI-IOV was carried out as described under Experimental Procedures. Lane 1 of panels A–C, purified protein 4.1 (4 μg) was phosphorylated in the absence of KI-IOV; lane 2 of panel A, KI-IOV was incubated with the phosphorylation reaction mixture in the absence of added exogenous kinase; lane 2 of panel B, the phosphorylation reaction mixture contained 1 mM EGTA but no protein kinase C activators (phosphatidylserine, diacylglycerol, and Ca^{2+}); lanes 3–5 of panels A and B and lanes 2–4 of panel C contained 6.8, 13.6, and 20.4 μg of KI-IOV, respectively.

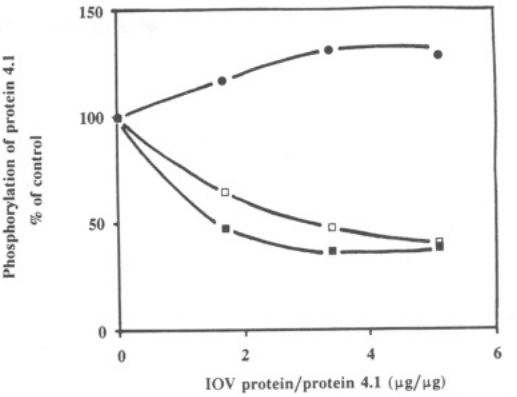

FIGURE 6: Effects of KI-IOV on the phosphorylation of protein 4.1. The phosphorylation of protein 4.1 by casein kinase A (■), protein kinase C (□), and cAMP-dependent protein kinase (●) in the absence (control) and presence of varying amounts of KI-IOV was further quantified by excising the ^{32}P-labeled protein 4.1 band from the gel shown in Figure 5 and counting in a scintillation spectrometer. The data are expressed as percent of the control.

the same as that shown in Figure 3 for the binding of casein kinase A phosphorylated protein 4.1 to unphosphorylated IOV. In an earlier report, we showed that phosphorylation of the cytoplasmic domain of band 3 did not affect its ability to bind ankyrin (Soong et al., 1987). Taken together, these data suggest that the interaction of the cytoskeleton with the membrane may be regulated by phosphorylation of the membrane-anchoring proteins, ankyrin and protein 4.1, but not by phosphorylation of the integral membrane proteins.

Effect of KI-IOV on the Phosphorylation of Protein 4.1. The possibility that association of protein 4.1 with stripped IOV might influence its ability to serve as phosphoryl acceptor in the kinase-catalyzed reaction was investigated. Protein 4.1 was preincubated with varying amounts of KI-IOV at room temperature for 3 h prior to the addition of kinase and initiation of the phosphorylation reaction. The amounts of IOV used were adjusted to give molar ratios of band 3 to protein 4.1 of 1, 2, and 3, on the basis of the assumption that band 3 constituted about 70% of the total IOV proteins. The incorporation of ^{32}P into protein 4.1 was analyzed by autoradiography following resolving the proteins in the incubation mixture by SDS–polyacrylamide gel electrophoresis. Figure 5 (panel A) shows the phosphorylation of protein 4.1 by casein kinase A in the absence and presence of increasing amounts of KI-IOV. In addition to protein 4.1, several proteins derived from the inside-out vesicles were also phosphorylated. This is to be expected as band 3 and glycophorins have been identified previously as substrates of casein kinase A (Simkowski & Tao, 1980). The band designated a represents the autophosphorylated casein kinase A, whereas that designated b is probably labeled phospholipids (Birchmeier & Singer, 1977). The phosphorylation of protein 4.1 was significantly reduced in the presence of IOV. Although human erythrocyte membrane contains endogenous kinase activities that can catalyze the phosphorylation of cytoskeletal proteins (Hosey & Tao, 1976), such activities are not present in the IOV preparations. As shown in Figure 5 (panel A, lane 2), phosphorylation of protein 4.1 and IOV-associated proteins was not observed in the absence of exogenous kinase.

Incubation with stripped IOV also resulted in a reduction of phosphorylation of protein 4.1 by protein kinase C (Figure 5, panel B). The phosphorylation of protein 4.1 was dependent on the presence of kinase C activators (panel B, lane 2). The addition of IOV, however, did not exhibit a similar inhibition of protein 4.1 phosphorylation by the cAMP-dependent protein kinase (Figure 5, panel C). On the contrary, a slight enhancement of phosphorylation was observed. Figure 5, panels B and C, also showed that none of the integral membrane proteins associated with IOV were phosphorylated by protein kinase C or the cAMP-dependent protein kinase.

The incorporation of ^{32}P into protein 4.1 in the absence and presence of varying amounts of IOV was also quantified by scintillation counting of the labeled protein band excised from the gel. The results presented in Figure 6 showed that the phosphorylation of protein 4.1 by protein kinase C and casein kinase A was reduced by about 60% at an IOV protein concentration 5 times that of protein 4.1. Since both band 3 and glycophorins are substrates of casein kinase A, the reduction in protein 4.1 phosphorylation could in part arise from substrate competition by these integral membrane proteins for casein kinase A activity. On the other hand, the inhibition of protein 4.1 phosphorylation by protein kinase C could not be the result of substrate competition since neither band 3 nor glycophorins were substrates of this kinase. Somewhat surprisingly, the phosphorylation of protein 4.1 by the cAMP-dependent protein kinase showed a slight increase (about 30%) in the presence of IOV. The reason for this increase is not known, although the binding of protein 4.1 to IOV could conceivably uncover new potential phosphorylation site(s).

Effects of the Cytoplasmic Domain of Band 3 on the Phosphorylation of Protein 4.1. Band 3 is an integral membrane glycoprotein that plays an important role in anchoring the cytoskeletal meshwork to the membrane by binding to ankyrin. In addition, band 3 serves as the major anion channel of erythrocytes (Knauf, 1979) and as a binding site for proteins 4.1 and 4.2, hemoglobin, and several glycolytic enzymes (Murthy et al., 1981; Haest, 1982; Cassoly & Salhany, 1983; Walder et al., 1984; Bennett, 1985; Harrison et al., 1991). Since band 3 represents the major protein in the stripped IOV, it is of interest to determine whether the binding of protein 4.1 to band 3 can in part account for the observed inhibition of phosphorylation of protein 4.1 by IOV. To examine this possibility, the cytoplasmic domain of band 3 was purified from acid-stripped, spectrin-depleted vesicles by cleavage with α-chymotrypsin (Bennett, 1983; Soong et al., 1987). The purified 43-kDa cytoplasmic domain of band 3 has been shown to bind

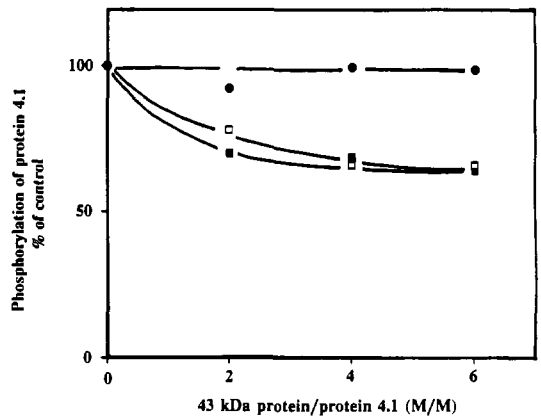

FIGURE 7: Effects of the cytoplasmic 43-kDa domain of band 3 on the phosphorylation of protein 4.1. Purified protein 4.1 (4 μg) was incubated in the absence and presence of 4.2, 8.4, and 12.6 μg of 43-kDa fragment for 1 h prior to phosphorylation by casein kinase A (■), protein kinase C (□), or cAMP-dependent protein kinase (●). The phosphorylation was conducted at 30 °C for 10 min, and the reaction mixture was electrophoresed on a 10% SDS–polyacrylamide gel. The ^{32}P-labeled protein 4.1 band was excised from the gel and the radioactivity determined by scintillation counting.

protein 4.1 and ankyrin (Pasternack et al., 1985; Bennett, 1985). Figure 7 shows the effect of the 43-kDa fragment on the phosphorylation of protein 4.1 by casein kinase A, cAMP-dependent protein kinase, and protein kinase C. Prior to initiation of phosphorylation, protein 4.1 was preincubated at 24 °C for 1 h in the presence of a 2–6-fold molar excess of the cytoplasmic domain of band 3. The results showed that phosphorylation of protein 4.1 by casein kinase A and protein kinase C was inhibited about 35% in the presence of a 6-fold molar excess of the 43-kDa cytoplasmic domain of band 3. By contrast, the phosphorylation of protein 4.1 by the cAMP-dependent protein kinase was not affected by the cytoplasmic domain of band 3.

Although the inhibition of casein kinase A catalyzed phosphorylation of protein 4.1 could also be due to substrate competition by band 3, the inability of higher concentrations of band 3 to extract a greater degree of inhibition suggested that only those potential phosphorylation sites that interact with band 3 were blocked. Thus, those sites which were not involved in band 3 binding might remain available for phosphorylation by casein kinase A. This possibility may explain why the inhibition of protein 4.1 phosphorylation at high concentrations of band 3 levels off at about 35% since not all phosphorylation sites are involved in binding to band 3.

Discussion

Mild digestion of protein 4.1 by α-chymotrypsin leads to the cleavage of the molecule at the three most susceptible sites, generating four peptide fragments with molecular masses of 30, 16, 10, and 24 kDa. The alignment of these peptides according to Leto et al. (1986) is shown in Figure 8. The amino-terminal 30-kDa domain is very hydrophobic, whereas the carboxy-terminal 24-kDa domain is quite acidic (Conboy et al., 1986). A comparison of phosphopeptide maps of protein 4.1 phosphorylated by casein kinase A, cAMP-dependent protein kinase, and protein kinase C indicates that these kinases phosphorylate distinct sites within protein 4.1 (Figure 8). The amino acid sequence of protein 4.1 has recently been deduced from its cDNA nucleotide sequence (Conboy et al., 1986), and the sites phosphorylated by the cAMP-dependent protein kinase have been identified by Horne et al. (1990) to be Ser-331 and Ser-467 located in the 16-kDa and 10-kDa chymotryptic fragments, respectively. We have essentially

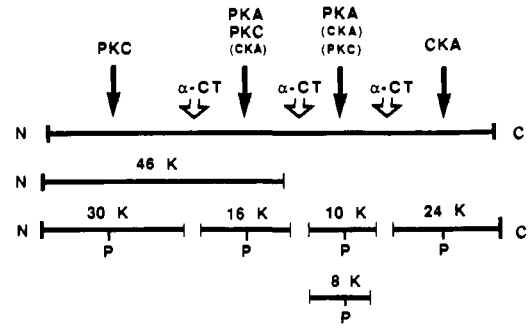

FIGURE 8: Alignment of chymotryptic peptides of protein 4.1 and phosphorylation sites. The chymotryptic peptides are aligned from N- to C-terminal according to Leto et al. (1986). The kinases enclosed in parentheses designate minor phosphorylation sites of these kinases. α-CT, α-chymotrypsin; PKA, cAMP-dependent protein kinase; CKA, casein kinase A; PKC, protein kinase C.

confirmed the observation that the cAMP-dependent protein kinase phosphorylation sites are localized within the 16- and 10-kDa domains and that seryl residues are the primary sites of phosphorylation. On the other hand, the major phosphorylation site(s) of casein kinase A is(are) located in the acidic C-terminal 24-kDa fragment. In addition, casein kinase A may also phosphorylate sites within the 16- and 10-kDa domains. The phosphopeptide map of purified protein 4.1 phosphorylated by protein kinase C differs somewhat from that of protein 4.1 labeled in the intact cell in the presence of 12-*O*-tetradecanoyl phorbol 13-acetate (Horne et al., 1985). In addition to the major site(s) within the 16-kDa domain identified earlier by Horne et al. (1985), significant phosphorylation has been detected in the 30-kDa fragment. Closer examination of the labeling pattern indicates that the 10-kDa domain may also contain phosphorylation site(s) for protein kinase C, though phosphorylation of this domain appears minor.

As noted earlier, protein 4.1 has two major functions: (1) to promote tight binding of spectrin to actin; (2) to anchor the cytoskeleton to the membrane by binding to integral membrane proteins or phospholipids. The membrane-binding function has been attributed to the amino-terminal 30-kDa domain whle the spectrin–actin-binding site to the 10-kDa domain or the 8-kDa domain derived from it (Leto et al., 1986; Conboy et al., 1986; Correas et al., 1986). In this study, we show that phosphorylation of protein 4.1 by protein kinase C reduces its binding to stripped IOV. Since the 30-kDa domain is a substrate of protein kinase C, it is tempting to speculate that phosphorylation of this domain may modulate the interaction of protein 4.1 with the membrane. Thus, cAMP-dependent protein kinase fails to modulate the binding of protein 4.1 to IOV due to its inability to phosphorylate the 30-kDa domain. Somewhat more difficult to evaluate is the result obtained with casein kinase A. As shown in Figure 3, phosphorylation of protein 4.1 by casein kinase A also exhibits a slightly reduced binding to IOV. The major site(s) of phosphorylation by this kinase is(are) the carboxyl-terminal 24-kDa domain, which raises the possibility that this domain may also participate in or contribute to membrane binding. Alternatively, it is conceivable that the effect of casein kinase A phosphorylation on membrane binding may be indirectly due to conformational changes in protein 4.1. The ability of casein kinase A, cAMP-dependent protein kinase, and protein kinase C to phosphorylate the 10-kDa domain may account for the attenuation of binding of protein 4.1 to spectrin or to spectrin–actin complex (Eder et al., 1986; Ling et al., 1988). The 16-kDa domain is the major substrate of the cAMP-dependent

protein kinase and one of the major phosphorylation sites of protein kinase C. Since phosphorylation of protein 4.1 by the cAMP-dependent protein kinase fails to elicit an effect on its binding to IOV, it suggests that the 16-kDa domain is probably not involved in membrane binding. This notion is contrary to the earlier suggestion by Danilov et al. (1990) that the 16-kDa domain may contain regulatory sites for band 3 binding.

Since the inhibition of IOV binding appears to correlate with the phosphorylation of specific membrane-binding domains of protein 4.1, this raises the possibility that the site(s) of phosphorylation may either be directly involved in membrane binding or be adjacent to the binding site. Thus, binding to the membrane could conceivably mask some of the phosphorylation sites and render them inaccessible to phosphorylation by kinases. This notion has been borne out by our observation that IOV (Figure 6) and band 3 (Figure 7) partially inhibit the phosphorylation of protein 4.1 by casein kinase A and protein kinase C, but not by the cAMP-dependent protein kinase. The extent of inhibition is somewhat influenced by the length of preincubation of protein 4.1 with IOV, suggesting that binding to IOV may account for the inhibitory effect (data not shown). That only the phosphorylation by casein kinase A and protein kinase C is inhibited is interesting and is consistent with the observation that these two kinases regulate the binding of protein 4.1 to membrane (Figure 3).

Although the physiological significance of protein phosphorylation in the regulation of cytoskeletal protein interactions remains uncertain, there is evidence suggesting that phosphorylation of membrane proteins may contribute to the deformability and mechanical strength of red cells (Youthavong & Limpaiboon, 1987). Since phosphorylation in general has been shown to decrease the interactions of the cytoskeletal proteins, this could lead to a more flexible, relaxed cytoskeletal structure that may be important for red cell shape maintenance and deformability (Chien & Sung, 1990). The ability of multiple kinases to catalyze the phosphorylation of proteins, such as ankryin and protein 4.1, at strategic junctions of the cytoskeletal network could provide multiple inputs and an effective mechanism for the regulation of the network.

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