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Phosphorylation Reduces the Affinity of Protein 4.1 for Spectrin†

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ABSTRACT: The phosphorylation of protein 4.1 by the membrane kinase and casein kinase A has been investigated. Each of these kinases catalyzed the incorporation of 2 mol of phosphate per mole of protein 4.1. The presence of both kinases in the reaction mixture did not lead to an increase in the incorporation of phosphates into the protein. An analysis of the acid hydrolysis products of the ^{32}P-labeled protein 4.1 indicated that the radioactivities were distributed between phosphothreonine and phosphoserine in a ratio of about 2 to 1. The effects of phosphorylation on the binding of protein 4.1 to spectrin were investigated by using sucrose density gradient centrifugation. The affinity of protein 4.1 for spectrin was reduced about 5-fold, from a K_D of 2×10^{-6} M to a K_D of 9.4×10^{-6} M, by phosphorylation. The phosphorylation of spectrin, on the other hand, appeared to increase slightly its affinity for protein 4.1. The results suggest that phosphorylation may lead to a relaxation of the cytoskeletal network and the formation of a more flexible membrane structure that is important to red cell function.

The human erythrocyte membrane contains an extensive cytoskeletal network that has been suggested to play an important role in the control of cell shape and deformability and the distribution of intramembrane particles and surface receptors (Marchesi, 1979, 1983; Branton et al., 1981; Tao & Conway, 1982). Detailed investigations of the composition, structure, and assembly of this membrane cytoskeletal network have shown that the network is formed by the interactions of three membrane proteins, spectrin, actin, and band 4.1 (Branton et al., 1981; Marchesi, 1983). Spectrin, the major protein of the erythrocyte membrane skeleton, is composed of two nonidentical subunits, α (M_r 240000) and β (M_r 220000), which associate to form double-stranded, fiberlike flexible heterodimers (Branton et al., 1981). These spectrin dimers can further assemble in a head to head arrangement into tetramers and higher oligomers (Morrow et al., 1981; Marchesi, 1983). The tetrameric form has been suggested to represent the major species of spectrin and the physiological functional unit in normal red cell ghosts (Liu & Palek, 1980;

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Ji et al., 1980). However, the possibility that spectrin oligomers may also be present in the cytoskeletal network has been raised (Marchesi, 1983). This latter notion is based on the perception that the local concentration of spectrin in the membrane is high enough (estimated to be about 10^{-4} M) to favor the formation of higher oligomers (Morrow et al., 1981; Marchesi, 1983). The actin in the membrane cytoskeleton is in the form of short filaments linked to one another by binding to the distal ends of the bivalent spectrin tetramers (Cohen, 1983). The interaction between spectrin and F-actin is markedly enhanced, as well as stabilized, by protein 4.1 (M_r 78 000–80 000), which also binds to the tail ends of spectrin tetramers (Fowler & Taylor, 1980; Pinder et al., 1984). Recent studies indicate that protein 4.1 may also interact directly with the membrane in the absence of peripheral membrane proteins (Anderson & Lovrein, 1984; Shiffer & Goodman, 1984; Pasternack et al., 1985). At least two membrane binding sites for protein 4.1 have been identified: one is band 3 (Pasternack et al., 1985), and the other is glycophorin A (Anderson & Lovrein, 1984).

Both spectrin and 4.1 are phosphorylated. The phosphorylation of spectrin and the physiological significance of this reaction have been studied extensively [for a review, see Marchesi (1983)]. So far, there is no convincing evidence to indicate that phosphorylation affects the function of spectrin. Our recent study likewise shows that phosphorylation of spectrin does not alter its affinity for ankyrin (Lu et al., 1985). Although 4.1 has been recognized quite early as a phosphoprotein and a component of the cytoskeletal network, the functional significance of phosphorylation of this protein has not been investigated. Various studies indicate that protein 4.1 may serve as a substrate for a number of protein kinases (Hosey & Tao, 1976; Ling & Sapirstein, 1984; Horne et al., 1985). An analysis of the membrane autophosphorylation reaction indicates that protein 4.1 may be phosphorylated by both cyclic AMP dependent and cyclic AMP independent protein kinases (Hosey & Tao, 1976). Subsequent studies show that the protein can be phosphorylated by a cytosolic cyclic AMP independent protein kinase purified from erythrocytes (Simkowski & Tao, 1980). Structural analysis indicates that the phosphorylation of 4.1 in ghost membranes in the presence of cyclic AMP occurs within a 10 000-dalton segment close to the acidic terminus of the molecule (Leto & Marchesi, 1984). More recently, Ling and Sapirstein (1984) and Horne et al. (1985) have reported that the labeling of protein 4.1 in intact erythrocytes with [^{32}P]orthophosphate is stimulated by phorbol ester, suggesting the possible involvement of protein kinase C in the phosphorylation of protein 4.1. The phorbol ester stimulated phosphorylation sites appear to be localized within a 16 000-dalton segment distinct from the cyclic AMP dependent and independent phosphorylation sites (Horne et al., 1985).

In this report, we have examined in greater detail the phosphorylation of protein 4.1 by erythrocyte membrane and soluble kinases in purified preparations. The effect of phosphorylation on the interaction between protein 4.1 and spectrin has also been studied. Our results indicate that phosphorylation of protein 4.1 decreases its affinity for spectrin. These data, together with our earlier observation that phosphorylation of ankyrin reduces its affinity for spectrin, suggest that the dynamics of the cytoskeletal network may be regulated by phosphorylation–dephosphorylation.

Experimental Procedures

Materials. Human blood was purchased from United Blood Services of Chicago and used within 2 weeks of the drawing date. Aprotinin, diisopropyl fluorophosphate (DFP),[1] and the phosphoamino acid standards were purchased from Sigma Chemical Co. Leupeptin and pepstatin were from Fluka Chemical Corp. and Transformation Research, Inc., respectively. [γ-^{32}P]ATP was obtained from Amersham Corp., and ^{125}I-labeled Bolton–Hunter reagent was supplied by ICN Pharmaceuticals, Inc. All other reagents were of analytical grade.

Preparation of Protein 4.1. Human erythrocyte membranes were prepared according to the procedure of Bennett and Stenbuck (1979). Protein 4.1 was extracted from the membranes by the method of Tyler et al. (1979). The crude extract (200 mL) containing protein 4.1 was dialyzed overnight against 4 L of a pH 7.6 buffer (buffer A) containing 20 mM Tris-HCl, 1 mM EDTA, 0.02% NaN_3, 0.5 mM 2-mercaptoethanol, 0.4 mM DFP, and 2 μg/mL samples of each of the protease inhibitors (aprotinin, pepstatin A, and leupeptin). The dialyzed extract was applied to a QAE-Sephadex column (3.2 × 20 cm) which had been equilibrated with buffer A supplemented with 150 mM KCl. The column was washed with at least 1 column volume of the equilibration buffer and eluted with a 150–450 mM KCl linear gradient prepared in buffer A. Peak fractions (A_{280nm}) were analyzed by SDS–polyacrylamide gel electrophoresis (Tao et al., 1980) for protein 4.1. Those fractions which contained the least amount of impurities were pooled and diluted with a pH 6.8 buffer containing 50 mM potassium phosphate, 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.02% NaN_3, 0.4 mM DFP, and 2 μg/mL samples of each of the protease inhibitors (buffer B) to adjust the KCl concentration to less than 150 mM. Further purification of protein 4.1 was conducted by chromatography on the hydroxylapatite column (1.5 × 5 cm). The column was equilibrated with buffer B containing 150 mM KCl, and protein 4.1 was eluted from the column with a linear gradient of 150–500 mM potassium phosphate. Those fractions containing only pure (as analyzed by SDS gel electrophoresis) protein 4.1 were pooled, dialyzed against buffer A, and stored at 0 °C at protein concentrations of 0.5–1.0 mg/mL. Typically, 800 mL of erythrocyte ghosts yielded about 5–10 mg of pure protein 4.1.

Preparation of Spectrin. Spectrin was extracted from human erythrocyte membranes according to the procedure of Marchesi (1974) except that 0.4 mM DFP and 2 μg/mL samples of each of the protease inhibitors were included in the extraction buffer. Spectrin was purified from the extract by gel filtration through a Sepharose CL-6B column. The elution of the column was conducted with a buffer containing 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA, 20 μg/mL PMSF, and 0.02% NaN_3. This procedure was repeated once, and those fractions which contained only pure spectrin (as analyzed by SDS–polyacrylamide gel electrophoresis) were pooled and stored at −20 °C at concentrations of less than 1.5 mg/mL in the elution buffer containing 2 μg/mL samples of each of the protease inhibitors. No precipitation or degradation of spectrin was observed during storage. The concentration of spectrin was determined by the absorbance at 280 nm, using the value $E^{1\%}_{1cm}$ = 8.8 (Kam et al., 1977), and by the methods described by Bradford (1976) and Lowry et al. (1951), using bovine serum albumin as a standard.

Preparation of Protein Kinases. The human erythrocyte membrane cyclic AMP independent protein kinase was extracted from erythrocyte ghosts with 0.5 M NaCl prepared

[1] Abbreviations: DFP, diisopropyl fluorophosphate; EDTA, ethylenediaminetetraacetate; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

in a pH 7.5 buffer containing 5 mM sodium phosphate, 1 mM EDTA, 15 mM 2-mercaptoethanol, 20 μg/mL PMSF, 0.4 mM DFP, and 2 μg/mL samples of each of the protease inhibitors. The NaCl concentration of the extract was adjusted to 0.3 M with the above buffer, and the diluted extract was applied to a phosphocellulose column. The kinase activity was assayed by using casein as a substrate as described by Tao et al. (1980).

The kinase eluted from the phosphocellulose column was concentrated by Diaflo ultrafiltration and applied to a Sephacryl S-200 column. The column was eluted with 0.5 M KCl in a buffer (buffer C) containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM 2-mercaptoethanol, 20 μg/mL PMSF, 0.4 mM DFP, and 2 μg/mL samples of each of the protease inhibitors. The active kinase fractions eluted from the column were pooled, concentrated, and diluted with 4 volumes of buffer C. The diluted sample was applied to a casein–Sepharose 4B affinity column. After successive washes with buffer C containing 0.1 and 0.3 M KCl, the kinase was eluted with 1 M KCl. The final kinase preparation was concentrated by Diaflo ultrafiltration, dialyzed against buffer C containing 0.15 M KCl and 50% glycerol, and stored at −20 °C. No loss of kinase activity was detected during storage for a period of at least 6 months. The enzyme preparation was judged to be homogeneous based on SDS–polyacrylamide gel electrophoresis.

The cytosolic cyclic AMP independent protein kinase or casein kinase A described earlier by Simkowski and Tao (1980) was also purified to homogeneity by using essentially the same procedures described above for the membrane kinase.

One unit of kinase activity was defined as that amount of enzyme which catalyzed the incorporation of 1 nmol of phosphate into casein per minute.

Phosphorylation of Protein 4.1. Phosphorylation of protein 4.1 was conducted at 37 °C in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 mM ATP or [γ-^{32}P]ATP, 0.3–0.6 mg/mL protein 4.1, 2–4 units/mL kinase, and 4 μg/mL samples of each of the protease inhibitors. The reaction was terminated by the addition of 0.5 volume of a SDS-containing electrophoresis sample buffer (Tao et al., 1980). Following gel electrophoresis, the incorporation of ^{32}P into protein 4.1 was analyzed by radioautography and by excising the protein band from the dried gel and counting in a liquid scintillation spectrometer.

Preparation of ^{125}I-Labeled Protein 4.1. Protein 4.1 was radioiodinated at 0 °C for 90 min in a reaction mixture containing 1 mCi of ^{125}I-labeled Bolton–Hunter reagent, 0.16 M sodium borate, pH 8.5, 250 mM NaCl, and 0.6–1.0 mg of protein 4.1 in a final volume of 0.2 mL. Unbound ^{125}I was removed by dialysis and by repeated washing with the binding buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.02% NaN_3, and 2 μg/mL samples of each of the protease inhibitors) in a microconcentrator. The final concentration of stock protein 4.1 solution was typically about 1.0 mg/mL with a specific activity of about 5×10^5 cpm/μg.

The phosphorylation of ^{125}I-labeled protein 4.1 with unlabeled ATP was conducted at 37 °C for 1 h as described in the preceding section. The reaction was terminated by dilution with the binding buffer, and excess ATP was removed by repeated washing with the binding buffer in a microconcentrator.

Binding Assay. The binding of ^{125}I-labeled protein 4.1 to spectrin was determined by sucrose density gradient centrifugation (Cohen & Langley, 1984). Briefly, phosphorylated or unphosphorylated protein 4.1 (90 pmol) was incubated at 0 °C for 30 min with 30 pmol of either phosphorylated or unphosphorylated spectrin in 100 μL of the binding buffer. Spectrin was phosphorylated with the casein kinase A to the extent of about 2 mol/mol of spectrin as described by Lu et al. (1985). The incubation mixture was carefully layered onto 5 mL of a linear gradient of 5–25% sucrose prepared in the binding buffer. Centrifugation was conducted at 4 °C in a Beckman SW 50.1 rotor at 32000 rpm for 16.5 h. Fractions of 0.12 mL were collected from the bottom of the tube and counted in a Packard 400 CGD automatic γ counter.

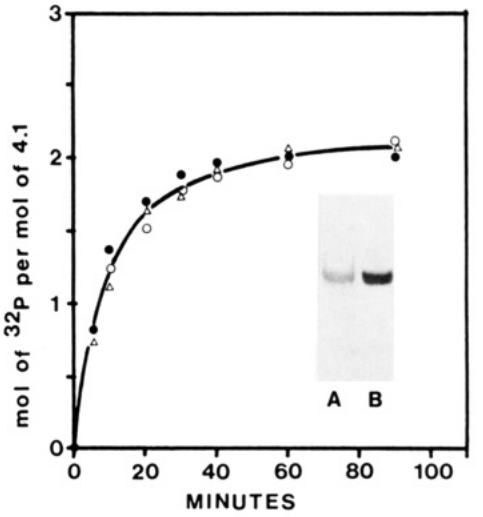

FIGURE 1: Time course of phosphorylation of protein 4.1 by the membrane kinase and casein kinase A. The phosphorylation of protein 4.1 was conducted in 150 μL of a reaction mixture containing 58 μg of protein 4.1 and 3.5 units/mL membrane kinase (○), casein kinase A (△), or both (●). At the time intervals indicated, aliquots of 15 μL were withdrawn for determination of ^{32}P incorporation. Other details are as described under Experimental Procedures. The inset represents a SDS–polyacrylamide gel electrophoretic profile of protein 4.1 phosphorylated by casein kinase A. (A) Coomassie blue stained gel; (B) radioautogram.

RESULTS

Comments on Protein 4.1 Preparation. Protein 4.1 was purified essentially according to the procedure described by Tyler et al. (1979) with slight modifications. Ion-exchange chromatography of the crude membrane extract was conducted by using QAE-Sephadex rather than DEAE-cellulose. The QAE-Sephadex was found to be more efficient than DEAE-cellulose at removing residual band 6 protein that was not completely removed during the initial extraction with 155 mM NaCl. The hydroxylapatite column chromatography step was employed only when the protein 4.1 preparation obtained from the QAE-Sephadex column was found to contain protein 4.2. This step effectively eliminated all contaminating protein 4.2. The degradation of 4.1 was not detected during storage over a period of about 4 weeks. An SDS–polyacrylamide gel electrophoretic pattern of the final 4.1 preparation is shown in Figure 1. The gel shows that the 4.1 preparation is free of any contaminating membrane proteins and degradation products.

Phosphorylation of Protein 4.1. In an attempt to gain insight into the significance of protein 4.1 phosphorylation, studies have been initiated to investigate in greater detail the reaction of the membrane kinase and casein kinase A toward this protein substrate. The radioautogram shown in the inset of Figure 1 confirmed that the kinase-catalyzed reaction resulted in the incorporation of ^{32}P label into protein 4.1. The optimal pH for the phosphorylation of protein 4.1 by either

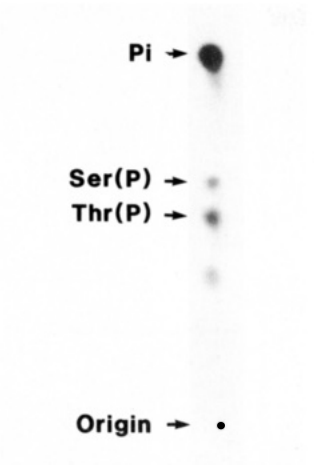

FIGURE 2: Identification of phosphoamino acids of phosphorylated protein 4.1. Protein 4.1 (150 μg) in 0.4 mL was phosphorylated with [γ-^{32}P]ATP in the presence of 2 units/mL membrane kinase. After 1 h of incubation at 37 °C, the reaction was terminated by the addition of 50% trichloroacetic acid to give a final concentration of 10%. The precipitate was collected by centrifugation, washed 5 times with ether–acetone (1:1), and dried under vacuum. The phosphoamino acids were separated by electrophoresis at pH 1.9 as described by Yan and Tao (1982). The radioactive spot under Thr(P) represents incompletely digested phosphopeptide.

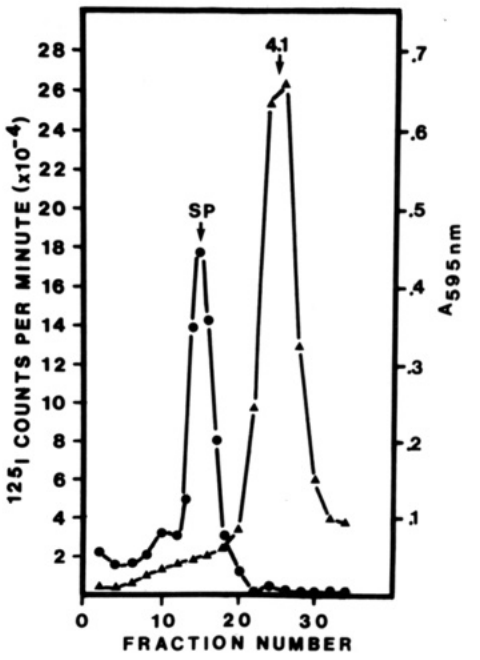

FIGURE 3: Sedimentation profiles of spectrin and ^{125}I-protein 4.1 in sucrose density gradients. Spectrin (240 pmol) and ^{125}I-protein 4.1 (90 pmol) were layered separately onto a 5–25% sucrose density gradient and centrifuged as described under Experimental Procedures. Spectrin was analyzed by the method of Bradford (1976), whereas ^{125}I-protein 4.1 was analyzed by counting in a γ spectrometer.

kinase has been determined to be about 7.5. Mg^{2+} was required for the reaction, and optimum phosphorylation was observed at about 5 mM concentration of this cation. With the exception of Mn^{2+} which could partially replace Mg^{2+} for activity, other divalent cations such as Ca^{2+}, Cu^{2+}, and Zn^{2+}, at concentrations of 1–5 mM, were found to be ineffective. The phosphorylation of protein 4.1 was strongly inhibited by KCl. The labeling of protein 4.1 in the presence of casein kinase A was inhibited approximately 50% by 20 mM KCl and completely by about 150 mM KCl. The same result was obtained for the membrane kinase. KCl appears to exhibit an anomalous effect on the kinase activities, however. As reported earlier, KCl was found to enhance the phosphorylation of casein (Hosey & Tao, 1977; Boivin et al., 1980). Since the effects of KCl vary with the substrate used, it suggests that the salt may be altering the structure of the substrate and affecting its interaction with the enzyme.

The membrane kinase and casein kinase A exhibit no significant differences in their activities toward protein 4.1. Figure 1 shows the time course of phosphorylation of protein 4.1 by the kinases. The result indicates that each kinase can catalyze the incorporation of a maximum of about 2 mol of phosphate per mole of protein 4.1. In view of this finding, it was of interest to determine whether the presence of both kinases in the reaction mixture could lead to an increase in the phosphorylation of protein 4.1. The result showed that the incorporation of [^{32}P]orthophosphate was not additive and that the same amount of radioactivities was incorporated as in experiments containing either one of the enzymes alone (Figure 1). A plausible interpretation of the above data was that the two enzymes might have the same phosphorylation sites on band 4.1. Indeed, our recent studies indicate that the two kinases have very similar properties and strongly suggest that they may be related, if not the same, enzymes. They were found to phosphorylate the same sites on spectrin (Tao et al., 1981), ankyrin (Lu et al., 1985), and T substrate (Yan & Tao, 1983).

An analysis of the acid hydrolysis products of ^{32}P-labeled protein 4.1 revealed that the major phosphorylation sites were seryl and threonyl residues (Figure 2). Interestingly, there was significantly more phosphothreonine than phosphoserine, with a ratio of about 2 to 1. Electrophoresis at pH 3.5 did not reveal the presence of phosphotyrosine (data not shown). The same results were obtained for both kinases.

Effects of Phosphorylation on the Interaction between Spectrin and Protein 4.1. The possibility that phosphorylation might affect the binding of protein 4.1 to spectrin was investigated. In this study, protein 4.1 was labeled with ^{125}I in order to facilitate analysis. Due to the large difference in the molecular mass between spectrin and band 4.1, these two proteins are clearly separated by rate zonal sedimentation on 5–25% sucrose density gradients as shown in Figure 3. The association of protein 4.1 with spectrin led to a dramatic increase in the sedimentation rate of protein 4.1, and the complex formed could be detected easily on the gradient. Figure 4A shows the sedimentation profile of an incubation mixture containing ^{125}I-labeled protein 4.1 and spectrin. As seen in the figure, a significant amount of the 4.1 radioactivities was found to cosediment with spectrin. That the radioactivities are associated with spectrin had been confirmed by analysis of the composition of the peak fractions by SDS–polyacrylamide gel electrophoresis. The spectrin on the gel was identified by staining with Coomassie blue whereas the ^{125}I-labeled protein 4.1 by radioautography. The slow sedimenting radioactive component was found to contain only protein 4.1. Interestingly, phosphorylation of protein 4.1 appeared to affect dramatically its interaction with spectrin. As shown in Figure 4B, phosphorylation of ^{125}I-labeled protein 4.1 effectively abolished its ability to bind to spectrin, and the sucrose density gradient exhibited only a single radioactive peak corresponding to the free protein 4.1.

In a similar study, we examined the association of protein 4.1, either phosphorylated or unphosphorylated, with phosphorylated spectrin. Our result indicates that the binding of protein 4.1 to spectrin does not appear to be dependent on the state of spectrin phosphorylation. As shown in Figure 5A, incubation of unphosphorylated protein 4.1 with phosphory-

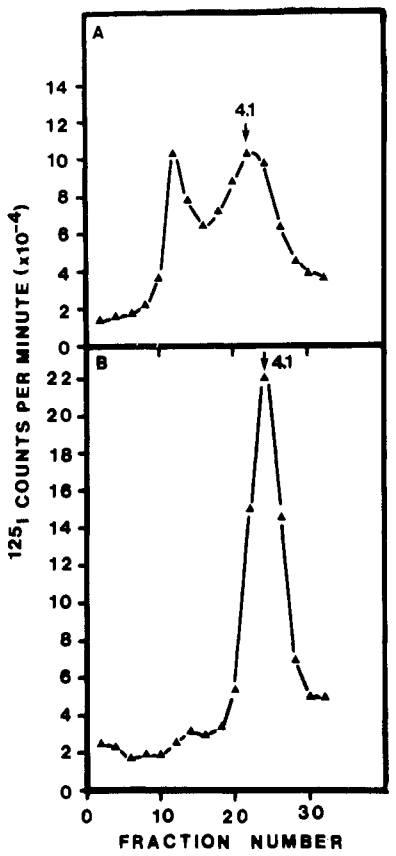

FIGURE 4: Binding of unphosphorylated (A) and phosphorylated (B) ^{125}I-protein 4.1 to spectrin. The experimental details are described in the text. The fast sedimenting radioactive peak represents the binding of ^{125}I-labeled 4.1 to spectrin.

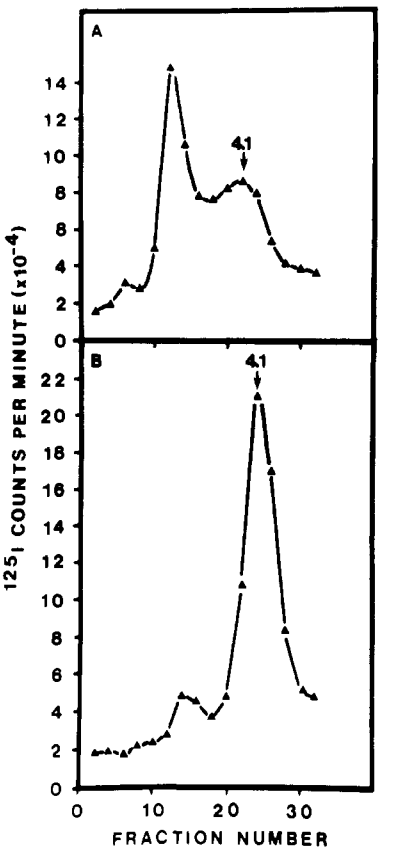

FIGURE 5: Binding of unphosphorylated (A) and phosphorylated (B) ^{125}I-protein 4.1 to phosphorylated spectrin. The experimental details are described under Experimental Procedures. The fast sedimenting radioactive peak represents the binding of ^{125}I-labeled 4.1 to phosphorylated spectrin.

lated spectrin resulted in a sedimentation profile that was similar to Figure 4A, which indicated the formation of a 4.1–spectrin complex. From the data, it would appear that phosphorylated spectrin exhibited a slightly higher affinity for protein 4.1. As seen in Figure 5A, the distribution of radioactivities in the fast sedimenting component was higher than that observed in Figure 4A. Figure 5B shows that the phosphorylated protein 4.1 also exhibits less affinity for the phosphorylated spectrin.

Figure 6 shows that protein 4.1 binds to spectrin with a relatively high affinity. From the Scatchard plots, a K_D of 2×10^{-6} M has been estimated for the interaction between protein 4.1 and spectrin, and a binding stoichiometry of about 2 mol of protein 4.1 per mole of spectrin heterodimer has been obtained. This binding stoichiometry is in agreement with that reported by Tyler et al. (1980) using immunoprecipitation assay for complex formation. The phosphorylation of protein 4.1, however, appeared to reduce significantly its affinity ($K_D = 9.4 \times 10^{-6}$ M), but not its binding stoichiometry, for spectrin. It should be noted, however, that the Scatchard plots may be subjected to error due to the incomplete separation of bound from free 4.1 in the sucrose gradient.

To rule out the possibility that some or all of the observed binding activities might be due to nonspecific association, the binding of heat-denatured protein 4.1 to spectrin was also investigated. Our results indicated that there was little, if any, complex formation between spectrin and protein 4.1 that had been heated at 60 °C for 10 min.

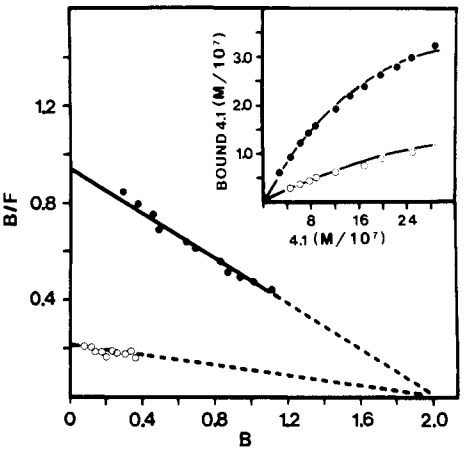

FIGURE 6: Scatchard plots of the binding of unphosphorylated and phosphorylated ^{125}I-protein 4.1 to spectrin. The binding studies were conducted as described under Experimental Procedures in the presence of varying concentrations of unphosphorylated (●) or phosphorylated (○) ^{125}I-protein 4.1. The distribution of protein 4.1 and spectrin in each peak fraction was analyzed by SDS–polyacrylamide gel electrophoresis. The amount of bound protein 4.1 was determined by adding the counts of those fractions corresponding to the spectrin–4.1 complex (for example, fractions 10–16 of Figure 4A) and dividing by the specific radioactivity of 4.1. Free protein 4.1 was estimated by subtracting the bound from the total cpm applied to the gradient. The data were plotted according to the Scatchard equation $B/F = -B/K_D + n/K_D$, where B is the moles of bound ^{125}I-protein 4.1 per mole of spectrin dimer, F is the concentration (micromolar) of unbound ^{125}I-protein 4.1, n is the total number of binding sites (moles of 4.1 per mole of spectrin), and K_D is the dissociation constant (molar).

DISCUSSION

The erythrocyte cytoskeleton has been suggested to play an important role in the maintenance of the shape and deformability of the red cell. Studies have shown that the erythrocyte cytoskeletal network proteins (spectrin and protein 4.1) and

the proteins (ankyrin and band 3) which are involved in the attachment of the network to the membrane are all phosphorylated (Guthrow et al., 1972; Fairbanks & Avruch, 1974; Hosey & Tao, 1976). These data, together with the observations that the shape, surface area, posttransfusional viability, and electromobility of erythrocytes are affected reversibly by the ATP level in the cell (Nakao, 1974), strongly implicate phosphorylation in the regulation of cytoskeletal function. However, studies with spectrin have not yielded any conclusive evidence supporting a role for phosphorylation in the regulation of this cytoskeletal network protein (Marchesi, 1983).

In contrast, we have recently found that phosphorylation of ankyrin reduces its affinity for spectrin tetramers but not dimers (Lu et al., 1985). Likewise, the present study shows that phosphorylated protein 4.1 exhibits less affinity than the unphosphorylated form for spectrin. Phosphorylation reduces the affinity of 4.1 for spectrin by about 5-fold from a K_D of 2×10^{-6} M to a K_D of 9.4×10^{-6} M. The Scatchard plots of the binding data were linear. Our results differed from those reported earlier by Tyler et al. (1980). These investigators showed that the Scatchard plots of protein 4.1 binding to spectrin were curvilinear and consistent with an interaction exhibiting positive cooperativity. A K_D value of about 10^{-7} M was estimated from the linear portion of the curve. The reason for this discrepancy is not clear, but it should be noted that Tyler et al. (1980) measured the binding of protein 4.1 to spectrin by precipitation with anti-spectrin–*Staphylococcus aureus* complex.

Interestingly, phosphorylation of spectrin appears to increase slightly (Figure 5) its affinity for protein 4.1. However, this increase was not sufficient to counteract completely the decrease in binding due to the phosphorylation of protein 4.1.

In this study, the phosphorylation of 4.1 and spectrin was conducted by using either membrane kinase or casein kinase A. Each of these enzymes catalyzed the incorporation of 2 mol of phosphate per mole of 4.1 or spectrin and produced the same effects on the binding activities of the cytoskeletal proteins. As noted earlier, protein 4.1 is also a substrate of the cyclic AMP dependent protein kinase (Hosey & Tao, 1976) and possibly also of protein kinase C (Ling & Sapirstein, 1984; Horne et al., 1985). The effects of phosphorylation by these kinases on the functional activities of protein 4.1 have not been investigated. It will be particularly interesting to determine the effect of phorbol ester stimulated phosphorylation on the binding properties of protein 4.1 since the sites phosphorylated under these conditions were distinct from those phosphorylated by the membrane kinase and the cyclic AMP dependent enzyme (Horne et al., 1985). The possibility that phosphorylation of protein 4.1 may affect its interactions with glycophorin A (Anderson & Lovrein, 1984) and band 3 (Pasternack et al., 1985) also needs to be examined.

The results presented in this study suggest an interesting regulatory process of the cytoskeletal network involving protein phosphorylation–dephosphorylation. Since phosphorylation decreases the affinities of protein 4.1 and ankyrin (Lu et al., 1985) for spectrin, it suggests that phosphorylation may provide a mechanism for creating a relaxed, flexible cytoskeletal structure which is important to the function of the red cell. Conversely, dephosphorylation could lead to a more rigid membrane structure due to an increase in the interactions among the network proteins and between the network and the cell membrane. Moreover, the phosphorylation–dephosphorylation cycle could also contribute to the dynamics of the cytoskeletal network. The above hypothesis provides a useful working concept to explain some of the observations concerning the role of ATP in the regulation of membrane structure and the shape and deformability of the red cell.

Registry No. Mg, 7439-95-4; Mn, 7439-96-5; protein kinase, 9026-43-1; casein kinase, 52660-18-1; phosphothreonine, 1114-81-4; phosphoserine, 407-41-0.

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Acetylcholine Receptor: Characterization of the Voltage-Dependent Regulatory (Inhibitory) Site for Acetylcholine in Membrane Vesicles from *Torpedo californica* Electroplax†

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ABSTRACT: Evidence for a voltage-dependent regulatory (inhibitory) site on the nicotinic acetylcholine receptor to which acetylcholine binds was obtained in membrane vesicles prepared from the *Torpedo californica* electric organ. Two rate coefficients, J_A and α, which pertain to the receptor-controlled ion flux, were measured. A 1000-fold concentration range of acetylcholine was used in a transmembrane voltage (V_m) range from 0 to −48 mV under a voltage-clamped condition at pH 7.4, 1 °C. The following observations were made. (i) At low acetylcholine concentrations, the value of J_A, the rate coefficient for ion translocation by the active (nondesensitized) state of the receptor, increased with increasing concentration. (ii) J_A decreased at high acetylcholine concentrations. (iii) In contrast, α, the rate coefficient for receptor desensitization, did not show such a decrease. (iv) When the transmembrane potential of the vesicle membrane was changed to more negative values, the value of K_R (the dissociation constant for binding of acetylcholine to the regulatory site) decreased by a factor of ~9 for a 25 mV change in V_m, while K_I (the dissociation constant for binding of acetylcholine to the receptor site that controls channel opening) did not show such a change and has a value of 80 μM. When V_m is −48 mV, K_R has a value of 8 μM. (v) The effect of a transmembrane voltage on the regulatory site was reversible and occurred within the time resolution (5 ms) of the quench-flow technique used in the measurements. These results can be explained by a simple model in which the function of the receptor is regulated by binding of acetylcholine to a voltage-dependent regulatory site, a site that is distinct from the sites responsible for receptor activation and desensitization, and from the inhibitory site for cationic noncompetitive inhibitors such as the local anesthetic procaine. The biological significance of the voltage-dependent regulatory site on the acetylcholine receptor, specific for acetylcholine, is discussed.

The acetylcholine receptor at the vertebrate neuromuscular junction and in the electric organ of electric fish transduces a chemical signal into electrical activity (Fatt & Katz, 1951; Nachmansohn, 1959). Katz and Thesleff (1957) suggested that three fundamental steps are involved in the transduction process: (1) acetylcholine binds to a receptor site that controls the formation of transmembrane channels, (2) the conformation of the receptor changes from a closed-channel state to an open-channel state that allows ions to flow through the receptor channel, and (3) a conformational change of the receptor, from an active to an inactive (desensitized) form, occurs. In the last few years, several models that could account for the signal transduction process mediated by the acetylcholine receptor have been proposed (Cash & Hess, 1980; Dunn & Raftery, 1982; Heidmann & Changeux, 1980; Kistler et al., 1982; Neubig & Cohen, 1980).

More recently, an additional regulatory step has been found to be involved in the functioning of the acetylcholine receptor in the *Electrophorus electricus* (Pasquale et al., 1983; Takeyasu et al., 1983) electric organ. Acetylcholine binds to the regulatory site, which is distinct from the sites responsible for activation and inactivation of the receptor; this leads to inhibition of the receptor function in a voltage-dependent manner. Furthermore, this voltage-dependent regulatory site for acetylcholine has been found to be distinct from a voltage-dependent local anesthetic site (Shiono et al., 1984).

In the present study, we characterize the voltage-dependent regulatory site for acetylcholine on the receptor from the *Torpedo californica* electric organ, further characterize the regulatory site of the receptor from the *E. electricus* electric organ, and suggest how this regulatory site for acetylcholine can play a decisive role in the transmission of signals between cells.

Experimental Procedures

Materials. Membrane vesicles were prepared from the *T. californica* electric organ (Pacific Biomarine Lab. Venice, CA) according to the method of Sobel et al. (1977) with slight

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