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Phosphorylation and Dephosphorylation of Spectrin

Grant Fairbanks, Joseph Avruch, Judith E. Dino, and Vikram P. Patel

Worcester Foundation for Experimental Biology, Inc., Shrewsbury, Massachusetts 01545 (G.F., J.E.D., V.P.P.); and Diabetes Unit, Massachusetts General Hospital, Boston, Massachusetts 02114 (J.A.)

The phosphorylation of spectrin polypeptide *2* is thought to be involved in the metabolically dependent regulation of red cell shape and deformability. Spectrin phosphorylation is not affected by cAMP. The reaction in isolated membranes resembles the cAMP-independent, salt-stimulated phosphorylation of an exogenous substrate, casein, by enzyme(s) present both in isolated membranes and cytoplasmic extracts. Spectrin kinase is selectively eluted from membranes by 0.5 M NaCl and co-fractionates with eluted casein kinase. Phosphorylation of band *3* in the membrane is inhibited by salt, but the band *3* kinase is otherwise indistinguishable operationally from spectrin kinase. The membrane-bound casein (spectrin) kinase is not eluted efficiently with spectrin at low ionic strength; about 80% of the activity is apparently bound at sites (perhaps on or near band *3*) other than spectrin. Partitioning of casein kinase between cytoplasm and membrane is metabolically dependent; the proportion of casein kinase on the membrane can range from 25% to 75%, but for fresh cells is normally about 40%. Dephosphorylation of phosphorylated spectrin has not been studied intensively. Slow release of ^{32}Pi from [^{32}P]spectrin on the membrane can be demonstrated, but phosphatase activity measured against solubilized [^{32}P] spectrin is concentrated in the cytoplasm. The crude cytoplasmic phosphospectrin phosphatase is inhibited by various anions – notably, ATP and 2,3-DPG at physiological concentrations. Regulation of spectrin phosphorylation in intact cells has not been studied. We speculate that spectrin phosphorylation state may be regulated 1) by metabolic intermediates and other internal chemical signals that modulate kinase and phosphatase activities per se or determine their intracellular localization and 2) by membrane deformation that alters enzyme–spectrin interaction locally. Progress in the isolation and characterization of spectrin kinase and phosphospectrin phosphatase should lead to the resolution of major questions raised by previous work: the relationships between membrane-bound and cytoplasmic forms of the enzymes, the nature of their physical interactions with the membrane, and the regulation of their activities in defined cell-free systems.

Key words: **dephosphorylation, spectrin, protein kinase, cAMP-independent, phosphoprotein phosphatase, phosphorylation**

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The phosphorylation of spectrin was observed initially in the context of descriptive red cell membrane biochemistry, but it is now studied with increasing intensity because of its probable importance in cellular regulation. The enzymes involved – protein kinase(s) and phosphoprotein phosphatase(s) – are of intrinsic interest because they are relatively accessible and present intriguing problems in regulation. However, the main impetus for the work has been the idea that phosphorylation and dephosphorylation direct major changes in the physical state of spectrin and that these effects account in part for the metabolic dependence of cell shape and membrane deformability. The development of this perspective was strongly influenced by Weed et al [1], who in 1969 presented a refined description of the effects of metabolic depletion on cell and membrane properties and inferred that protein organization at the membrane–cytoplasm interface undergoes major changes as ATP levels decline. The first direct evidence for spectrin phosphorylation in human erythrocytes appeared two years later in another context [2]. During the same period, the fractionation and characterization of soluble protein kinases from rabbit reticulocytes [3, 4] and mature red cells [4, 5] was undertaken. This work ultimately contributed to the development of general criteria that distinguish the three major classes of protein kinases [6]. Both cAMP-dependent and cAMP-independent activities were detected in hemoglobin-free human erythrocyte ghosts [7]. And endogenous, or "autophosphorylation," reactions in which the membrane-bound enzymes utilize [γ-^{32}P]ATP in phosphorylating spectrin and other membrane proteins were described by several laboratories [8–13].

Most studies of spectrin phosphorylation have been confined to phenomenology and have yielded little insight into its functional significance. There have been several attempts to demonstrate altered phosphorylation in association with abnormal membrane function in hemolytic disease, but no convincing evidence for such a relationship has yet appeared [14, 15]. Recent work does, however, implicate spectrin phosphorylation directly in the regulation of protein organization in normal membranes: Singer and associates [16, 17] presented evidence that spectrin phosphorylation is required in the MgATP-dependent shape change of ghosts from crenated spheres to smooth biconcave discs. Another important contribution was made by Pinder et al [18–20], who examined the interaction between spectrin and skeletal muscle actin in solution and observed that phosphorylation of spectrin both increases its binding to actin and greatly enhances its capacity to induce actin polymerization. These results have been elaborated in the form of a working hypothesis: that the spectrin array on the membrane is cross-linked by actin monomers or very short filaments and that these associations are regulated by spectrin phosphorylation [20, 21].

A simplified diagram of the spectrin phosphorylation–dephosphorylation system is shown in Figure 1. Some of its components and interactions are known, but many are hypothetical. Because spectrin is multiply phosphorylated [15, 22], the equilibrium between phosphorylated and dephosphorylated forms may be much more complex than indicated. The phosphorylation sites are clustered on polypeptide *2* [23], but need not be functionally equivalent; the possibility that multiple enzymes are involved also remains to be tested. We envision that changes in the level of spectrin phosphorylation modulate actin–spectrin cross-linking on the membrane, and that this cross-linking (together with other factors) regulates cell shape, membrane deformability, and lateral mobility of integral proteins [1, 24]. In this scheme, turnover of spectrin-bound phosphate could

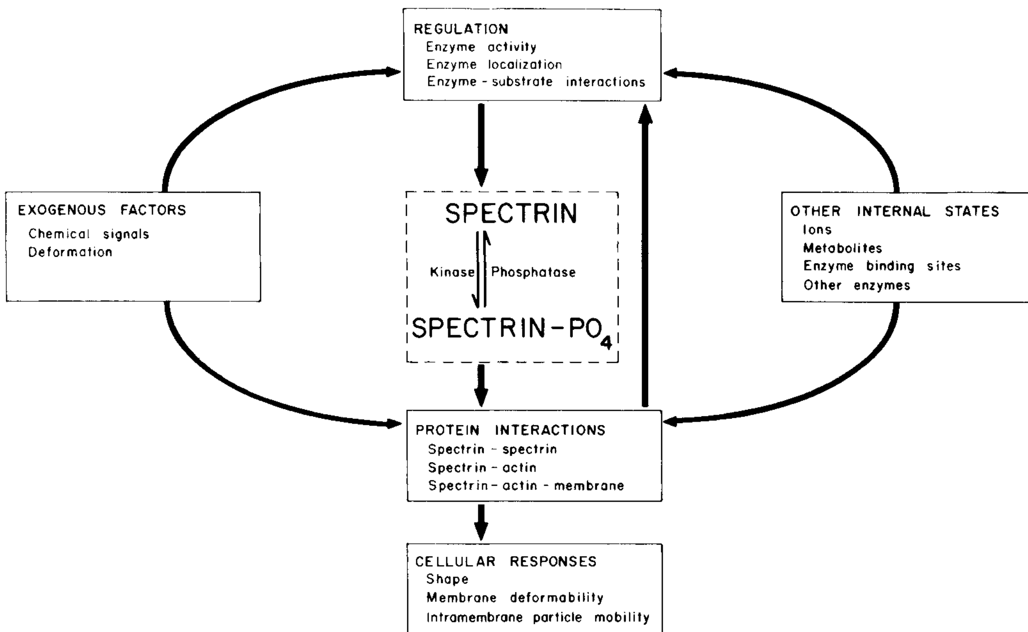

Fig. 1. The spectrin phosphorylation–dephosphorylation system. This heuristic model embodies the hypothesis that cell shape and membrane material properties are determined by the organization of peripheral proteins on the cytoplasmic face of the membrane and that spectrin phosphorylation modulates the protein interactions involved. Hypothetical factors in the regulation of interconversion of phosphorylated and dephosphorylated spectrin are 1) external influences, 2) various internal states, and 3) direct feedback from the spectrin–actin meshwork.

have the critical function of allowing reordering of the spectrin–actin meshwork after derangement by hemolysis or other trauma.

Virtually nothing is known about the regulation of spectrin phosphorylation except for its MgATP requirement. As indicated in Figure 1, it is conceivable that both external signals and internal states influence the interconversion rates. Furthermore, it is important to observe that the actions of these factors are not necessarily restricted to modulation of protein kinase or phosphoprotein phosphatase activity per se. Spatial disposition may play a major role if enzyme(s) bind to the membrane at sites other than their substrate, spectrin, or if enzyme partitioning between membrane and cytoplasm is variable. In Figure 1, possibilities for regulation at the level of enzyme–substrate interaction are shown as 1) direct feedback from the spectrin–actin meshwork, 2) deformation as an external stimulus, and 3) enzyme-binding sites on the membrane as internal regulatory elements.

Our primary objective in this paper is to review briefly the phenomenology of spectrin phosphorylation and dephosphorylation and to describe the current status of research on the properties of spectrin kinase and phosphospectrin phosphatase. We conclude with further speculation on modes of regulation that are consistent with the data available and are sufficiently attractive in principle to stimulate further investigation.

SPECTRIN PHOSPHORYLATION

As illustrated in Figure 2, both cytoplasmic and membrane proteins are labeled in intact red cells incubated with ^{32}Pi in a physiological buffer system. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the whole hemolysate resolves about 20 phosphopeptides with molecular weights above 18,000. The labeling patterns of the cytoplasmic and membrane fractions show no significant overlaps, demonstrating that each phosphopeptide is apportioned uniquely to one of the two cellular compartments. These data also suggest that, among all high molecular weight phosphoproteins in the cell, spectrin *2* bears the largest pool of exchangeable phosphate groups.

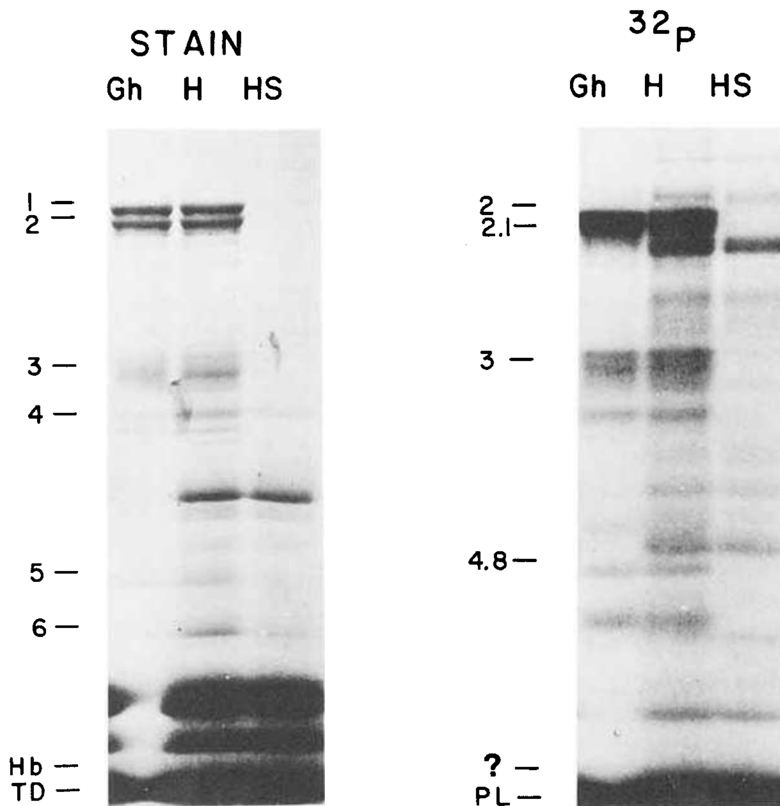

Fig. 2. Protein phosphorylation in intact human erythrocytes. Fresh cells were washed and suspended at 10% hematocrit in a modified Krebs-Ringer-bicarbonate buffer [49] containing 0.5 mM $CaCl_2$, 20 mM NaHEPES (pH 7.4), 5 mM glucose, 10 mM inosine, 1 mM adenine, and 20 μg Gentamicin/ml plus carrier-free ^{32}Pi at approximately 400 μCi/ml. The suspension was incubated at 37°C with gentle shaking and continuous gassing with humidified 5% CO_2/95% N_2. After 18 h, the cells were washed and hemolyzed [50] for membrane preparation. Samples of the hemolysate (H), hemolysate supernatant (cytoplasmic extract – HS), and washed membranes (Gh) were subjected to electrophoresis in Maizel/Laemmli slab gels [51]. The Coomassie blue staining patterns (left panel) are compared with the [^{32}P] distributions in the same lanes revealed by autoradiography (right panel). Protein from approximately 2.3 μl packed cells was applied to each lane; in H and Gh, this represented 8 μg membrane protein. Methods used in sample preparation and autoradiography of slices from stained slabs will be described in a future report.

Phosphorylation of endogenous substrates in isolated membranes requires addition of Mg^{++} and nucleoside triphosphate (either ATP or GTP, with the γ-^{32}P-labeled nucleotide as tracer). Incubation conditions have varied greatly in many respects, but, in all published labeling patterns, [^{32}P] spectrin is prominent [8–13]. The nature of the phosphorylation sites has not yet been investigated extensively, but it is known that most of the ^{32}P in labeled membranes can be recovered in the stable esters, phosphoserine and phosphothreonine [9, 10]. Our own contributions [11, 12, 26] have stressed the effects of cations and cAMP. Based on high-resolution fractionation of the substrates and the observation of differential responsiveness to cAMP, monovalent ions, and Ca^{++}, we suggested that at least two protein kinase activities are expressed independently in isolated membranes [11, 12]. The principal finding was that phosphorylation of spectrin *2* and band *3* is unaffected by cAMP, which selectively stimulates incorporation into several minor components, including *2.1* (mol. wt. ~ 200,000) and *4.5* (mol. wt. ~ 50,000), as indicated in Figure 3. Because band *2.1* migrates at the leading edge of *2*, resolution of the cAMP-dependent and cAMP-independent components of labeling in that zone requires selective elution of spectrin [12] or extended running time in electrophoresis [27]. Hosey and Tao [13, 27, 28] have contributed the useful observation that [γ-^{32}P] GTP can be utilized in the cAMP-independent phosphorylation of bands *2* and *3*, but not in the cAMP-dependent reactions.

Spectrin phosphorylation is stimulated by increasing the ionic strength up to an optimum in the range 0.1–0.2 M NaCl or KCl [11, 15]. This distinguishes it from all the other phosphorylation reactions (including those with band *3* and lipid), which are progressively inhibited with increasing ionic strength. In our early experiments we observed that addition of 1 mM Ca^{++} stimulated spectrin *2* phosphorylation 50–150% [12]. The

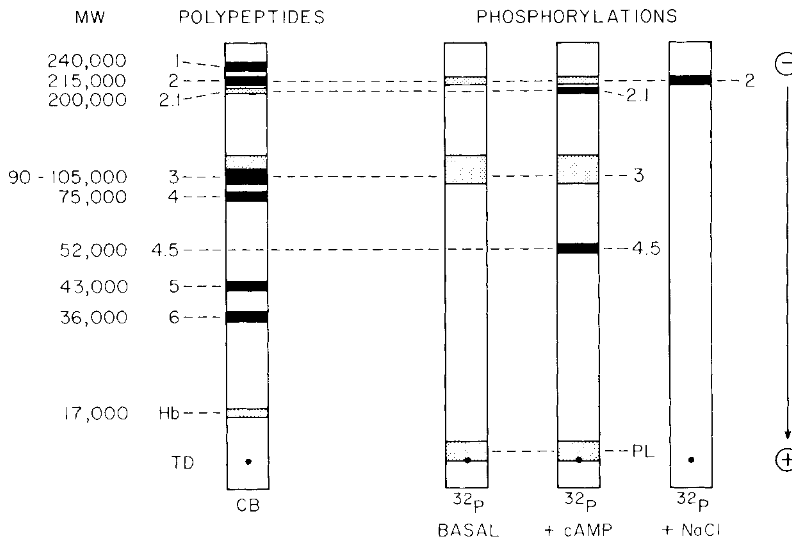

Fig. 3. Schematic representation of endogenous phosphorylation reactions. The band patterns shown are obtained by electrophoretic fractionation of membranes in sodium dodecyl sulfate–polyacrylamide gels after phosphorylation in 10-second reactions with 2 μM [γ-^{32}P] ATP in 1 mM $MgCl_2$ – 20 mM imidazole chloride (pH 7.4) [11, 12]. Addition of 20 μM cAMP or 0.2 M NaCl to the basal reaction mixture alters phosphorylation as diagrammed. Band *4.5* corresponds to the band labeled *4.8* in reports by Hosey and Tao [13, 28] and in Figure 2. [From ref. 26].

reactions were initiated by adding ghosts (to 1 mg/ml protein) to the mixture of Mg[γ-^{32}P] ATP, salts, and buffer. We suspect that Ca^{++} stimulation under these conditions is a transient effect resulting from Ca^{++}-spectrin interaction. Other laboratories have reported only inhibition by Ca^{++} [8, 9]; when we regulated the concentration of free Ca^{++} using an EGTA buffer system, the ion exerted inhibition of spectrin phosphorylation over the entire range tested – 10^{-7}–10^{-3}M [unpublished observations]. Because increased internal Ca^{++} is associated with dramatic alterations of shape in both metabolically depleted cells and reconstituted ghosts [1], the interactions of Ca^{++} with the spectrin phosphorylation–dephosphorylation system need to be examined in greater detail.

PROPERTIES OF SPECTRIN KINASE

The interpretation of the endogenous phosphorylation reactions is fraught with ambiguities because both protein kinases and substrates are semi-immobilized in the membrane, where their interactions may be subject to complex control. The isolation and characterization of spectrin kinase, the analysis of its regulation in a defined system, and the study of its mode(s) of binding to the membrane all require a quantitative assay of enzyme activity. Histone and casein kinase activities have been detected in both the cytoplasmic and membrane fractions of human erythrocytes [7, 13, 26, 29, 30]. These exogenous reactions have properties that correspond in general with the major distinguishing features of the endogenous reactions [26]. The cAMP-dependent histone kinases have not been studied extensively, because their function in a cell lacking significant levels of adenylate cyclase [31–33] is obscure. Our attention is directed to the cAMP-independent, salt-stimulated casein kinase, which appears to represent the enzyme phosphorylating spectrin *2* and, possibly, band *3* [26, 28].

Hosey and Tao have reported [29] that the membrane-associated casein kinase is unaffected by purified regulatory subunit of cAMP-dependent protein kinase under conditions that abolish activity directed against histone. We have made similar observations using the heat-stable inhibitor protein of rabbit skeletal muscle [34], which inhibits only the histone kinase activity [unpublished observations]. Both results establish that the casein kinase of the membrane is a true cAMP-independent, or type III [6], protein kinase.

Attempts to purify this enzyme have so far met with limited success. This is attributable, at least in part, to the capricious instability of the casein kinase, its tendency to aggregate at low ionic strength, and the fact that it is a minor component of the membrane. (Electrophoretic analysis of salt extracts indicates that the molar excess of spectrin over spectrin kinase must be at least 10:1 [unpublished observations].) Other laboratories have encountered the same frustrations [28, 29].

The observations that 1) spectrin phosphorylation is stimulated by salt and that 2) spectrin kinase is not eluted efficiently with spectrin at low ionic strength [11] led us to investigate systematically the effects of salt on enzyme–membrane interaction. Some results of this line of work are presented in Figure 4. As demonstrated earlier [26, 29], the casein kinase of the human red cell membrane is rapidly and selectively eluted by washing with 0.5 M NaCl at 0–4°C. It is evident in Figure 4 that the salt-elution profile for the enzyme is quite broad compared to that for glyceraldehyde-3-phosphate dehydrogenase. There is no evidence to suggest that the enzyme itself is heterogeneous, but its environment on the membrane may vary significantly. (The "tail" on the elution profile at low salt concentrations may reflect the presence of some readily dissociated aggregates, like

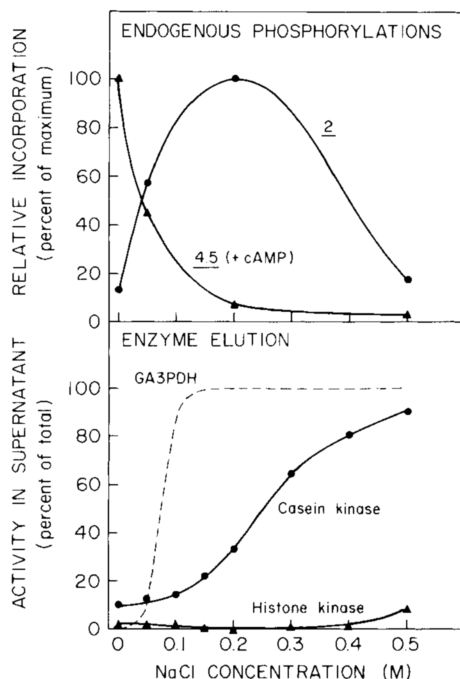

Fig. 4. Salt concentration dependence of endogenous phosphorylation reactions and protein kinase elution. Upper curves are derived from published data on the phosphorylation of spectrin *2* and band *4.5* (Fig. 3) [11]. Lower curves are enzyme elution profiles obtained in 20-min extractions of ghosts diluted 1/10 in ice-cold 5 mM sodium phosphate (pH 8) – 1 mM dithioerythritol plus salt at the indicated final concentrations. Protein kinase activities were measured as described previously [26]. Elution is expressed as percent of total activity released to the 3.5 × 10^5 g-min supernatants.

those demonstrated in Figure 5. However, it is unlikely that aggregation is responsible for the retention of casein kinase by membranes, because lysis in isotonic $NaHCO_3$–NH_4Cl does not alter partitioning between membrane and cytoplasm [unpublished observations].) It is of interest that salt concentrations that are effective in eluting casein kinase are inhibitory in the endogenous phosphorylation of spectrin *2* (Fig. 4). This suggests that the salt optimum for phosphorylation [11] may be attributed to local enhancement of enzyme–substrate interaction rather than the release of enzyme to the solution phase.

Extraction with 0.5 M NaCi has been the basis for resolving the cAMP-independent and cAMP-dependent protein kinases of the membrane [26, 28, 29]. We have extracted over 85% of the casein kinase activity while leaving at least 85% of the histone kinase in the membranes [26]. After removal of salts, the extracted membranes exhibited unimpaired phosphorylation of bands *2.1* and *4.5* in the cAMP-dependent endogenous reactions, but spectrin phosphorylation was reduced to about 15% of the original level. Significantly, when the dialyzed extract was restored to the membranes in its original proportion, spectrin *2* phosphorylation was elevated to about 50% of the level in unextracted membranes, whereas none of the cAMP-dependent reactions was altered [unpublished observations]. Selective phosphorylation by a salt extract was also demonstrated in semi-quantitative experiments by Hosey and Tao [28]. These findings constitute the best evidence available that the salt-extractable casein kinase is identical to spectrin kinase. Clearly, however, the

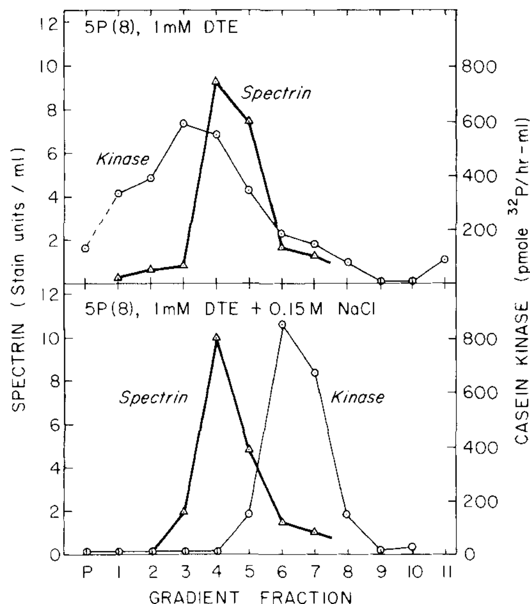

Fig. 5. Zone sedimentation analysis of spectrin and casein kinase in a low ionic strength extract. Ghosts were prepared from fresh red cells as described previously [50], except that hemolysis was carried out by 1/30 dilution into stirred 5 mM sodium phosphate (pH 8)–1 mM EDTA–0.25 mM dithioerythritol at 37°C. The extract was prepared by 1/5 dilution of packed washed ghosts into 0.1 mM EDTA (pH 8) at 37°C followed by homogenization and centrifugation for 6 × 10^6 g-min [50]. Samples (0.5 ml) were layered on 20–40% glycerol gradients (4.2 ml) containing 5 mM sodium phosphate (pH 8)–1 mM dithioerythritol, with or without saline as indicated, and centrifuged for 20 h at 50,000 rpm using a Spinco SW56Ti rotor. Gradient fractions were assayed for casein kinase activity [26]. Spectrin concentrations were determined by densitometric scanning of stained sodium dodecyl sulfate–polyacrylamide gels.

possibility that spectrin kinase simply cofractionates with other cAMP-independent protein kinase(s) cannot be excluded until the enzyme is purified to homogeneity.

In our experiments, we noted that salt extraction and reconstitution depleted and restored the capacity to phosphorylate band *3* just as described above for spectrin. Hosey and Tao [28] also noted that the salt-extracted enzyme selectively phosphorylated both spectrin *2* and band *3* in membranes in which the endogenous enzymes were inactivated by prior treatment with heat or 2,3-dimethylmaleic anhydride. The possibility that the salt-stimulated spectrin kinase phosphorylates band *3* in a salt-*inhibited* reaction is thus raised; resolution of the question awaits progress in the isolation and characterization of the salt-extracted enzyme(s).

Selective elution of spectrin, actin, and minor membrane proteins at low ionic strength has also been helpful in analyzing the organization of the spectrin phosphorylation system [11]. In repeated measurements we have determined that only about 20% of the membrane-associated casein kinase is eluted with spectrin [unpublished observations]. This correlates perfectly with the low spectrin phosphorylation in the extracts [11] and strongly suggests that most spectrin kinase is not bound to its substrate in the membrane but occupies separate sites nearby. There is no evidence that more than one enzyme is involved: The eluted and retained enzymes have the same salt optimum in the reaction with

casein; and both the enzyme eluted at low ionic strength and the total salt-extracted enzyme sediment as a single peak (apparent mol. wt. 30,000–45,000) in sucrose gradients containing salt [Fig 5, ref 29 and unpublished observations]. When spectrin eluates are fractionated by zone sedimentation in low salt, as shown in Figure 5 (upper panel), the casein kinase sediments much more rapidly and appears heterogenous. In this respect also it resembles the salt-extracted enzyme, which aggregates variably when the ionic strength is reduced [29; unpublished observations]. The sedimentation profiles of spectrin and casein kinase differ markedly, suggesting that specific interactions between the two are minimal in the low ionic strength eluate.

Table I summarizes the available information on the properties of the putative spectrin kinase present in isolated membranes. The extent to which the properties of casein kinase faithfully reflect those of spectrin kinase is a fundamental question that must be addressed in the future. Other important issues to be considered are the utilization of [γ-^{32}P]GTP by spectrin kinase and the relationship between the membrane-bound and cytoplasmic forms of cAMP-independent protein kinase.

In their analysis of the endogenous phosphorylation reactions, Hosey and Tao [13] observed that [γ-^{32}P]GTP would serve as a phosphate donor only in the cAMP-independent phosphorylation of spectrin *2* and band *3*. These results are consistent with the distinctions derived from experiments on enzyme extraction and reconstitution [26, 28]. The use of [γ-^{32}P]GTP with crude red cell fractions is problematic because of the high activity of nucleoside diphosphokinase [35], which rapidly forms [γ-^{32}P]ATP from endogenous ADP. However, labeled ATP is not produced in this fashion in assays of purified reticulocyte protein kinase [4] or in incubation of isolated membranes [28; unpublished observations]. According to Hosey and Tao [28, 29], the cAMP-independent casein kinase of the membrane cannot utilize [γ-^{32}P]GTP in phosphorylating casein but readily accepts GTP as a phosphate donor in the endogenous reaction and in the phosphorylation of heat-inactivated membranes by extracted enzyme. The mechanism by which interaction with the membrane confers the ability to accept GTP as phosphate donor is unkonwn.

Because of the metabolic dependence of casein kinase partitioning [26] (see below), the relationship between the membrane-bound and cytoplasmic forms is of great interest. The casein kinases from the two cellular compartments exhibit similar properties in zone sedimentation and gel filtration [29, 36; unpublished observations]. However, neither form

TABLE I. Properties of Membrane-Bound Casein (Spectrin) Kinase*

cAMP-independent
Salt-stimulated
Extracted by 0.5 M NaCl
Aggregates in < 0.1 M NaCl
MW 30-45,000 in > 0.1 M NaCl
Optimum pH ~ 7.5
Optimum [Mg^{++}] 2–5 mM
K_m(ATP) ~ 65 μM
GTP utilization?
Relationship to cytoplasmic casein kinase?

*K_m(ATP) and optimums for pH and Mg^{++} are data of Hosey and Tao [29] on the rabbit enzyme. The most rigorous mol. wt. estimate was also made by Hosey and Tao [29]: the value of 30,000 is based on their results in gel filtration and zone sedimentation.

has yet been isolated in high purity, so definitive information on possible heterogeneity is lacking. Hosey and Tao [27] partially purified cytoplasmic casein kinase and used it to phosphorylate membranes with [γ-^{32}P] GTP as phosphate donor. This led to the phosphorylation of several minor components in addition to spectrin *2* and band *3* and suggested that the cytoplasmic casein kinase lacks the strict specificity for the two major substrates that is characteristic of the membrane-bound enzyme. It will be important to repeat this experiment using highly purified enzyme.

SPECTRIN DEPHOSPHORYLATION

Dephosphorylation of [^{32}P] spectrin has been observed with both isolated membranes and intact cells subjected to metabolic depletion [17, 37–39]. In general, the dephosphorylation reaction has not been studied with the intensity it merits considering its co-equal status with protein kinase-mediated phosphorylation. In analyzing the kinetics of phosphorylation at 2 μM ATP, we noted that incorporation reached a plateau within 5 min and declined only slightly between 5 and 10 min [11]. As shown in Figure 6, dephosphorylation in isolated membranes is more readily demonstrated with prolonged incubation. However, in the experiment shown, [^{32}P] was released from spectrin *2* and band *3* at an initial rate of only about 50% per hour – less than one-twentieth the rate at which the proteins were phosphorylated prior to continuing incubation.

We have also explored some of the properties of red cell phosphoprotein phosphatase using isolated [^{32}P] spectrin as the substrate [40]. Very high levels of phosphospectrin phosphatase activity were detected in diluted crude cytoplasmic extracts, whereas less

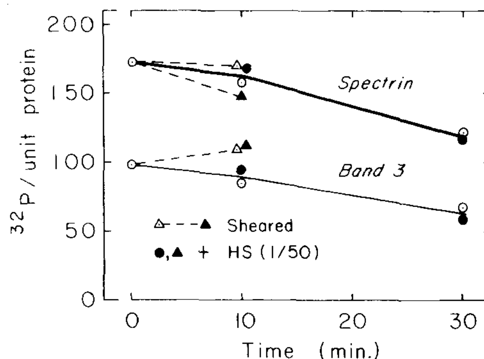

Fig. 6. Dephosphorylation of major membrane phosphopeptides. Ghosts were prepared by 1/100 dilution of washed cells in 10 mM Tris-HCl (pH 7.4)–0.25 mM dithioerythritol, washed once with the same buffer, and once with 20 mM Tris-HCl (pH 7.4)– 0.25 mM dithioerythritol. Phosphorylation of ghosts at 70/106 dilution was performed in 4.7 mM magnesium acetate–22.5 μM [γ-^{32}P] ATP. After incubation for 5 min at 37°C, the suspension was diluted with 30 volumes of cold 20 mM Tris-HCl. The ghosts were sedimented and washed once with the same buffer. Reaction mixtures for dephosphorylation contained 85 μl packed [^{32}P]-labeled ghosts plus 15 μl buffer or dialyzed hemolysate (final concentration 1/50 relative to packed cells). Incubations at 37°C were terminated by rapid addition of 1 ml 10 mM sodium borate (pH 8.3)–1 mM EDTA–5 mM N-ethylmaleimide–0.25 M sucrose–1.6% sodium dodecyl sulfate and heating for 2 min at 95°C. Two samples were sheared during the 10-min incubation using a motor-driven homogenizer with Teflon pestle rotating at 1,400 rpm. After reduction with 40 mM dithioerythritol (pH 8) for 2 min at 95°C, the samples were applied to Maizel/Laemmli [51] cylindrical gels. Counts in the spectrin and band *3* zones were normalized to corresponding areas in densitometer tracings of the stained gels.

than 1% of the total cellular activity was recovered in hemoglobin-free membranes (Table II). Triton X-100 did not significantly activate dephosphorylation of exogenous [^{32}P] spectrin by membranes. Interestingly, dialyzed cytoplasm had no effect on spectrin dephosphorylation in situ under standard conditions but appeared to accelerate dephosphorylation in suspensions that were homogenized during the first 10 min of incubation (Fig. 6). The physical basis of this effect is under study: Shearing appears to enhance the accessibility of [^{32}P] bound to spectrin on the membrane; it might act by expanding holes in the membrane to admit the added enzyme or by changing the conformation of the [^{32}P] spectrin *2* chain in the region of the phosphorylation sites. The data extant leave open the possibility that the cytoplasmic phosphatase is a nonspecific enzyme inactive against native spectrin, while a membrane-bound enzyme not detected in our assay catalyzes dephosphorylation of spectrin in the membrane. Further characterization of the enzyme(s) should resolve this question.

Some potentially significant responses to red cell metabolites were observed in our study of the cytoplasmic phosphatase (Table III). Like other phosphoprotein phosphatases [41], the enzyme is inhibited by fluoride ions. In addition, it is strongly inhibited by inorganic and organic phosphates, particularly those containing multiple phosphate groups. The effects of these compounds have so far been tested only in the absence of Mg^{++}, which in other systems moderates inhibition strikingly [42]. The effect of Mg^{++} may account for the apparent discrepancy between the ATP inhibition we observed in the reaction with solubilized [^{32}P] spectrin and the reported dephosphorylation of spectrin in membranes incubated with MgATP [37–39]. In any case, the concentrations of free 2,3-DPG and ATP in oxygenated red cells are sufficiently high [43] to suppress phosphospectrin phosphatase completely if its regulation in vivo resembles that in the cell-free assay system.

REGULATION

There is no direct evidence that either spectrin kinase or phosphospectrin phosphatase in the human red cell changes activity in response to extracellular signals. Hence, our discussion of regulation is confined to potential roles of metabolic intermediates in modulating enzyme activity and localization and to possible effects of local deformation on enzyme–substrate interaction.

TABLE II. Properties of Human Erythrocyte Phosphospectrin Phosphatase*

Assay conditions		
Substrate:	[^{32}P] spectrin, 1 mg/ml (~ 1X re packed cells)	
Crude enzyme:	Hemolysate supernatant, 0.1 mg/ml protein (~ 1/6000X re packed cells)	
Activity:	Initial rate ~ 60% [^{32}P] release/h at 37°C	
Optimum milieu		
pH ~ 7		
Salt ~ 0.1 M NaCl or KCl		
Subcellular distribution		
Hemolysate super + 1st wash		100%
2nd wash		0
Ghosts		~0.04

*Data from ref 40 and unpublished observations.

Changes in the oxygenation state of hemoglobin alter levels of free ATP, 2,3-DPG and Mg^{++} [43] and may be reflected in the state of spectrin phosphorylation. On the venous side of the circulation, binding of 2,3-DPG to deoxyhemoglobin sharply reduces the level of free 2,3-DPG while elevating the concentration of free Mg^{++}. Under this condition, the casein (spectrin) kinase would be activated due to the increase in Mg^{++} below its optimum concentration (Table I) [29] and, possibly, release from modest inhibition by 2,3-DPG [29]. At the same time, the phosphospectrin phosphatase activity would also be sharply activated as free ATP and 2,3-DPG were sequestered and prevented from exerting their strong inhibition (Table III). The net result could be sharply accelerated turnover of spectrin-bound phosphate. This prediction has yet to be tested, but is of interest because a plausible function for this mode of regulation can be put forth. The necessary assumptions are 1) that spectrin phosphorylation promotes its binding to actin, thereby contributing to the establishment of long-range order in the spectrin–actin lattice [19–21]; and 2) that passage through capillaries is sufficiently traumatic to cause perturbations in membrane organization that require periodic repair. The acceleration of spectrin phosphate turnover in the low-shear environment of the venous circulation would then allow the rearrangement of the spectrin–actin lattice and a realignment of its elements with underlying proteins [25, 44, 45] to establish the optimal state for the maintenance of membrane integrity and deformability under subsequent shear deformation.

We have suggested elsewhere [26] that spectrin phosphorylation might be regulated by translocations of spectrin kinase between membrane and cytoplasm. This speculation was based on the observation that partitioning of casein kinase in the red cell is apparently under metabolic control. Membranes isolated from fresh red cells normally contain about 40% of the total cellular casein kinase activity. However, overnight metabolic depletion elevates membrane retention of casein kinase to a maximum of 75%, whereas incubation with adenine, inosine, and glucose, which elevates intracellular ATP levels [46], reduces the fraction of total enzyme on the membrane to about 25% [26; unpublished observations]. When the incubations are done in a nitrogen atmosphere, these shifts in enzyme localization occur without change in total cellular casein kinase activity.

TABLE III. Inhibitors of Phosphospectrin Phosphatase*

> 75% Inhibition	25–75% Inhibition	< 25% Inhibition
ATP, GTP (0.1 mM)		
ADP, GDP (1 mM)	AMP, GMP (1 mM)	Adenosine (1 mM)
2,3-DPG (5 mM)	2,3-DPG (1 mM)	2,3-DPG (0.5 mM)
PPi (0.1 mM)		
Pi (10mM)	Pi (1 mM)	Pi (0.1 mM)
F^- (10 mM)		F^- (0.1 mM)
	Mg^{++} (10 mM)	Mg^{++} (1 mM)
	Mn^{++} (10 mM)	Mn^{++} (1 mM)
	Ca^{++} (10 mM)	Ca^{++} (1 mM)
		EGTA (1 mM)
		EDTA (1 mM)

*Erythrocyte cytoplasm diluted 1/12000 was incubated at 37°C with 42 μg [^{32}P]spectrin in 0.05 ml mixtures containing 20 mM NaHEPES (pH 7.0)–0.1 M KCl–4 mM dithiothreitol–1 mM EDTA plus additions at the concentrations indicated (EDTA omitted in tests of EGTA and divalent cations). Inhibition was calculated as percent reduction in [^{32}Pi] release relative to controls incubated without additions.

Figure 7 shows the results of two attempts to correlate the endogenous phosphorylation reactions in isolated membranes with the increase in protein kinase activity induced by metabolic depletion. Because the membranes from depleted cells contain higher levels of casein kinase, and because the acceptor capacity of the substrate, spectrin, is presumably elevated due to progressive dephosphorylation [37,38], we expected to observe a significant increase in the rate of spectrin phosphorylation in the endogenous reaction. In fact, we consistently produce the paradoxical finding illustrated – a slight reduction in spectrin phosphorylation in the face of a 40–70% increase in membrane-bound casein kinase [unpublished observations]. The accessibility of a membrane inner surface marker, glyceraldehyde-3-phosphate dehydrogenase, is nearly 100% for both membrane preparations, so the results cannot be attributed to preferential resealing of membranes from depleted cells.

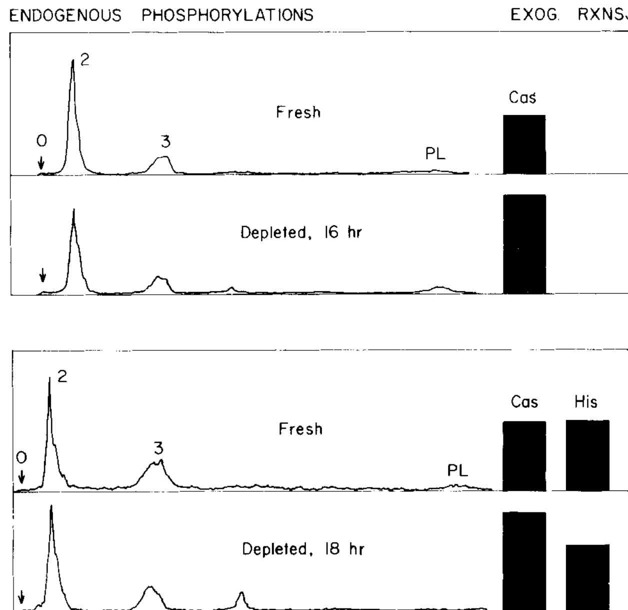

Fig. 7. Endogenous phosphorylation reactions in membranes from fresh and metabolically depleted cells. Red cells were depleted metabolically by overnight incubation in modified Krebs–Ringer–bicarbonate as described in Figure 2, but in the absence of adenine, inosine, and glucose. Fresh cells were drawn from the same donor on the second day and washed in the same medium. Leaky ghosts were prepared from the washed cells by hemolysis in 5 mM sodium phosphate–1 mM EDTA–0.25 mM dithioerythritol at 37°C, as described in Figure 5. The membranes were phosphorylated in 20-sec reactions with 2 μM [γ-^{32}P] ATP in 5 mM magnesium acetate–20 mM NaHEPES (pH 7.5). The reactions were terminated by adding 1.1% sodium dodecyl sulfate–5.6 mM EDTA–0.24 M sucrose–14 mM dithioerythritol (final concentrations) and incubating in a boiling water bath for 2 min. The solubilized proteins were resolved by electrophoresis in Tris-acetate-EDTA gels at pH 7.4 [50]. The [^{32}P] labeling patterns are densitometer scans of autoradiographs of dried longitudinal slices [11]. Casein and histone kinase activities (Cas and His, respectively, at the right) in the membranes were measured as described previously [26] and are displayed as vertical bars with arbitrary units.

These findings raise questions that cannot be answered on the basis of current knowledge of the spectrin phosphorylation–dephosphorylation system:

1. Is the extra membrane-bound casein kinase a distinct enzyme that is unable to utilize spectrin or other major membrane proteins as its substrate? This would imply that the cytoplasmic form of casein kinase is not identical to spectrin kinase.
2. Is the extra enzyme bound in a fashion that renders it nonfunctional? Possibilities include aggregation or localization at sites remote from the phosphorylated segment of spectrin *2*.
3. Does metabolic depletion induce membrane alterations that hinder the normal interaction between spectrin and its kinase? Palek and associates [47, 48] have demonstrated changes in susceptibility to oxidative cross-linking in membranes of cells depleted under nitrogen. We postulate that membrane reorganization under these conditions leads to a state in which phosphate acceptor sites on the protein are less accessible to the kinase.

Clearly, the ultimate resolution of the apparent paradox of Figure 7 will require further comparative studies of the membrane and cytoplasmic casein kinase(s) together with a definitive analysis of enzyme binding to the membrane under various conditions.

Both models considered above involve chemical signals that modify enzyme activity or localization. The schemes relegate spectrin phosphorylation to a secondary role in relation to other cellular functions because they do not envision a mechanism for feedback regulation. In fact, if spectrin phosphorylation indeed controls red cell shape and membrane material properties, the regulatory apparatus should have the capacity to sense deviations from optimal shape or alterations in membrane organization induced by deformation. We find it intriguing that most of the membrane-bound spectrin kinase activity is apparently not associated with spectrin itself but is bound to some other site on the membrane. Given this mode of organization, it is conceivable that physical stress on the membrane alters the intermolecular distance between spectrin and spectrin kinase or shifts spectrin conformation to promote or inhibit the phosphorylation reaction. Such local effects would permit the spectrin-phosphorylation system to serve as a sensor for membrane deformation and would provide feedback regulation without the elaboration of chemical signals.

Although much progress has been made in describing some properties of the enzymes involved in phosphorylation and dephosphorylation, as well as in defining membrane elements that respond to alterations in phosphorylation state, most of the data are indirect and compromised by major ambiguities. As we have repeatedly stated above, isolation and characterization of membrane and cytoplasmic forms of casein kinase and phosphospectrin phosphatase is indispensable. Ultimately, this will lead to definitive identification of the enzymes involved and will permit the analysis of enzyme regulation in defined systems in which metabolite interconversion is precluded. Work in our laboratories is directed at the characterization of casein (spectrin) kinase binding sites on the membrane and at analysis of effects of membrane deformation on spectrin phosphorylation. Further knowledge of these may clarify our ideas concerning regulation at the level of enzyme–substrate interaction. We are hopeful that, just as the study of the red cell membrane has contributed greatly to present understanding of membrane structure in general, continued investigation of the spectrin phosphorylation–dephosphorylation system may lead to useful insights into the more complex mechanisms of cAMP-independent regulation of protein phosphorylation in nucleated cells.

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