Erythroid Membrane-bound Protein Kinase Binds to a Membrane Component and Is Regulated by Phosphatidylinositol 4,5-Bisphosphate*

Chantal E. Bazenet, Jennifer L. Brockman, Dwyane Lewis, Catherine Chan, and Richard A. Anderson†
From the Department of Pharmacology and the Cell and Molecular Biology Program, University of Wisconsin Medical School, Madison, Wisconsin 53706

In the erythrocyte, a membrane-bound serine/threonine protein kinase (a casein kinase) has been shown to phosphorylate a number of membrane proteins, modulating their function. Here we report that the membrane-bound protein kinase binds to membranes by an association with a minor membrane component contained in preparations of glycophorin (possibly a minor glycophorin). The binding of the kinase to glycophorins does not significantly modify kinase activity. However, upon binding, the kinase activity is potently inhibited by phosphatidylinositol 4,5-bisphosphate, and the affinity of the kinase for the glycophorins is increased. Other phospholipids or polyanions such as inositol 1,4,5-trisphosphate or 2,3-diphosphoglycerate do not affect protein kinase activity when the kinase is bound to membranes but do inhibit the solubilized membrane-bound kinase. In the erythrocyte, there is a cytosolic form of the casein kinase which is very similar, having the same molecular weight and substrate specificity as the membrane-bound casein kinase. The cytosolic casein kinase is inhibited by 2,3-diphosphoglycerate but much less so by glycophorin preparations containing phosphatidylinositol 4,5-bisphosphate. When the sequences of both casein kinases were compared by two-dimensional peptide mapping, it was found that the two kinases were very similar but not identical.

The turnover of phosphoryl groups on membrane proteins and especially the inositol lipids represents a large fraction of the total metabolic energy expended by the erythrocyte. Phosphorylation of membrane skeletal proteins has been studied in detail, demonstrating that a number of associations are regulated by protein phosphorylation (1). However, at the cellular level there appears to be little convincing evidence that protein phosphorylation has any effect on mature erythrocyte function, such as maintaining the discocyte shape (2). Certainly, ATP is required for maintaining the discoid shape and phosphorylation appears to be phosphorylation of the inositol lipids. Indeed, a reduction in the membrane content of the polyphosphoinositides, in particular phosphatidylinositol 4,5-bisphosphate (PIP₂), appears to be linked to the formation of the echinocyte shape (4, 5).

In the erythrocyte, the function of the rapid turnover of phosphoryl residues on the inositol lipids is unknown and perplexing. In other cells, the polyphosphoinositides act as precursors to the second messengers inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, which cause calcium release from internal stores and activate protein kinase C, respectively. In erythrocytes, the polyphosphoinositides do not act as second messenger precursors. Indeed, the cleavage of the polyphosphoinositides by phospholipase C, which is stimulated by calcium, is irreversible since the inositol lipids cannot be synthesized in the erythrocyte. As such, if the turnover of phosphoryl residues on the inositol lipids is important for the normal function of the cell (which appears to be the case), then the polyphosphoinositides must have their impact on the plasma membrane. Examples of regulation of this kind have been identified; in nucleated cells, both profilactin and gelsolin associate with PIP₂ on the plasma membrane.

In the erythrocyte, protein 4.1 requires PIP₂ for a high affinity interaction with the membrane (5). In this case, a class of membrane proteins seems to interact specifically with PIP₂ (6–9), and this results in high affinity binding to protein 4.1 on the plasma membrane. This class of transmembrane glycoproteins, glycophorins, is structurally similar and sequence homologous (10–13). These transmembrane proteins have properties unique from most integral membrane proteins in that once extracted from membranes they are soluble in aqueous buffers in the absence of detergent. This property results from formation of a very stable protein-phospholipid (PL) micelle structure containing 15–20 glycophorin and 40–70 PL molecules (5, 14–16). Previously, it has been demonstrated that this structure retains transmembrane proteins in a biologically active state (5). Finally, when glycophorin-PIP₂ micelles were covalently linked to Sepharose CL-4B, this was an effective affinity matrix for purifying protein 4.1 (17).

* This work was supported by the University of Wisconsin Medical School, National Institutes of Health Grant GM 38906, Basil O'Connor Starter Scholar Research Award 5-659, and American Cancer Society Grant BC-630. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Pharmacology, University of Wisconsin Medical School, 1300 University Ave., Madison, WI 53706. Tel.: 608-262-3753.

‡ The abbreviations used are: PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₃, phosphatidylinositol 4-phosphate; PL(s), phospholipid(s); 2,3-DPG, 2,3-diphosphoglycerate; PMSF, phenylmethylsulfonyl fluoride; DPF, dipropylfluorophosphosphate; PC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatic acid; EGTA, [ethylenebis(oxyethylenemethyl)iminato]tetraacetic acid; I2O(s), inside-out membrane vesicle(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MB, membrane-bound.
The erythrocyte membrane-bound protein kinase (a casein kinase) phosphorylates a number of membrane proteins, modulating their function. These include spectrin, ankyrin, aducin, proteins 3, 4.1, 4.9, and other proteins; the kinase is also autophosphorylated (1, 18–21). The phosphorylation of ankyrin and protein 4.1 by the membrane-bound protein kinase appears to lower the affinity of their interactions with spectrin, possibly resulting in disassembly of the membrane skeleton (18, 19). The phosphorylation of ankyrin reduces its affinity for spectrin tetramers and oligomers (20). Phosphorylation of protein 4.1 reduces the affinity of the spectrin–protein 4.1 association (18). The effect this has on the ability of protein kinase which is cytosolic (22, 23). The cytosolic casein kinase does not appear to be regulated by the mechanism of regulation is not known.

Erythrocytes contain a counterpart of the membrane-bound protein kinase which is cytosolic (22, 23). The cytosolic casein kinase has an identical molecular mass (33–34 kDa) and has the same substrate specificity (18–24). Indeed, the cytosolic and membrane-bound protein kinases have been used interchangeably when studying the effects of protein kinase phosphorylation on membrane proteins (18, 20, 21, 24).

Although the effects of several protein kinases on the function of erythrocyte membrane proteins have been studied, most of these protein kinases have no apparent mechanism for activation intrinsic to the erythrocytes (1). However, the cytosolic casein kinase does appear to be regulated by the concentration of 2,3-DPG (23), an important modulator of erythrocyte function. In the case of the membrane-bound protein kinase, the mechanism of regulation is not known. Here we report that the membrane-bound protein kinase associates with a membrane component isolated in preparations of glycophorin (possibly a minor glycophorin) and is inhibited by PIP$	ext{$_2$}$.

The cytosolic protein kinase, although structurally similar, does not bind to membranes and does not interact with glycophorin–PIP$	ext{$_2$}$.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fresh human blood was obtained from the Wisconsin Blood Center within 4 days of the drawing date. [γ-$^{32}$P]ATP was purchased from either Du Pont–New England Nuclear or Amersham Corp. Leupeptin, pepstatin A, aprotinin, PMSF, DFP, $\text{L}$-$\text{L}$-phenylalanine, antipain, benzamidine, and benzamidine were purchased from Sigma. 3,5-Diiodosalicylic acid purchased from Kodak was re-crystallized twice from methanol, and the lithium salt was prepared by neutralization with lithium hydroxide followed by recrystallization from water. PIP$	ext{$_2$}$ and PIP were prepared from bovine brain by phosphatidylinositide (PI) hydrolysis (25, 26). Other phospholipids, PC (phosphatidylcholine), PE (bovine brain), PS (bovine brain), PA (egg), PI (bovine brain), and sphingomyelin (bovine brain) were from Avanti Polar Lipids. All other reagents were analytical grade.

**Preparation of Erythrocyte Membranes and Proteins**—Human erythrocytes were isolated and membranes prepared as before (18, 20, 27, 28) by hypotonic lysis in 10 volumes of 5 mM sodium phosphate, 1 mM EDTA, 1 mM diethiothreitol, 0.1 mM DFP, 20 μg/ml PMSF, 1 μg/ml peptatin A, benzamidine, and $\text{L}$-$\text{L}$-phenylalanine at pH 8.0. The membrane-bound protein kinase was extracted from these membranes with 0.5 M NaCl, 5 mM sodium phosphate, 5 mM EDTA, 10 μg/ml each of $\text{L}$-$\text{L}$-phenylalanine and benzamidine, and 3 μg/ml each of leupeptin, bestatin, and peptatin A at pH 7.5 at 0°C for 30 min (19). Inside-out membrane vesicles were prepared from membranes washed free of hemoglobin by extraction with 0.1 mM EDTA, 0.1 mM DFP, pH 8.5, at 37°C for 30 min and then washed once in the same buffer. The resulting inside-out membrane vesicles (IOVs) were extracted with 0.5 M NaCl as above. Initial experiments were done with protein kinase isolated by the method of Tao et al. (19).

More recent experiments have used a modification of the method of Eder et al. (18). Briefly, the 0.5 M NaCl supernatant from either membranes or IOVs was centrifuged at 250,000 × g and dialyzed against 150 mM KCl, 10 mM sodium phosphate, 2 mM diethiothreitol, 1 mM EDTA, 20 μg/ml PMSF, 0.1 mM DFP, 10% (v/v) glycerol, pH 7.5 (buffer A) and applied to a phosphocellulose column. The membrane-bound protein kinase was eluted with a 150–600 mM NaCl gradient. The fractions containing membrane kinase activity were pooled, dialyzed against 20 mM Tris, 0.5 mM EGTA, 0.1 mM PMSF, 0.1 mM DFP, 10% glycerol, pH 7.0 (buffer B), and applied to a DEAE-Sepharose column and eluted with a 0–250 mM NaCl gradient. The membrane-bound protein kinase eluted at the 80% pure. The cytosolic casein kinase was prepared according to Boivin and Gandal (23) and Simkowski and Tao (22) with modifications. The lysis in 6 liters of packed cells, was precipitated at 45% saturation of ammonium sulfate (pH 6.8). The pellet was washed once with the same solution, dialyzed against buffer A, applied to a phosphocellulose column (30 ml), and eluted with a 0.2–1.0 M NaCl gradient. Fractions containing kinase activity were pooled and applied to a Q-Sepharose column equilibrated with buffer B. The Q-Sepharose column was eluted with a 0–250 mM KCl gradient. The purified cytosolic casein kinase on SDS-PAGE is also a 33- and 34-kDa doublet, and this method yields a protein preparation that is greater than 95% pure.

**Protein Kinase Assays**—Protein kinase assays were similar to the method of Tao et al. (19). The protein kinases were combined with substrate (casein or spectrin) with or without other components such as glycoporphin micelles, phospholipids, or IOVs. All components were in isotonic KCl buffer, NaCl, 15 mM MgCl$_2$, 0.1 mM EDTA, 0.1 mM DFP and PMSF, 0.1 mM EGTA, pH 7.5. The phosphorylation was started by addition of 0.2 mM phosphorylation buffer (0.2 M Tris·HCl, 20 mM MgCl$_2$), either 2.5 μM [γ-$^3$P]ATP (30 Ci/mmol) or 0.25 mM [γ-$^{32}$P]ATP (30 or 300 Ci/ml), at pH 7.5. The final volume of the reaction was 50 μl; the amount of substrate (casein or spectrin) was per reaction. The course of phosphorylation for quantitative experiments was always in the linear range. The phosphorylation was stopped and quantified by one of two methods: (a) addition of 4 × concentrated SDS PAGE sample buffer (28), the solution was then applied to SDS-PAGE, after fixing and staining the gel, the spectrin bands were excised and counted; (b) addition of trichloroacetic acid to 5% and isolation of the $^{32}$P-labeled proteins by precipitation on filter paper according to Tao et al. (19). For quantitation, the gel slices were first solubilized in 0.5 ml of 1%–2% trisubstituted (RIP) and then counted in a β-counter with 2 ml of scintillation fluid. The $^{32}$P on the filter papers was also counted in 2 ml of scintillation fluid. One unit of kinase activity was defined according to Tao et al. (19).

**Glycophorin Micelles, Liposomes, and Affinity Chromatography**—Glycophorin was isolated by the lithium diiodosalicylic-pheno1 method (29); the intrinsic phospholipids were extracted, and glycophorin was desialated as before (5, 7, 27). For the chromatographic separation of the glycophorins, the crude lithium diiodosalicylic acid preparation of the glycophorins was applied to a 2.5 × 100-cm column containing Bio-Gel A-1.5 as described previously (14, 30). The fractions were analyzed by SDS-PAGE and silver staining; fractions enriched in a specific glycophorin were pooled and precipitated with ethanol. The glycophorins in the fractions were then extracted of intrinsic phospholipid and desialated (5, 7). The glycophorins were then applied to 10 mg/ml, dialyzed against isocotic KCl buffer, and stored as glycoporphin-phospholipid mixed micelles and phospholipid liposomes containing glycophorin as prepared before without modification (5, 15, 27, 31).

**Affinity Chromatography**—The glycoporphin-Affi-Gel 15 affinity matrix was prepared as described previously (17). Briefly, micelles of glycrophorin containing in phospholipid (mainly PS and PIP) were desialated and covalently attached to the Affi-Gel 15 beads in 150 mM KCl, 20 mM HEPES, pH 8.0. This matrix was washed with 1 M KCl and then equilibrated with isocotic KCl buffer containing 2 mM Mgl$_2$. A 0.5 M NaCl extract from ghost membranes was dialyzed against isocotic KCl containing 2 mM Mgl$_2$ and applied to a glycoporphin-Affi-Gel 15 column equilibrated with the same buffer. The column was washed, and bound protein eluted with 1 M KCl (17). Fractions were analyzed by SDS-PAGE with silver staining. As a control, the 0.5 M NaCl extract was applied and eluted from an Affi-Gel 15 column to which glycophorin was added; no protein kinase activity was detected.

**Inhibition of Membrane-bound Protein Kinase Binding to IOVs**—IOVs prepared from fresh erythrocytes retain the membrane-bound protein kinase activity (1, 19). To quantitate glycoporphin PL competition with IOVs for membrane-bound protein kinase binding, glycoporphin-PL micelles were combined with 60 μg of IOV protein (150 μl) in isocotic KCl for 1 h on ice or for 15 min at 20°C. The IOVs were then centrifuged through a 10% sucrose cushion separating
the IOVs completely from the glycophorin micelles (5). The ratio of P2s to glycophorin for these experiments was 41–51 nmol of P2/mg of glycophorin, except P2p and P2s, which were 42 and 192 nmol/mg of glycophorin, respectively. In micelles containing P2s, increasing P2s from 164 nmol/mg of glycophorin did not alter inhibition of binding or activity. The IOVs were resuspended in isotonic KCl with 0.01% Triton X-100; 20 μg of spectrin was added as substrate. Substrate phosphorylation was started as above. The total 32P incorporation into spectrin in 15 min was used to determine kinase activity. Incorporation of 32P into band 3 and ankyrin was quantitatively the same as into spectrin, indicating that substrate effects are not involved.

**RESULTS**

**Affinity Chromatography of the Membrane-bound Protein Kinase on Glycophorin-Affi-Gel 15**—In the process of studying the phosphorylation of protein 4.1 and its interaction with membranes, it was found that a protein kinase copurified with protein 4.1 (33). In an attempt to separate protein 4.1 and the kinase activity, the isolated protein 4.1 was applied to a 7–15% SDS-PAGE, fixed, and stained with Coomassie Blue. The stained bands corresponding to the protein kinases were excised and labeled with 32P as before (23, 32). The electrophoresis and thin-layer chromatography (TLC) were as before (23), but TLC plates were glass backed with a 0.1-mm thickness of cellulose (EM reagents). 100 emu of 32P/dalton of protein molecular mass was loaded/plate.

To demonstrate more clearly that the protein kinase does not require protein 4.1 for binding to glycophorin-Affi-Gel 15, the protein kinase was extracted from intact erythrocyte membranes with 0.5 M NaCl at 0 °C, conditions that do not extract protein 4.1. This extract, containing a large number of proteins, was applied to a glycophorin-Affi-Gel 15 column (see "Experimental Procedures") and eluted with 1 M KCl. When the fractions were assayed for protein kinase activity, using casein or spectrin as substrate, a 1-ml column (0.1 mg/ml of glycophorin) retained 82% of the protein kinase activity when 0.2 unit of protein kinase activity was applied (average of three experiments). Analysis of fractions by SDS-PAGE showed that four proteins were retained by the column: two bands (100 and 105 kDa) that are the protein adducin (1), a 55-kDa band that was not identified, and a 33–34-kDa doublet. The 33–34-kDa doublet has the same molecular mass as the kinase that copurifies with protein 4.1. A characteristic of many protein kinases is autophosphorylation. To determine if the 33–34-kDa bands were phosphorylated (perhaps auto-phosphorylated), an aliquot of each fraction was incubated with 50 μM [γ-32P]ATP (300 Ci/mol) and applied to a 7–15% acrylamide SDS-PAGE; the autoradiograms of the SDS-PAGE of the 0.5 M NaCl extract from membranes, the flow-through from the glycophorin column, and the 1 M KCl elution are shown in Fig. 1. Kinase activity toward other proteins in the 0.5 M NaCl extract is largely removed by the glycophorin column (Fig. 1, lanes A and B). The 1 M KCl elution shows phosphorylation of only two bands, the 100-kDa subunit of adducin and a 34-kDa band that corresponds with the protein kinase activity.

A membrane-bound (MB) protein kinase has been purified previously by Tao et al. (19) which has characteristics indistinguishable from those of the protein kinase that binds glycophorins. To determine if the protein kinase retained by glycophorin-Affi-Gel is similar to the MB protein kinase, we have purified the MB protein kinase by the method of Tao et al. (19). When compared, the protein kinase that binds to glycophorin-Affi-Gel and the MB protein kinase were found to be indistinguishable. Both kinases are eluted from membranes by high ionic strength, phosphorylate casein and spectrin preferentially, bind to and are eluted from DEAE-cellulose and phosphocellulose at the same ionic strength, are doublets on SDS-PAGE with molecular masses of 33 and 34 kDa, and are autophosphorylated (Ref. 19 and Fig. 1). When the protein kinase is eluted from the glycophorin affinity column and compared with the MB protein kinase by one-dimensional peptide mapping (32), the kinases have identical 32P-peptide maps (Fig. 1, lanes E–L). By these criteria, the protein kinase that binds to the glycophorin affinity column is indistinguishable from the MB protein kinase isolated by Tao et al. (19) and therefore will be referred to as the MB protein kinase.

These results suggest that the MB protein kinase associates with a glycophorin or a component within the glycophorin-PL micelle and perhaps binds to the membrane by an association with this same component. To test this hypothesis,
the ability of glycophrin preparations retaining intrinsic PL to compete with inside-out erythrocyte membrane vesicles for kinase binding was measured. To assay for the MB protein kinase, spectrin or casein was used as substrate. Sperctrin and casein are substrates that are specifically phosphorylated by the MB protein kinase, not by cAMP-dependent protein kinase, which is also known to be associated with human erythrocyte membranes (for review, see Ref. 1). Indeed, the only protein kinase associated with erythrocyte membranes which phosphorates spectrin is the MB protein kinase; thus, spectrin phosphorylation is a selective method to quantitate MB protein kinase.

Competition of MB protein kinase binding to IOVs by glycophrin micelles was measured by combining IOVs and micelles; after an incubation (see "Experimental Procedures"), the IOVs were sedimented through a 10% sucrose cushion. Using this method, the IOVs were separated completely from the glycophrin-PL micelles as demonstrated previously (5). Separation was also checked by applying the IOVs to an SDS-PAGE and staining with silver. The denatured glycophrin stains very intensely with silver and migrates with a different apparent molecular weight than does native glycophrin. By this criteria, no measurable micellar glycophrin was retained by the IOVs.

The amount of MB protein kinase retained by IOVs was assayed by dissolving the IOV pellet in buffered 0.01% Triton X-100 and then determining kinase activity toward spectrin. The addition of Triton X-100 to the IOVs had two effects. (a) The membranes were solubilized, and thus kinase was completely accessible to substrate. (b) Addition of Triton X-100 to membranes increased IOV-associated casein kinase activity toward spectrin. Addition of Triton X-100 appears to eliminate kinase activity due to membrane binding. This cannot be explained by exposure of trapped kinase by Triton X-100-induced lysis of IOVs since glycophrin-intrinsic PL micelles can completely remove all MB protein kinase activity from the IOVs (Fig. 2). As demonstrated in Fig. 2, increasing the concentration of the glycophrin micelles containing intrinsic PL eluted greater than 95% of the MB protein kinase activity from the IOVs. To demonstrate that the kinase activity was in the supernatant, the supernatants containing glycophrin micelles were assayed after addition of Triton X-100 to the supernatant. In a representative experiment (see Fig. 9), glycophrin micelles with intrinsic PL (25 mg/ml) eluted 83% of the MB protein kinase activity from IOVs (500 mg/ml). Upon addition of Triton X-100, 68% of the protein kinase activity was recovered in the supernatant. This experiment demonstrates that the majority of the MB protein kinase has been eluted from the membrane and was recovered in the supernatant. Further, this experiment also suggests that the intact structure of both IOVs and glycophrin-PL micelles is required for inhibition of kinase activity.

To determine the role played by intrinsic PLs retained by isolated glycophrin micelles, the PLs were extracted, and both glycophrin micelles and the extracted PLs were tested for the ability to elute MB protein kinase activity from the membrane. Glycophrin micelles lacking phospholipid were found to compete with MB protein kinase binding but much less effectively than glycophrin containing intrinsic phospholipid (Fig. 2). The phospholipid fraction, containing mainly phosphatidylycerine (PS), phosphatidylserine (PS), and phosphatidylcholine (PC), had a 4:1:1 m ratio (6-9), was suspended in buffer, sonicated, and combined with IOVs. These phospholipids depleted IOVs of 50% of the MB protein kinase activity only at 33 mg/ml PIP2. This amount of PIP2 is equivalent to that contained in 1 mg/ml of glycophrin retaining intrinsic phospholipid. This suggests that the MB protein kinase requires both glycophrin and PIP2 for a high affinity association.

Since intrinsic PL in the glycophrin micelle enhanced the affinity of MB protein kinase for glycophrin, micellar glycophrin was reconstituted with a variety of PLs to determine if a specific PL was required. The glycophrin-PL micelles were then tested for their ability to deplete the MB protein kinase content of membranes. As demonstrated in Fig. 2, glycophrin-PL micelles must contain PIP2 to be effective at depleting membranes of MB protein kinase activity. However, for maximal affinity, the glycophrin-PL micelles must have PIP2 and in addition 1.4 mg/ml of a negatively charged PL (PS or PA). However, PA and PS only enhanced the effect of PIP2; alone they are not effective at inhibiting MB protein kinase binding to IOVs.

Since the glycophrin-PL micelles were effective at competing with membranes for MB protein kinase binding, the glycophrin-PL micelles were assayed for their effect on MB protein kinase activity. The results show that the catalytic activity of the MB protein kinase is inhibited by glycophrin-PL micelles containing intrinsic PL. As with inhibition of binding to membranes, extraction of the intrinsic PL from the glycophrin destroyed the ability of the glycophrin micelles to inhibit kinase activity. Further, the extracted intrinsic PLs and glycophrins without PL did not inhibit protein kinase activity. When micellar glycophrin was reconstituted with PL by detergent dialysis (5), inhibition of MB protein kinase activity was also recovered but only when PIP2 was reconstituted into the glycophrin micelles (Table I). Other PLs reconstituted into glycophrin-PL micelles did not inhibit the MB protein kinase activity. However, when both PIP2 and either FS or PA were incorporated into micelles, the catalytic activity of the MB protein kinase was potently inhibited by glycophrin-PL micelles.

To ascertain if glycophrin and PIP2 in an environment more like the native membrane also regulate the MB protein...
absence of PIP2, there was a relatively small decrease in kinase activity. However, liposomes containing glycophorin and PIP2 showed a more pronounced inhibition of MB protein kinase activity. This inhibition is dependent upon the presence of PIP2 in the liposomes; other PLs did not elicit this effect. Further, both the glycophorins and PIP2 must be present in liposomes for inhibition of MB protein kinase activity. PIP2 alone in liposomes does not inhibit the MB protein kinase, even at high concentrations of liposomes or at high mol % of PIP2 in liposomes (up to 5 mol %).

Glycophorin preparations contain at least four membrane proteins (10-12, 14, 28, 29, 32). To determine if a specific component was required for the interaction with the MB protein kinase, the glycophorin preparation was fractionated by gel permeation chromatography (14, 29). The resulting pooled fractions (Fig. 5) were reconstituted with PIP2, PS, and PA as above and assayed for their effectiveness at inhibiting MB protein kinase. Fractions C and D containing the minor glycophorins B, C, and D and other components in lesser amounts were the most effective at inhibiting MB protein kinase activity. Although this experiment does not conclusively identify the membrane component that interacts with the MB protein kinase, it does demonstrate that the MB protein kinase interacts with a specific component in the preparation. Glycophorin A does not appear to interact with the kinase since it is the major component of fraction A but does not inhibit kinase activity effectively.

The erythrocyte has two forms of casein kinase: a membrane-bound and a cytosolic form. Both protein kinases have very similar properties. They have an identical molecular mass by SDS-PAGE of 33-34 kDa, both are eluted from ion exchange columns at the same salt concentration, and both appear to have the same substrate specificity (18-24). The difference is that one protein kinase is membrane associated, and the other is soluble. The erythrocyte cytosolic casein kinase has been shown to be inhibited by 2,3-DPG with a Ki of 4.6 mM (23). The MB protein kinase is also inhibited by

### Table I

| Phospholipid | Protein kinase activity (pmol 32P/min) |
|--------------|---------------------------------------|
| No additions | 27.8 ± 1.1                            |
| Intrinsic PL  | 1.3 ± 0.4                              |
| No PL*       | <0.01                                 |
| PE           | 24.5 ± 0.8                             |
| PC           | 27.3 ± 1.6                             |
| PI           | 25.6 ± 1.3                             |
| PA           | 23.4 ± 0.9                             |
| PS           | 25.5 ± 1.1                             |
| PIP          | 18.7 ± 0.1                             |
| PIP2         | 8.4 ± 0.6                              |
| PIP2 + PS    | 0.9 ± 0.5                              |
| Intrinsic PL*| 26.1 ± 1.9                             |
| Intrinsic PL*| 23.7 ± 2.9                             |

* Glycoporphins with intrinsic PL extracted.

a Intrinsic phospholipid extracted from glycophorin.

b Phospholipid in the absence of glycophorin was added, nmol/reaction.
Regulation of Membrane and Cytosolic Casein Kinases by PIP₂

Fig. 5. MB protein kinase appears to be modulated specifically by a membrane component. Left, silver-stained SDS-PAGE showing pooled glycophorin fractions from a Bio-Gel A-1.5 column. The letters to the left show the glycophorin type and the oligomerization state. The glycophorins (350 mg) were fractionated according to Pursmeyer and Marchesi (30). The fractions were analyzed by SDS-PAGE, and fractions of glycophorins were combined into four pools (A–D). The glycophorin fractions were dialyzed and reconstituted with PIP₂ (33 nmol/mg), PS (132 nmol/mg), and PA (33 nmol/mg) as before (23, 31).

To determine how similar or dissimilar the cytosolic and MB casein kinases are, both kinases were isolated and their sequences compared by two-dimensional 125I peptide mapping. Both tryptic and chymotryptic peptide maps were done on two different preparations of both the MB and cytosolic casein kinases. The chymotryptic peptide maps of the cytosolic and MB protein kinase are shown in Fig. 7. Clearly the two protein kinases are very similar; indeed, the cytosolic protein kinase has all of the peptides found in the MB protein kinase. However, for both tryptic and chymotryptic peptide maps, the cytosolic protein kinase has additional peptides that are not contained within the peptide map of the MB protein kinase.

Fig. 6. Comparison of inhibition by glycophorin-PIP₂ containing micelles of cytosolic and MB casein kinases. The greater protein kinase activity (open symbols) is the MB protein kinase. The lesser protein kinase activity (closed symbols) is the cytosolic protein kinase. The symbols are glycophorin-PL micelles containing C, PS, PA, and PS. The concentration of casein was 625 µg/ml, MB and cytosolic protein kinases were 0.6 and 0.2 units/ml, respectively. Phosphorylation was in 100 µl with a reaction mixture containing 50 µM [γ-³²P]ATP (300 Ci/mol). The reaction was stopped after 15 min with 5% trichloroacetic acid and quantitated according to “Experimental Procedures.”

2,3-DPG with an I₅₀ of 1–2 mM but only when the kinase is solubilized from membranes; when associated with membranes, it is not inhibited by 2,3-DPG. Inositol 1,4,5-trisphosphate also inhibited the solubilized MB protein kinase but at concentrations higher than observed in cells (I₅₀ of 0.2 mM). Inositol 1,4,5-trisphosphate, like 2,3-DPG, did not inhibit the MB protein kinase when the kinase was bound to membranes (results not shown). These results suggest that the cytosolic and MB protein kinases are functionally similar and can be regulated by some of the same modulators. However, as shown in Fig. 6, purified cytosolic casein kinase was not inhibited by glycophorin-PIP₂ micelles as effectively as was the MB protein kinase.

DISCUSSION

The molecular basis for modulation of MB protein kinase activity appears to be 2-fold. (a) There appears to be an interaction with a component within the glycophorin preparation, perhaps a minor glycophorin. (b) There is a ternary interaction with PIP₂ which is of higher affinity and inhibits kinase activity. This interaction is enhanced by other negatively charged PLs such as PS or PA. The glycophorins, when isolated, retain intrinsic PLs, and the PL composition in such glycophorin micelles is selective for PS, PIP, and PIP₂. There is evidence that the PL composition of glycophorin micelles results from an association of glycophorins with PS and PIP₂ in the membrane (6–9). Liposomes containing PC, PS, PA, and PIP₂ but without glycophorin did not inhibit the MB protein kinase; however, PIP₂ micelles alone do weakly inhibit kinase activity. This inhibition requires a much higher concentration of PIP₂ than is required in the presence of the glycophorins. Since the glycophorins are known to interact with acidic PLs, specifically PS and PIP₂ (5–9), mechanically, a glycophorin or a similar protein within the glycophorin preparation may serve to bind the MB protein kinase, PIP₂, and PS, bringing the components into spatial proximity.

Both the cytosolic and solubilized MB protein kinases are inhibited by 2,3-DPG, suggesting a functional similarity. The stereochemistries of the phosphate residues on the 2- and 3-hydroxyls of 2,3-DPG and of the phosphate residues on the 4- and 5-hydroxyls of the myo-inositol ring of PIP₂ are identical. Since this is the case, a functional feature that is intrinsic to both the membrane-bound and the cytosolic pro-
Regulation of Membrane and Cytosolic Casein Kinases by PIP$_2$

FIG. 7. Two-dimensional peptide maps of the cytosolic (A) and the MB (B) casein kinases, with a composite drawing (C) showing the similar (hatch-marked) and dissimilar peptides.

The regulation of membrane and cytosolic casein kinases is regulation by 2 adjacent phosphate residues of the correct stereochemistry. The association of the MB protein kinase with a membrane component may simply potentiate this regulation. Since 2,3-DPG, inositol 1,4,5-trisphosphate, and PIP$_2$ inhibit the MB casein kinase, an association with a membrane component (possibly a glycoporphin) could place the protein kinase close to the bilayer in an environment with a high local concentration of PIP$_2$. This may also explain why glycoporphin micelles with intrinsic PL or reconstituted with PIP$_2$ and PS are such potent inhibitors of MB protein kinase activity. In this structure, unlike the membrane, the protein kinase associates with a component that spatially is very close to PIP$_2$. In the membrane, the local concentration of PIP$_2$ is likely not as high, thus the binding affinity and inhibition of activity may be lower than that observed for the interaction with the glycoporphin micelle. However, other associations not yet defined could also orient the protein kinase such that regulation of protein kinase activity by PIP$_2$ in the membrane is more pronounced.

The peptide maps demonstrate that the MB kinase is a subset of peptides derived from the cytosolic kinase. This suggests that all of the tyrosine-containing sequence found in the MB kinase is also found in the cytosolic kinase. Thus, if the two kinases differ in sequence and since they have an identical molecular mass, then a region of the MB protein kinase sequence would be required to have no tyrosine (silent to $^{125}$I-peptide mapping). To obtain the peptide map that we have for the cytosolic protein kinase, the silent region in the cytosolic protein kinase which would have tyrosines; this would result in new peptides. Such a sequence change is possible but seems unlikely.

Post-translational modification of the cytosolic kinase, such as phosphorylation, would also result in peptides unique from the MB kinase. Both the MB protein kinase and the cytosolic protein kinase are autophosphorylated (18–24); whether or not they are phosphorylated in vivo is not known. However, phosphorylation of the protein kinase at one or more sites could explain the differences and similarities between the two kinases.

The third possibility is that there is a protein of identical molecular mass which copurifies with the cytosolic protein kinase, possibly forming an association. Intuitively this seems unlikely but cannot be ruled out. Indeed, statistically a 33-kDa protein should contain about 16 tyrosines (assuming 1 tyrosine in 20 amino acids); this should give rise to about 16 $^{125}$I-containing peptides. A low exposure of the peptide map of the MB protein kinase shows about 16 peptides. The map shown is at higher exposure and contains about 30 peptides.
that likely arise from incomplete chymotrypsin cleavage. The peptide map of the cytosolic protein kinase at a lower exposure than that shown contains about 33 peptides; these are equally distributed between the MB protein kinase map and the new peptides arising from the cytosolic protein kinase. Statistically this would argue that the new peptides arise from a contaminating protein. However, these new peptides have a pattern similar to the MB protein kinase except that the peptides have decreased electrophoretic and chromatographic mobility, consistent with post-translational modification by phosphorylation.

Demonstrating which of these possibilities is correct will require further studies. However, the similarities and differences in the structure of the two protein kinases may be the bases to explain why the cytosolic kinase does not associate with the membrane but is regulated by 2,3-DPG and why the MB protein kinase associates with the membrane and is regulated by both 2,3-DPG (when solubilized) and glycoporphin-PIP2. Indeed, if the sequences of the two kinases are identical, which is possible, then it is likely that the two forms of the kinase can be interconverted. Functionally, this could be an important regulatory mechanism.

REFERENCES

1. Mische, S. M., and Morrow, J. S. (1988) Protoplasma 145, 167-175
2. Backman, L. (1988) Nature 334, 653-654
3. Nakao, M., Nakao, T., and Yamazoe, S. (1986) Nature 187, 945-947
4. Ferrell, J. E., Jr., and Heusist, W. H. J. (1984) J. Cell Biol. 98, 1992-1998
5. Anderson, R. A., and Marchesi, V. T. (1985) Nature 318, 495-498
6. Shukla, S. D., Coleman, R., Finesso, J. B., and Michell, R. H. (1979) Biochem. J. 179, 441-444
7. Armitage, I., Shapiro, D. L., Furthmayr, H., and Marchesi, V. T. (1977) Biochemistry 16, 1317-1320
8. Van Zoelen, E. J. J., Zwaal, R. F. A., Reuvens, F. A. M., Demel, R. A., and Van Deenen, L. L. M. (1977) Biochim. Biophys. Acta 494, 482-492
9. Buckley, J. T. (1978) Can. J. Biochem. 56, 349-351
10. Blanchard, D., Dahm, W., Hummel, M., Latron, F., Beyreuther, K., and Cartron, J.-P. (1987) J. Biol. Chem. 262, 5806-5811
11. High, S., and Tanner, M. J. A. (1987) Biochem. J. 243, 277-280
12. Reid, M. E., Anstee, D. J., Tanner, M. J. A., Ridgwell, K., and Nurse, G. T. (1987) Biochem. J. 244, 123-128
13. Siebert, P. D., and Fukuda, M. (1986) J. Biol. Chem. 261, 12433-12436
14. Furthmayr, H. (1981) in Biology of Carbohydrates (Ginsburg, V., ed) Vol. 1, pp. 123-197, John Wiley & Sons, New York
15. Anderson, R. A. (1986) in Membrane Skeletons and Cytoskeletal Membrane Associations (Bennett, V., Cohen, C. M., Lux, S., and Palek, J., eds) pp. 225-241, Alan R. Liss, Inc., New York
16. Anderson, R. A. (1989) in Red Blood Cell Membranes: Structure, Function and Clinical Implications (Agre, P., and Parker, J. C., eds), pp. 187-235, Marcel Dekker, Inc., New York
17. Horne, W. C., Leto, T., and Anderson, R. A. (1989) Methods Enzymol. 173, 380-392
18. Eder, P. S., Soong, C., and Tao, M. (1986) Biochemistry 25, 17644-1765
19. Tao, M., Conway, R., and Cheta, S. (1980) J. Biol. Chem. 255, 2563-2568
20. Lu, P.-W., Soong, C. J., and Tao, M. (1985) J. Biol. Chem. 260, 14938-14944
21. Soong, C. J., Lu, P. W., and Tao, M. (1987) Arch. Biochem. Biophys. 254, 509-517
22. Simkowski, K. M., and Tao, M. (1989) J. Biol. Chem. 260, 6406-6411
23. Boivin, P., and Galand, C. (1979) Biochem. Biophys. Res. Commun. 90, 7-16
24. Erusalimsky, J. D., Balas, N., and Milner, Y. (1983) Biochim. Biophys. Acta 756, 171-181
25. Schacht, J. (1978) J. Lipid Res. 19, 1063-1067
26. Palmer, F. B. St. C. (1981) J. Lipid Res. 22, 1296-1300
27. Anderson, R. A., and Marchesi, V. T. (1984) Nature 307, 645-650
28. Speicher, D. W., Morrow, J. S., Knowles, W. J., and Marchesi, V. T. (1982) J. Biol. Chem. 257, 9093-9101
29. Marchesi, V. T., and Andrews, E. P. (1971) Science 174, 1247-1249
30. Furthmayr, H., and Marchesi, V. T. (1983) Methods Enzymol. 96, 268-280
31. Mims, L. T., Zampighi, G., Nozaki, Y., Tanford, C., and Reynolds, J. A. (1981) Biochemistry 20, 833-840
32. Cleveland, D. W., Fischer, S. C., Kirschner, M. W., and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102-1106
33. Leto, T. L., Correas, I., Tobe, T., Anderson, R. A., and Horne, W. C. (1986) in Membrane Skeletons and Cytoskeletal Membrane Associations (Bennett, V., Cohen, C. M., Lux, S., and Palek, J., eds) pp. 201-209, Alan R. Liss, Inc., New York
Erythroid membrane-bound protein kinase binds to a membrane component and is regulated by phosphatidylinositol 4,5-bisphosphate.

C E Bazenet, J L Brockman, D Lewis, C Chan and R A Anderson

J. Biol. Chem. 1990, 265:7369-7376.