Phosphorylation of Ankyrin Decreases Its Affinity for Spectrin Tetracer

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The effects of phosphorylation on the interaction between spectrin and ankyrin were investigated. Spectrin and ankyrin were phosphorylated using purified human erythrocyte membrane and cytosolic (casein kinase A) kinases. These two kinases have similar properties as well as activities toward spectrin and ankyrin. Both kinases catalyzed the incorporation of about 2 mol of phosphate/mol of spectrin and about 7 mol of phosphate/mol of ankyrin. These phosphates were incorporated primarily into seryl and threonyl residues of the proteins. The phosphopeptide maps of ankyrin phosphorylated by the membrane kinase and casein kinase A were identical.

Binding studies indicate that ankyrin exhibits different affinities for spectrin dimers ($K_D = 2.5 \pm 0.9 \times 10^{-9} \text{ M}$) and tetramers ($K_D = 2.7 \pm 0.8 \times 10^{-7} \text{ M}$). These dissociation constants were not appreciably affected by the phosphorylation of spectrin. On the other hand, phosphorylation of ankyrin was found to significantly reduce its affinity for either phosphorylated or unphosphorylated spectrin tetramers ($K_D = 1.2 \pm 0.1 \times 10^{-8} \text{ M}$) but not spectrin dimers ($K_D = 2.5 \pm 0.4 \times 10^{-8} \text{ M}$). The same results were obtained using either the membrane kinase or casein kinase A as the phosphorylating enzyme. The above observation suggests that ankyrin phosphorylation may provide an important mechanism for the regulation of the erythrocyte membrane cytoskeletal network.

Underlying the erythrocyte membrane are a group of peripheral proteins which aggregate to form a cytoskeletal network. This cytoskeletal network is thought to play an important role in controlling the shape and deformability of erythrocytes (1, 2). The major constituents of the cytoskeleton are spectrin, band 4.1, and actin. Spectrin is a heterodimer consisting of an $\alpha$ ($M_t = 240,000$) and a $\beta$ ($M_t = 220,000$) subunit (1, 2). Spectrin dimers can further associate to form tetramers and other higher molecular aggregates (3). However, there is evidence to suggest that the predominant form of spectrin in the membrane is the tetramer (4, 5). The cytoskeleton is linked to the membrane, at least in part, by the association of ankyrin with spectrin and band 3, an integral membrane protein (6). Ankyrin, also referred to as band 2.1, is a high molecular weight protein ($M_t = 210,000$) constructed of a single polypeptide chain.

Studies from several laboratories have shown that both spectrin and ankyrin are phosphoproteins. The phosphorylation of these proteins can be demonstrated in intact cells as well as in membrane preparations (7-9). Subsequent studies have identified several erythrocyte protein kinases which are capable of utilizing spectrin as a substrate in vitro (10). However, only two of these kinases, one isolated from the membrane (membrane kinase) and the other from the cytosol (casein kinase A), are found to yield phosphopeptide patterns of spectrin similar to that of spectrin phosphorylated in intact cells (10).

Studies on the functional significance of spectrin phosphorylation have yielded conflicting results (11-13). Birchmeier and Singer (11) initially reported that shape changes in erythrocyte ghosts were related to the phosphorylation of the $\beta$ subunit of spectrin. Later on, Pinder et al. (12) showed that phosphorylated spectrin caused a dramatic increase in G-actin polymerization. Phosphorylation, however, did not affect the dimer-tetramer equilibrium of spectrin (14). In contrast, Brenner and Korn (13) showed that the binding of F-actin to spectrin was independent of spectrin phosphorylation and that purified spectrin, irrespective of its phosphorylation states, did not bind G-actin. This latter observation is in agreement with the finding of Cohen and Branton (15). Finally, Anderson and Tyler (16) examined the turnover of spectrin-bound phosphates in intact cells and concluded that there was no correlation between spectrin phosphorylation or dephosphorylation and shape changes.

In this work, we have examined the phosphorylation of spectrin and ankyrin by the membrane kinase and casein kinase A in purified preparations. The results indicate that both spectrin and ankyrin contain multiple phosphorylation sites. The phosphorylation of ankyrin appears to affect its binding to spectrin tetramer but not to the dimer. In contrast, the phosphorylation of spectrin does not appreciably affect its binding to either phosphorylated or unphosphorylated ankyrin.

EXPERIMENTAL PROCEDURES

Materials—Human blood was obtained from the University of Illinois Hospital blood bank and used within 2 weeks of the drawing date. [γ-32P]ATP and [3H]labeled Bolton-Hunter reagent were purchased from Amersham Corp. Leupeptin, pepstatin A, aprotinin, and DFP were purchased from Sigma. All other reagents were of analytical grade.

Preparation of Erythrocyte Membranes—Human erythrocytes were washed as described earlier (17). The washed erythrocytes were sedimented three times at $1 \times g$ in 3 volumes of a buffer containing 160 mM NaCl, 5 mM Na phosphate, pH 7.5, and 0.75% (w/v) dextran 500 (18). Erythrocyte ghosts were prepared by hypotonic lysis in 4 volumes of a pH 7.5 buffer containing 7.5 mM Na phosphate, 1 mM EDTA, 20 μg/ml PMSF, 0.4 mM DFP, and 2 μg/ml each of the protease inhibitors (leupeptin, pepstatin A, and aprotinin) and 100 μg/ml each of PMSF, diisopropyl fluorophosphate, PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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washed free of hemoglobin with 7.5 mM Na phosphate buffer, pH 7.5, containing 1 mM EDTA.

Preparation of Ankyrin—The solubilization of ankyrin from erythrocyte ghost membranes was conducted essentially as described by Bennett and Stenkub (18) except that the buffers used were supplemented with 0.4 mM DFP, 0.02% NaN₃, and 2 μg/ml each of the protease inhibitors. The dialyzed extract was applied to a QAE-Sephadex column (3.2 × 20 cm) which had been equilibrated with Buffer B supplemented with 150 mM KCl. The column was washed with 150 mM KCl in Buffer B and eluted with a linear KCl gradient of 0.2–0.6 M in a total volume of 1.2 liters. Peak fractions (Aₓmm) were analyzed by SDS-polyacrylamide gel electrophoresis (17). Those fractions which contained ankyrin were pooled and concentrated in an Amicon ultrafiltration cell equipped with a YM-10 membrane. Ankyrin was further purified by sedimentation in a linear 5–20% sucrose gradient prepared in Buffer B containing 150 mM KCl. The centrifugation was conducted at 38,000 rpm, 4°C, in a Beckman SW 41 rotor for 20 h. Fractions of 0.5 ml each were collected from the bottom of the tube. Those fractions which contained the above criteria of impurities were pooled and applied onto a hydroxyapatite column (1.5 × 3 cm) which had been equilibrated with 20 mM K phosphate, pH 6.7, 0.5 mM KCl, 0.2 mM dithiothreitol, 1 mM EDTA, 0.02% NaN₃, 0.4 mM DFP, 20 μg/ml PMSF, and 2 μg/ml each of the protease inhibitors. A linear phosphate gradient (50–300 mM phosphate) was then applied to the column. Fractions containing ankyrin were pooled, dialyzed against 20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol, 0.02% NaN₃, 20 μg/ml PMSF, 0.4 mM DFP, and 2 μg/ml each of the protease inhibitors and stored at −20°C. The purity of the final ankyrin preparation was analyzed by SDS-polyacrylamide gel electrophoresis and found to be about 95% pure.

Preparation of Spectrin—Spectrin was extracted from human erythrocyte membranes at 37°C for 30 min with 0.1 mM EDTA, pH 8.0, containing 2 μg/ml each of the protease inhibitors and 0.4 mM DFP, as described by Marchesi (19) with slight modifications. The extract which contained primarily spectrin and actin was purified by gel filtration through a Sepharose CL-6B column. The elution of the column was conducted with 10 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 1 mM EDTA, 20 μg/ml PMSF, and 0.2% NaN₃. This procedure was repeated once, and the purity of each fraction collected from the second passage was analyzed by SDS-polyacrylamide gel electrophoresis. Those fractions which contained spectrin and actin were pooled and stored at −20°C at concentrations of less than 1.5 mg/ml in the elution buffer containing 2 μg/ml each of the protease inhibitors. The concentration of spectrin was determined by absorbance at 280 nm, using the value E₁ₓnm = 8.8 (5), and by the methods described by Bradford (20) and Lowry et al. (21), using bovine serum albumin as a standard. The spectrin used in the binding assay was stored in a buffer (binding buffer) 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 each of the protease inhibitors.

Binding Assay—The binding of 125I-ankyrin to spectrin was analyzed by nondenaturing density gradient gel electrophoresis according to the method described by Weaver et al. (22). Varying amounts of phosphorylated and unphosphorylated 125I-ankyrin were incubated for 1 h at 0°C with 40.5 μg of phosphorylated or unphosphorylated spectrin in 60 μl of the binding buffer. The binding of 125I-ankyrin to spectrin was analyzed by electrophoresis in a 2–4% acrylamide gradient slab gel at 60 V for 4 h in a cold room. After the electrophoresis was terminated, the gel was stained with Coomassie Brilliant Blue, dried under vacuum, and exposed to X-ray films. The radioactive bands were also excised from the dried gel and counted in a γ-counter.

Preparation of Protein Kinase—The heat-denatured membrane cyclic AMP-independent protein kinase was extracted from ghosts at 0°C for 30 min with 0.5 M NaCl in a buffer containing 5 mM Na phosphate, pH 7.5, 1 mM EDTA, 15 mM 2-mercaptoethanol, 20 μg/ml PMSF, 0.4 mM DFP, and 2 μg/ml each of the protease inhibitors. Under the storage conditions described above, no precipitation of spectrin was observed. The kinase was eluted from the column with a linear (0.3–1.0 M) NaCl gradient.

The peak fractions containing kinase activity, as assayed using casein as substrate (17), were pooled, concentrated by Diaflo ultrafiltration, dialyzed against Buffer C containing 0.15 M NaCl and 0.5% glycerol, and stored at −20°C. No significant loss of kinase activity was observed during storage for a period of at least 6 months. The enzyme preparation was judged to be homogeneous based on SDS-polyacrylamide gel electrophoresis.

RESULTS

Phosphorylation of Spectrin—Fig. 1 shows the SDS-gel electrophoretic pattern of the spectrin preparations used in this study. The spectrin preparations are free of any contaminating membrane proteins and degradation products.

The effect of various agents on the phosphorylation of ankyrin and spectrin by the kinases has been examined in order to establish conditions for maximal incorporation of phosphate into these two membrane proteins. The phosphorylation of spectrin exhibits an optimum Mg²⁺ concentration of about 5 mM. Since a moderate concentration of salt was needed to maintain the solubility of spectrin (24), the effect of NaCl on the phosphorylation was also examined. NaCl, in the range of 50–100 mM, was found to have no appreciable effect on the rate of spectrin phosphorylation. At concentrations greater than 150 mM, however, inhibition of the phosphorylation reaction was observed. This inhibition could be attributed, in part, to the interference of high salt concentrations on the interaction between the kinase and spectrin as suggested earlier by Conway and Tao (25). The phosphorylation of spectrin exhibits a broad pH activity profile. No significant difference in incorporation was observed between
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Fig. 1. SDS-polyacrylamide gel electrophoresis of spectrin and ankyrin phosphorylated by casein kinase A. The electrophoresis of spectrin (6 µg) and ankyrin (7 µg), phosphorylated as described under "Experimental Procedures," was conducted on a 5% polyacrylamide gel slab. The specific activities of [γ-32P]ATP used for the phosphorylation of spectrin and ankyrin are 750 and 350 cpm/pmol, respectively. A, stained gel; B, radioautogram.

pH 6 and 8.5. In this study, the reaction was routinely conducted at pH 7.5.

Fig. 1 shows that the incorporation of phosphate occurs only on the β subunit of spectrin. However, under extreme conditions, such as at pH 9, a small degree of phosphorylation of the α subunit was also detected. The phosphorylation assays were conducted in the presence of protease inhibitors. No detectable degradation of spectrin was observed during incubations. An analysis of the acid hydrolysis products of 32P-labeled spectrin reveals the presence of 32P labels in seryl and threonyl residues (data not shown). The ratio of 32P labels in seryl and threonyl residues was estimated to be about 3:2. The membrane kinase and casein kinase A exhibit no significant differences in their activities toward spectrin. Under optimum condition, both kinases catalyze the incorporation of 1.7–2.0 mol of phosphate/mol of β subunit. This value is somewhat greater than that estimated previously by Harris and Lux (26) and by Tao et al. (10). This difference, however, is not due to a difference in the endogenous phosphate contents of the isolated spectrin. The spectrin preparation used in this study had been routinely analyzed for protein-bound phosphates using the malachite green method of Kicklner (27) and found to be about 4–5 mol/mol of spectrin. This value is similar to that reported earlier for the spectrin preparation employed by Harris et al. (28) in their study.

Phosphorylation of Ankyrin—The procedure of Bennett and Stenbuck (18) for the preparatin of ankyrin has been modified in our laboratory. We have employed QAE-Sephadex in place of DEAE-cellulose for the initial fractionation step as this appears to give us a better separation of ankyrin from other membrane proteins. An additional step involving hydroxylapatite chromatography is introduced in order to remove minor degradation products of ankyrin. Especial precaution has also been taken to prevent proteolysis of ankyrin during fractionation. By including the various protease inhibitors such as DFP, aprotinin, leupeptin, and pepstatin A in the buffers, we have been able to minimize proteolysis; and the ankyrin preparation obtained can be stored for a prolonged period of time with no detectable degradation. An SDS-gel electrophoretic pattern of ankyrin is shown in Fig. 1 (Ankyrin, lane A). The gel contains a barely visible minor protein contaminant migrating in the region of about 155,000 daltons. This same protein component is also reported in the ankyrin preparation of Bennett and Stenbuck (18). Our purification procedure generally yielded 3–5 mg of ankyrin/unit of blood.

Since there is a paucity of information concerning the phosphorylation of ankyrin by purified kinases, in this study we have investigated in greater detail the activity of the membrane kinase and casein kinase A toward ankyrin. Fig. 1 (Ankyrin, lane B) shows that the phosphorylation of ankyrin with either enzyme results in the labeling of only the protein band corresponding to ankyrin. The phosphorylation of ankyrin exhibited a pH optimum of about 7.5 and a Mg2+ optimum of 5 mM KCl, at 40 mM, was found to be slightly stimulatory; whereas at concentrations greater than 0.1 M, inhibition of phosphorylation was observed. These parameters are the same for both the membrane kinase and casein kinase A.

Fig. 2 shows the time course of phosphorylation of ankyrin by the membrane kinase and casein kinase A under optimum conditions. The data show that each kinase can catalyze the incorporation of about 7 mol of phosphate/mol of ankyrin. Preliminary estimate indicates that each ankyrin contains three endogenous protein-bound phosphates.

The kinetic parameters were determined by measuring the kinase activity at varying concentrations of one substrate in the presence of different fixed levels of the other. The data for the membrane kinase-catalyzed reaction shown in Fig. 3 are presented as double reciprocal plots of the initial velocity versus the concentrations of ankyrin at different fixed concentrations of ATP, and vice versa. Essentially the same results were obtained for casein kinase A. The data suggest that the reaction mechanism of the kinases is the same as that of the wheat germ kinase (28) and the cyclic AMP-dependent protein kinase (29) and is consistent with a sequential bireactant reaction kinetics involving the formation of a ternary enzyme-substrate complex (30). From replots of the slopes and intercepts of the double reciprocal plots shown in Fig. 3, K, values of 10 µM and 0.18 mg/ml were obtained for ATP and ankyrin, respectively. These values are the same for both the membrane kinase and casein kinase A.

An analysis of the phosphoamino acids (31) obtained from
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Fig. 3. Double reciprocal plots of initial velocity versus substrate concentration. The reactions were carried out in the presence of 4 units/ml membrane kinase. The incubation was conducted at 37 °C for 4 min. A, double reciprocal plots of initial velocity versus ankyrin concentration at different fixed levels of ATP (●—●, 5 μM; ○—○, 10 μM; ▲—▲, 25 μM; and △—△, 50 μM). B, double reciprocal plots of the initial velocity versus ATP concentration at different fixed levels of ankyrin (●—●, 0.1 mg/ml; ○—○, 0.2 mg/ml; ▲—▲, 0.3 mg/ml; and △—△, 0.4 mg/ml).

Acid hydrolysis of 32P-labeled ankyrin indicates that phosphorylation occurs primarily on seryl and threonyl residues (data not shown). The possibility that phosphotyrosine may also be formed has been examined by conducting the high voltage electrophoresis at pH 3.5 according to the procedure of Hunter and Sefton (32). Our result failed to reveal the presence of phosphotyrosine (data not shown). The same results were obtained for both the membrane kinase and casein kinase A.

Since both the membrane kinase and casein kinase A can catalyze the incorporation of approximately 7 mol of phosphate into each mol of ankyrin, it is of interest to determine whether these two kinases phosphorylate the same or different sites on the ankyrin molecule. Experiments in which the phosphorylation of ankyrin was conducted in the presence of both kinases showed that the amount of phosphate incorporated was not additive. Under these conditions, the number of phosphate incorporated was the same as that using either kinase alone (data not shown). A comparison of the phosphopeptide maps of ankyrin phosphorylated by the membrane kinase and casein kinase A also revealed no significant difference in their 32P-labeled patterns (Fig. 4). The results suggest, although do not prove, that the two kinases may have the same specificities toward ankyrin and that they recognize the same phosphorylation sites.

Effects of Phosphorylation on the Interaction between Spectrin and Ankyrin—Studies have shown that ankyrin contains two important binding sites: one is for spectrin and the other is for band 3 (22, 33). Thus, ankyrin serves to link the membrane cytoskeleton to the overlying membrane. Since both spectrin and ankyrin are phosphoproteins, it was of interest to determine whether phosphorylation could affect their interactions.

The binding of 125I-ankyrin to spectrin was investigated using nondenaturing gel electrophoresis according to the method of Morrow and Marchesi (3). This gel system is particularly useful since it resolves spectrin into its various oligomeric forms; therefore, it allows us to measure the binding of ankyrin, not only in the dimeric form, but more importantly also to the tetrameric form of spectrin. As indicated
earlier, spectrin tetramer has been suggested to represent the native form of spectrin in the membrane cytoskeleton. We have confirmed the earlier observation of Morrow and Marchesi (3) that the aggregation of spectrin to form tetramers and higher oligomers is a time- and temperature-dependent process.

A representative experiment illustrating the binding of $^{125}$I-ankyrin to spectrin is shown in Fig. 5. It can be seen from the figure that ankyrin can bind to the various aggregated forms of spectrin. The extent of binding to each of these forms, however, was dependent on ankyrin concentrations. At low concentrations, nearly all of the ankyrin was found to be associated with the tetramer and higher oligomers, even though the dimeric form was the predominant species present. Increasing the concentration of ankyrin resulted in an increase in the binding to the dimeric species. Ankyrin was found to exhibit a greater affinity for spectrin tetramer ($K_D = 2.7 \pm 0.8 \times 10^{-7} \text{ M}$) than for spectrin dimer ($K_D = 2.5 \pm 0.9 \times 10^{-6} \text{ M}$). The dissociation constants were calculated from the slopes of double reciprocal plots of ankyrin binding to spectrin dimer and tetramer according to the method of Weaver et al. (22). The values are averaged from five determinations. The binding of ankyrin to higher oligomers was not determined due to poor resolution of these oligomers. Our results are in general agreement with those reported earlier by Weaver et al. (22).

In a similar study, we examined the binding of ankyrin to phosphorylated spectrin. Spectrin was phosphorylated by either the membrane kinase or casein kinase A to the extent of about 2 mol/mol of spectrin. It should be noted that phosphorylation did not affect the dimer-tetramer equilibrium, and the distribution of these molecular species on the gel was the same as the unphosphorylated preparation. Our results indicate that phosphorylation of spectrin also does not affect significantly its interaction with ankyrin. The dissociation constants of ankyrin for phosphorylated spectrin dimer and tetramer were approximately the same as those obtained for unphosphorylated spectrin (data not shown). On the other hand, phosphorylation of ankyrin (about 7 mol of phosphate/mol of ankyrin) was found to affect its affinity for spectrin tetramer but not the dimer (Fig. 6). The $K_D$, based on five

Fig. 5. Binding of $^{125}$I-ankyrin to spectrin. Varying amounts of $^{125}$I-ankyrin (8 x 10^6 cpn/mg) were incubated with spectrin as described under “Experimental Procedures.” Lanes 1–4 and lanes 6–8 contain, respectively, the following amounts of $^{125}$I-ankyrin: 1.6, 4.8, 7.0, and 10.2 and 12.8, 16.0, and 19.2 μg. Lane 5 contains 40.5 μg of spectrin, whereas lane 9 contains only $^{125}$I-ankyrin (19.2 μg). The following are the distribution of the various molecular forms of spectrin in lane 5: dimer, 55%; tetramer, 35%; and oligomers, 10%. A, stained gel; B, radioautogram; T, tetramer; D, dimer.

Fig. 6. Binding of phosphorylated $^{125}$I-ankyrin to spectrin. Varying amounts of the phosphorylated $^{125}$I-ankyrin (phosphorylated with ATP and casein kinase A) were incubated with spectrin as described under “Experimental Procedures.” Lanes 1–3 and lanes 5–7 contain, respectively, the following amounts of phosphorylated $^{125}$I-ankyrin: 4.4, 5.9, and 8.1 and 9.6, 12.6, and 16.3 μg. Lanes 4 and 8 contain only spectrin (40 μg) and $^{125}$I-ankyrin (19.2 μg), respectively. Lane 4 contains 59% dimer, 30% tetramer, and 10% oligomers. A, stained gel; B, radioautogram; T, tetramer; D, dimer.

Fig. 7. Double reciprocal plots of the binding of phosphorylated $^{125}$I-ankyrin to unphosphorylated spectrin. The phosphorylation of $^{125}$I-ankyrin with ATP and casein kinase A was conducted as described under “Experimental Procedures.” The data were plotted according to the relationship [spectrin]/[bound ankyrin] = $K_D$/[free ankyrin] + 1 for binding to spectrin dimers (O) and tetramers (△).
determinations, for the complex between phosphorylated ankyrin and spectrin tetramer was increased about 4-fold to a value of 1.2 ± 0.1 x 10^-6 M, whereas the Ko, 2.5 ± 0.4 x 10^-6 M, for the complex between phospho-ankyrin and spectrin dimer was not significantly affected (Fig. 7). The same results were obtained for the complexes between phospho-ankyrin and phosphospectrin. Thus, the interaction between spectrin and phospho-ankyrin was also independent of the phospho-tyrosine phosphorylation states of spectrin. The results described above are the same irrespective of whether the membrane kinase or casein kinase A was used to catalyze the phosphorylation reaction.

Quantitation of the amounts of ankyrin bound to spectrin dimer and tetramer showed that the tetramer bound about twice the amount of ankyrin as did the dimer, confirming an earlier report by Weaver et al. (22). The binding molar ratio was not affected by phosphorylation of either ankyrin or spectrin, or both. It should also be noted that the labeling of ankyrin with 32P did not affect its phosphate accepting capacity.

**DISCUSSION**

Although the phosphorylation of erythrocyte membrane proteins has been widely observed and investigated, the significance of the phosphorylation reaction remains unknown. Among the major erythrocyte membrane proteins, spectrin, band 3, ankyrin and bands 4.1, 4.5, and 4.8 have all been identified as substrates of protein kinases (7). However, the study of phosphorylation has focused primarily on spectrin in light of the importance of this protein in the cytoskeletal network assembly of the erythrocyte membrane.

Spectrin is a phosphoprotein containing 4–5 mol of bound phosphate. Since a similar amount of protein-bound phosphate was found in spectrin isolated from both fresh and outdated erythrocytes, it would appear that these endogenous phosphates had low turnover rate. The present study indicates that an additional 2 mol of phosphates can be incorporated into spectrin by either the membrane kinase or casein kinase A. We have preliminary evidence to indicate that the extent of spectrin phosphorylation by the purified kinases is dependent upon the salt concentration in the reaction mixture. The amount of phosphate incorporated into spectrin was decreased significantly in the presence of 150 mM or greater of NaCl.

In contrast to spectrin, the isolated ankyrin was found to contain a significantly greater number of phosphorylation sites available for the membrane kinase and casein kinase A. That ankyrin may contain multiple phosphorylation sites has been suggested earlier by the observation of Weaver and Marchesi (34). The phosphopeptide maps of ankyrin phosphorylated by the membrane kinase and casein kinase A are identical. These data together with the observation that the same amounts of phosphate are incorporated into ankyrin in reactions containing either each of the kinases alone or both kinases suggest that the two kinases exhibit the same specificity toward ankyrin. The phosphates incorporated into ankyrin are found primarily on seryl and threonyl residues with a somewhat greater amount of labels found to be associated with phosphothreonine. This distribution of phosphate between the two amino acids in ankyrin is contrary to that found in spectrin. It remains to be determined as to whether the membrane kinase or casein kinase A, or both, are responsible for the phosphorylation of ankyrin in the intact cells. It should be noted, however, that there is considerable evidence to indicate that the membrane kinase and, perhaps, also casein kinase A may play an important role in the phosphorylation of spectrin in vivo (10, 35).

Since both spectrin and ankyrin contained a significant number of phosphorylation sites, it was of interest to determine whether phosphorylation could affect the interaction between these two proteins. Our results clearly show that phosphorylation of ankyrin decreases its affinity for either phosphorylated or unphosphorylated spectrin. Somewhat surprisingly, this effect appears to be confined mainly to the interaction between ankyrin and spectrin tetramer. The affinity of phosphorylated ankyrin for phosphorylated and unphosphorylated-spectrin tetramer was found to decrease by about 4-fold. This observation that only the binding to spectrin tetramer is affected is particularly interesting in light of the available evidence which suggests that the tetramer is the predominant form of spectrin in the membrane cytoskeletal network in situ. In contrast, the phosphorylation of spectrin appears to have little or no appreciable effect on its interaction with either phosphorylated or unphosphorylated ankyrin. These data lend further support to an earlier report which indicates that spectrin phosphorylation does not affect its binding to cell membrane via ankyrin (16). Hence the phosphorylation of spectrin appears to have little, if any, role in the regulation of the assembly of the cytoskeletal network and the interaction of the network with the membrane.

Although the significance of membrane protein phosphorylation remains unknown, there is sufficient evidence to indicate that phosphorylation may play an important role in erythrocyte shape changes and deformability through the regulation of the interactions of the various cytoskeletal network components. The results presented in this study could conceivably provide an attractive mechanism to explain the dynamics of the cytoskeletal network. Since phospho-ankyrin binds spectrin tetramer less tightly, the phosphorylation of ankyrin could lead to a weakening of the interaction between the cytoskeletal network and the membrane. As a result, the membrane cytoskeleton could assume a more relaxed and flexible structure. Conversely, dephosphorylation of ankyrin could lead to a more rigid network due to a stronger association with the cell membrane through ankyrin. The possibility that phosphorylation may affect the binding of ankyrin to spectrin is further strengthened by studies which show that the spectrin-binding site of ankyrin is located within a 32,000-dalton region near the end of the molecule (22). This 32,000-dalton region is shown to be phosphorylated (22). The above proposed mechanism represents a working hypothesis based on the available data. Obviously, more experimentation is needed in order to confirm or reject the validity of the hypothesis.

**REFERENCES**

Phosphorylation of Ankyrin and Spectrin