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 phosphoinositol 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). However, the only link so far established between cell 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), 1 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 protein kinases 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 cell, 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 (5-9, 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.

1 The abbreviations used are: PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP₁, phosphatidylinositol 4-phosphate; PIP₃, phosphatidylcholine; PC(s), phospholipid(s); 2,3-DPG, 2,3-diphosphoglycerate; PME₃F, p-methyllumethyldisulfon fluoride, DFP, diisopropyl fluorophosphate; PC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; EGTA, ethylenbis(oxyethylenenitrilo)tetraacetic acid; IOV(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 appears to lower the affinity of their interactions with membranes washed free of hemoglobin by extraction with 0.1 M NaCl supernatant from either Du Pont-New England Nuclear or American Corp. Leupeptin, pepstatin A, antipain, bestatin, 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 as glycophorin micelles, phospholipids, or IOVs. All components were desialated as before (5, 15, 27, 31). Fractions were desialated and covalently attached to the Affi-Gel 15 beads in each of leucotin, bestatin, and pepstatin A at pH 7.5. The final volume of the reaction was 50 μl; the amount of phosphorylation for quantitative experiments was always in the linear range. The phosphorylation was stopped and quantitated 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 in 2 ml of scintillation fluid. One unit of kinase 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-phenol 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-phenol 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 eluates were then extracted into chloroform/methanol and desialated (5, 7). The glycophorins were concentrated to 10 mg/ml, dialyzed against isotonic KCl buffer, and stored on ice. Glycophorin-phospholipid mixed micelles and phospholipid liposomes containing glycophorin were prepared as before without modification (5, 15, 27, 31).

Affinity Chromatography—The glycophorin-Affi-Gel 15 affinity matrix was prepared as described previously (17). Briefly, micelles of glycophorin containing intrinsic phospholipid (mainly PS and PIP) were desialated and covalently attached to the Afi-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 isotonic KCl buffer containing 2 mM MgCl2. A 0.5 M NaCl extract from ghost membranes was dialyzed against isotonic KCl containing 2 mM MgCl2 and applied to a glycophorin-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 Afi-Gel 15 column to which glycine was coupled; no protein kinase activity was retained.

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 glycophorin PL competition with IOVs for membrane-bound protein kinase binding, glycophorin-PL micelles were combined with 60 μg of IOV protein (150 μl) in isotonic 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 PLs to glycophorin for these experiments was 41-51 nmol of PL/mg of glycophorin, except PIP₂ and PS, which were 42 and 192 nmol/mg of glycophorin, respectively. In micelles containing PS, increasing PS to 164 nmol/mg of glycoporphin did not alter inhibition of binding or activity. The IOV pellets were resuspended in isotonic KCl with 0.01% Triton X-100; 20 µg of spectrin was added to separate phosphorylation from binding and was completed from the spectrin, indicating that substrate effects are not involved.

Cellulose Peptide Mapping of Cytosolic and Membrane-bound Casein Kinase—Membrane-bound and cytosolic casein kinase were 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 (28, 32). The electrophoresis and thin-layer chromatography (TLC) were as before (28), but TLC plates were glass backed with a 0.1-mm thickness of cellulose (EM reagents). 100 cpm of [³²P]-peptide with 50 µM [γ³²P]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 is 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 the protein kinase that binds to the glycophorin column.

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 (28, 32). The electrophoresis and thin-layer chromatography (TLC) were as before (28), but TLC plates were glass backed with a 0.1-mm thickness of cellulose (EM reagents). 100 cpm of [³²P]-peptide with 50 µM [γ³²P]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 glycophorin. 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 glycophorin 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. Spectrin 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 phosphorylates 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 glycophorin 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 glycophorin-PL micelles as demonstrated previously (5). Separation was also checked by applying the IOVs to an SDS-PAGE and staining with silver. The desalted glycophorin stains very intensely with silver and migrates with a different apparent molecular weight than does native glycophorin. By this criteria, no measurable micellar glycophorin 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 inhibition of kinase activity due to membrane binding. This cannot be explained by exposure of trapped kinase by Triton X-100-induced lysis of IOVs since glycophorin-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 glycophorin 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 glycophorin micelles were assayed after addition of Triton X-100 to the supernatant. In a representative experiment (see Fig. 9), glycophorin micelles with intrinsic PL (25 μg/ml) eluted 98% of the MB protein kinase activity from IOVs (500 μg/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 glycophorin-PL micelles is required for inhibition of kinase activity.

To determine the role played by intrinsic PLs retained by isolated glycophorin micelles, the PLs were extracted, and both glycophorin micelles and the extracted PLs were tested for the ability to elute MB protein kinase activity from the membrane. Glycophorin micelles lacking phospholipid were found to compete with MB protein kinase binding but much less effectively than glycophorin containing intrinsic phospholipid (Fig. 2). The phospholipid fraction, containing mainly phosphatidylycerine (PS), phosphatidylinositol 4-phosphate (PIP), and PIP$_2$ at a 4.2:1:1.2 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 35 μM PIP$_2$. This amount of PIP$_2$ is equivalent to that contained in 1 mg/ml of glycophorin retaining intrinsic phospholipid. This suggests that the MB protein kinase requires both glycophorin and PIP$_2$ for a high affinity association.

Since intrinsic PL in the glycophorin micelle enhanced the affinity of MB protein kinase for glycophorin, micellar glycophorin was reconstituted with a variety of PLs to determine if a specific PL was required. The glycophorin-PL micelles were then tested for their ability to deplete the MB protein kinase content of membranes. As demonstrated in Fig. 2, glycophorin-PL micelles must contain PIP$_2$ to be effective at depleting membranes of MB protein kinase activity. However, for maximal affinity, the glycophorin-PL micelles must have PIP$_2$ and in addition 1-4 mol of a negatively charged PL (PS or PA). However, PA and PS only enhanced the effect of PIP$_2$ alone they are not effective at inhibiting MB protein kinase binding to IOVs.

Since the glycophorin-PL micelles were effective at competing with membranes for MB protein kinase binding, the glycophorin-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 glycophorin micelles containing intrinsic PL (Fig. 3). As with inhibition of binding to membranes, extraction of the intrinsic PL from the glycophorin destroyed the ability of the glycophorin micelles to inhibit kinase activity. Further, the extracted intrinsic PLs and glycophorins without PL did not inhibit protein kinase activity. When micellar glycophorin was reconstituted with PL by detergent dialysis (5), inhibition of MB protein kinase activity was also recovered but only when PIP$_2$ was reconstituted into the glycophorin micelles (Table I). Other PLs reconstituted into glycophorin-PL micelles did not inhibit the MB protein kinase activity. However, when both PIP$_2$ and either FS or PA were incorporated into micelles, the catalytic activity of the MB protein kinase was potently inhibited by glycophorin-PL micelles.

To ascertain if glycophorin and PIP$_2$ in an environment more like the native membrane also regulate the MB protein
kinase, glycophorin was reconstituted into PL liposomes. The composition was, as mol %, 75% PC, 20% PS, and 3% PA, with or without added PIP$_2$ (Fig. 4). When MD protein kinase was combined with liposomes containing glycophorin in the absence of PIP$_2$, there was a relatively small decrease in kinase activity. However, liposomes containing glycophorin and PIP$_2$ showed a more pronounced inhibition of MB protein kinase activity. This inhibition is dependent upon the presence of PIP$_2$ in the liposomes; other PLs did not elicit this effect. Further, both the glycophorins and PIP$_2$ must be present in liposomes for inhibition of MB protein kinase activity. PIP$_2$ alone in liposomes does not inhibit the MB protein kinase, even at high concentrations of liposomes or at high mol % of PIP$_2$ 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 PIP$_2$, PS, and PA as above and assayed for their effectiveness at inhibiting the 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 $K_i$ of 4.6 mM (23). The MB protein kinase is also inhibited by...
Regulation of Membrane and Cytosolic Casein Kinases by PIP₂

7374 Regulation of Membrane and Cytosolic Casein Kinases by PIP₂

FIN. 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 Furth-
mayr and Marchesi (30). The fractions were analyzed by SDS-PAGE, and fractions of glycophorins were combined into four pools (A–D). The glycophorin frac-
tions were dialyzed and reconstituted with PIP₂ (33 nmol/mg), PS (132 nmol/mg), and PA (33 nmol/mg) as before (23, 31). Right, fractions A (O), B (O), C (Δ), and D (A) were then assayed for the ability to inhibit the MB protein kinase activity.

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.

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 glycophors, 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 glycophors 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 glycophors. Since the glycophors 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 stereochimistries 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-

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.

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 O, PS; □, PA and PS; Δ, PIP₂, 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.

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.

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 glycophors, 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 glycophors 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 glycophors. Since the glycophors 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 stereochimistries 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-

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 Furth-
mayr and Marchesi (30). The fractions were analyzed by SDS-PAGE, and fractions of glycophorins were combined into four pools (A–D). The glycophorin frac-
tions were dialyzed and reconstituted with PIP₂ (33 nmol/mg), PS (132 nmol/mg), and PA (33 nmol/mg) as before (23, 31). Right, fractions A (O), B (O), C (Δ), and D (A) were then assayed for the ability to inhibit the MB protein kinase activity.

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.

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 glycophors, 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 glycophors 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 glycophors. Since the glycophors 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 stereochimistries 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$_2$

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 MB protein kinase would have to be replaced by a sequence 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 glyco- phosphoryn-PIP<sub>2</sub>. 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
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


Access the most updated version of this article at http://www.jbc.org/content/265/13/7369

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/13/7369.full.html#ref-list-1