In this study, we found that Hg²⁺ and Cd²⁺ enhanced the phosphorylation of human erythrocyte membrane proteins, especially band 4.2 protein, which was hardly phosphorylated in the absence of the metal ions. p-Chloromercuribenzenesulfonate and p-chloromercuribenzoate had effects similar to those of Hg²⁺ and Cd²⁺ on band 4.2 protein phosphorylation, while other metal ions and sulfhydryl agents, such as N-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid), or iodoacetate, did not. The Hg²⁺-stimulated phosphorylation of band 4.2 protein required a millimolar concentration of Mg²⁺, and it was inhibited by Ca²⁺ dose-dependently. Phosphoserine was identified from a hydrolysate of the phosphorylated band 4.2 protein by high-voltage electrophoresis. A specific protein inhibitor against CAMP-dependent protein kinase decreased the Hg²⁺-stimulated phosphorylation of band 4.2 protein. This protein had more binding sites for [³²P]ATP than any other membrane proteins. A spectrin complex from the Hg²⁺-treated membranes contained the band 4.2 protein, which was not detected in the complex from untreated membranes. Furthermore, protein kinase, which could phosphorylate the band 4.2 protein, was also contained in the cytoskeletal fraction from the Hg²⁺-treated membranes. These results suggest that Hg²⁺ may bind certain sulfhydryl groups of band 4.2 and other proteins to make band 4.2 protein susceptible to the endogenous CAMP-dependent protein kinase.

In this paper, we describe the effects of heavy metal ions on the phosphorylation of human erythrocyte membrane proteins, especially on band 4.2 protein, and discuss heavy metal ion-induced change of membranous protein interaction.

**MATERIALS AND METHODS**

**Reagents**—[γ-³²P]ATP was prepared according to Weiss et al. (12). [³²P]Phosphoric acid and [γ-³²P]GTP were obtained from New England Nuclear. [³⁵S]HCl was purchased from Radiochemical Centre (Amersham, United Kingdom). Phosphoserine, phosphothreonine, and a protein inhibitor of rabbit skeletal muscle were from Sigma. Phosphotyrosine was synthesized and purified according to Mitchell and Lunan (13). Sepharose 2B was obtained from Pharmacia (Uppsala, Sweden). Human erythrocyte membranes were prepared according to the method of Dodg et al. (14) using 5 mM Tris-Cl buffer (pH 8.0). Erythrocyte membranes of hereditary spherocytosis were obtained by Prof. Y. Nozawa and Dr. Y. Okano of the Gifu University School of Medicine.

All other chemicals used were commercial preparations of the highest purity.

**Membrane Phosphorylation**—Membrane phosphorylation and preparation of samples for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed according to the method given in Ref. 15 with some modifications as follows. A reaction mixture (100 µl) containing membranes (180 µg of protein), 20 mM Tris-Cl (pH 8.0), 8 mM MgCl₂, 10 µM phenylmethylsulfonyl fluoride, and sulfhydryl reagent at the indicated final concentration was preincubated at 37 °C for 2 min, and the reaction was started by the addition of 10 µl of [γ-³²P]ATP (10,000–20,000 cpm/pmol, final concentration 25 µM). Incubation was carried out at 37 °C for 5 min, and the reaction was stopped by the addition of 10 µl of 20% SDS containing 20 mM dithiothreitol followed by heating at 75 °C for 1 min. Ten µl of 40% sucrose containing a tracking dye (pyronin Y) were added to the mixture before electrophoresis.

**SDS-PAGE and Autoradiography**—Twenty-five µl of each sample prepared as above were subjected to SDS-PAGE using a 5.6% slab polyacrylamide gel layer (5 mm thick) at the bottom. The gel was run out into the bag by electrophoresis (12 mA/column, 7 h). Band 4.2 protein in the gel was cut out and swollen in 40 mM Tris acetate buffer (pH 7.4) containing 1% SDS and 1 mM EDTA. The swollen gel was cracked into small pieces and packed in a glass column fitted with a 5% polyacrylamide gel layer (5 mm thick) at the bottom. The column was filled with 40 mM Tris acetate buffer (pH 7.4) containing 0.2% SDS and 1 mM EDTA and fixed with small a cellophane bag filled up with the same buffer at the bottom end. Band 4.2 protein in the gel was run out into the bag by electrophoresis (12 mA/column, 7 h). The protein solution was dialyzed against ethanol, and the precipitate formed during the dialysis was collected by centrifugation. The precipitate was hydrolyzed in 6 N HCl at 105 °C for 2 h as reported by Allerton and Perlman (18). High-voltage thin-layer electrophoresis and autoradiography were carried out according to Stein.
Cold phosphoamino acids were used as carriers, and their electrophoretic positions were identified by ninhydrin staining.

Binding of $^{35}$S$^{+}$ to Erythrocyte Membrane Proteins—A reaction mixture (100 μl), which contained membranes (180 μg of protein), 20 mM Tris-HCl (pH 8.0), 8 mM MgCl$_2$, and 10 μM phenylmethanesulfonyl fluoride, was preincubated at 37°C for 2 min, and then, binding of Hg$^{+}$ was started by the addition of 10 μl of 1.1 mM $^{35}$HgCl$_2$ (1.5 mCi/μg). Incubation was carried out at 37°C for 5 min. The pellet was suspended in 1 ml of ice-cold 10 mM Tris-HCl (pH 7.4), and the suspension was centrifuged at 13,500 rpm to collect the membranes. These procedures were repeated three times to remove free $^{35}$HgCl$_2$. The membrane pellet was solubilized in 80 μl of 40 mM Tris acetate buffer (pH 7.4) containing 3% SDS by heating at 75°C for 1 min. Twenty-five μl of the sample were subjected to slab gel electrophoresis as described above. The gel was stained with fast green FCF according to Gorovsky et al. (20), and scanning of protein bands was carried out. The gel was dried and cut at the ends of each protein band. Then, the amounts of $^{35}$Hg bound to the protein bands were determined.

Isolation of Spectrin Complex—A low ionic strength extraction of erythrocyte membrane proteins was performed according to Steck and Yu (21) with some alteration as follows. Fifteen ml of erythrocyte membrane suspension (3.8 mg of protein/ml) in 5 mM Tris-HCl (pH 8.0) were poured into 200 ml of ice-cold water, and the mixture was incubated at 0°C for 4 h with occasional shaking. The extract was obtained by centrifugation of the mixture at 25,000 × g for 30 min. The extract was then added to one-ninth volume of 1 M NaCl, and the mixture was concentrated to a final volume of 5.2 ml using Lyphogel (Gelman). The condensed extract was applied onto a Sepharose 2B column (1.5 × 32 cm) previously equilibrated with 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl. Gel filtration was carried out with the same buffer. All the above procedures were carried out at 0–4°C.

Protein Determination—Proteins were determined by the method of Lowry et al. (22) with bovine serum albumin as a standard.

RESULTS

Effects of Hg$^{+}$ and Cd$^{2+}$ on Membrane Phosphorylation—Hg$^{+}$ was found to have a marked stimulatory effect on the endogenous phosphorylation of human erythrocyte membrane proteins (Fig. 1 and Table I). Cd$^{2+}$ also enhanced the membrane phosphorylation at the 10 μM range of the final concentration. On the other hand, divalent metal ions, such as Pb$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Sn$^{2+}$, Ni$^{2+}$, and Mn$^{2+}$, showed no such effect. Among many phosphorylated proteins, band 4.2 protein, which was hardly phosphorylated in the absence of Hg$^{+}$ or Cd$^{2+}$, was heavily phosphorylated in the presence of these heavy metal ions. Phosphorus incorporated into the band 4.2 protein shown in Table I was 0.2 μmol of P$_i$/mol of protein based on the molecular weight of band 4.2 protein estimated to be 72,000. The stimulation of the phosphorylation of band 4.2 protein in the presence of Hg$^{2+}$ or Cd$^{2+}$ was also observed in the membranes of hereditary spherocytosis erythrocytes, on which there have been various reports describing changes on endogenous phosphorylation (23–26) or band 4.2 protein (27, 28), and in the diamide-treated membranes, in which endogenous phosphorylation activity was lowered as reported by Hosey et al. (29) (data not shown). In Fig. 1, the same amount of protein (38 μg) from each sample was subjected to slab gel electrophoresis, and no detectable proteolysis of the membrane proteins was observed from the protein staining patterns.

Effects of Sulfhydryl Reagents on Membrane Phosphorylation—It was well known that Hg$^{2+}$ and Cd$^{2+}$ bind covalently to sulfhydryl groups of proteins and to other biological materials. Therefore, we investigated whether or not other sulfhydryl reagents, such as pCMBS and pCMBS, had the same effect on membrane phosphorylation.

As shown in Fig. 2, pCMBS and pCMBS also exerted stimulatory effects on protein phosphorylation, including the band 4.2 protein. The pCMBS and pCMBS used in this experiment contained a negligible amount of inorganic mercury (less than 0.1% w/w) by atomic absorption spectrophotometry. From these results, it was suggested that the phosphorylation of band 4.2 protein was caused by some modification of the sulfhydryl group of membranous proteins. In the case of these organic sulfhydryl reagents, a higher concentration was required than that of mercuric ion. The reason may be that the association constants of organic mercurials are much smaller than that of mercuric ion (30) or that Hg$^{2+}$ can react with some sulfhydryl groups in the membranous proteins to which it is difficult for organic mercurial compounds to access.

Effects of Mg$^{2+}$ and Ca$^{2+}$ on Band 4.2 Protein Phosphorylation—As shown in Fig. 3, a millimolar range of Mg$^{2+}$ was required for mercuric ion-stimulated band 4.2 protein phosphorylation, and the reaction was inhibited by Ca$^{2+}$ dose-dependently. These effects of Mg$^{2+}$ and Ca$^{2+}$ are consistent with the actions of these ions on protein kinase(s) of human erythrocyte membranes (9, 11, 31, 32). The data suggest that mercuric ion-stimulated $^{32}$P incorporation into band 4.2 protein was a result of enzymatic reactions of membranous protein kinase(s). These effects of Mg$^{2+}$ and Ca$^{2+}$ were also observed on Hg$^{2+}$-stimulated phosphorylation of other proteins (bands 2, 2.1, 4.1, and 4.5) (data not shown).

| Protein band | Addition | Addtion |
|--------------|----------|---------|
| None         | 20 μM HgCl$_2$ | 10 μM CdCl$_2$ |
| 1            | 500 (100) | 700 (140) | 900 (180) |
| 2            | 29,800 (100) | 63,800 (214) | 75,100 (252) |
| 2.1          | 3,400 (100) | 8,000 (235) | 6,900 (203) |
| 3            | 20,800 (100) | 18,600 (89) | 35,700 (172) |
| 4.1          | 7,200 (100) | 13,000 (181) | 14,300 (197) |
| 4.2          | 900 (100) | 98,300 (10,922) | 49,800 (5,533) |
| 4.5          | 4,900 (100) | 29,500 (602) | 21,200 (433) |
| 5            | 6,200 (100) | 8,700 (140) | 7,200 (116) |
| 6            | 500 (100) | 700 (140) | 600 (120) |
| 7            | 5,000 (100) | 5,300 (106) | 4,800 (96) |

Table I  Effects of Hg$^{2+}$ and Cd$^{2+}$ on phosphorylation of human erythrocyte membranous proteins

$^{32}$P incorporation into each protein band of a dried SDS-polyacrylamide electrophoresis gel was measured by a liquid scintillation counter. The experimental conditions were the same as for Fig. 1. The values are expressed as counts/minute of $^{32}$P incorporated. The values in parentheses are per cent of control.
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4.1 3-
4.2 –
4.5 –
5 –

FIG. 2. Autoradiogram showing the effects of pCMBS and pCMB on the phosphorylation of human erythrocyte membranes. A, control; B and C, pCMBS, 0.1 and 1.0 mM, respectively; D and E, pCMB, 0.1 and 1.0 mM, respectively.

FIG. 3. Effects of Mg2+ and Ca2+ on the Hg2+-stimulated phosphorylation of band 4.2 protein. 32P incorporation into band 4.2 protein was monitored spectrophotometrically on an autoradiogram. Left, human erythrocyte membranes were incubated in the presence of 20 μM HgCl2 and the indicated concentration of MgCl2; right, the membranes were incubated in the presence of 5 mM MgCl2, 20 μM HgCl2, and the indicated concentration of CaCl2.

Identification of Phosphorylated Amino Acids—We investigated the phosphoamino acids of band 4.2 protein after the incorporation of 32P from [γ-32P]ATP in the presence of Hg2+. By high-voltage electrophoresis, only [32P]phosphoserine was identified from a hydrolysate of 32P-labeled band 4.2 protein (Fig. 4). This result also suggested that membranous protein kinase(s) catalyzed Hg2+-stimulated 32P incorporation into band 4.2 protein. We do not know at present the number of phosphorylated sites on band 4.2 protein.

Effects of cAMP and a Protein Inhibitor—Since it is known that at least two kinds of protein kinases are present on the erythrocyte membranes, namely cAMP-dependent and cAMP-independent protein kinases (5–11), we next studied which enzyme catalyzed the reaction, especially the phosphorylation of band 4.2 protein.

Cyclic AMP enhanced the incorporation of 32P from [γ-32P]ATP to certain proteins (bands 2.1, 4.1, and 4.5) in human erythrocyte membranes, but this effect could not be detected in the presence of 20 μM Hg2+ (data not shown). The Hg2+-stimulated phosphorylation of band 4.2 protein was inhibited by a rabbit muscle protein inhibitor of cAMP-dependent protein kinase (Fig. 5). These results may suggest that the mercuric ion dissociates the membrane-bound cAMP-dependent protein kinase into a regulatory subunit and a catalytic subunit, and the free catalytic subunit, which can be inhibited by the protein inhibitor of cAMP-dependent protein kinase (9), phosphorylated some endogenous proteins such as band 4.2 protein, etc.

32Hg2+ Binding to Membrane Proteins—The covalent binding capacity of Hg2+ with the membrane proteins was investigated using 32Hg2+. As shown in Table II, Hg2+ bound to almost all proteins, but the relative amount of bound 32Hg2+/protein weight was varied. Among membrane proteins, band 4.2 protein had the largest binding sites for 32Hg2+. From these results, it may be considered that Hg2+ binding to band 4.2 protein promotes the accessibility of this protein to a protein kinase through the change of its conformation or protein-protein interactions on the membranes.

Hg2+-induced Change of Interactions of Membrane Proteins—We studied the Hg2+-induced change of the arrange-
ment of band 4.2 protein on the human erythrocyte membranes. For this purpose, low ionic strength extraction of the membranes was carried out, and a spectrin complex was isolated from the intact (Fig. 6A) or Hg$^{2+}$-treated (Fig. 6B) membranes as the first peak from a Sepharose 2B column. As shown in Fig. 6, spectrin complex from the intact membranes contained bands 1, 2, 4.1, and 5 as the main bands. On the other hand, the spectrin complex from the Hg$^{2+}$-treated membranes showed band 4.2 in addition to the above-mentioned bands. These results suggest that Hg$^{2+}$ treatment caused a rearrangement of cytoskeletal structures through some changes of protein interactions on the membranes. Furthermore, a protein kinase activity toward band 4.2 protein was detected only in the spectrin complex fraction from Hg$^{2+}$-treated membranes (Fig. 7). Therefore, Hg$^{2+}$ might lead band 4.2 protein and a protein kinase to associate with a cytoskeleton. However, the molecular details of the Hg$^{2+}$-induced membrane organization change are still unclear, and further studies are necessary.

**DISCUSSION**

We found that divalent cations such as Hg$^{2+}$ and Cd$^{2+}$ stimulated the membrane phosphorylation of human erythrocytes. Detailed analysis of the phosphorylated proteins revealed that, in addition to the ordinal substrates, band 4.2 protein, which was hardly phosphorylated in the absence of these divalent cations, was most heavily phosphorylated. From the results of the effects of Mg$^{2+}$ and Ca$^{2+}$, the identification of $^{32}$P-incorporated amino acids, and the effect of a protein inhibitor, the phosphorylation of band 4.2 protein seemed to be catalyzed by the membrane-bound cAMP-dependent protein kinase. This speculation is consistent with our previous paper (9), which indicated that, in heat-treated membranes, band 4.2 protein was a good substrate of the purified catalytic subunit of cAMP-dependent protein kinase from human erythrocyte membranes.

There are several possible explanations for the stimulation of the phosphorylation by the heavy metal ions. The first possibility is that the heavy metal ions stimulate erythrocyte membrane protein kinases including cAMP-dependent protein kinase. This is not plausible because Hg$^{2+}$-stimulated phosphorylation by the membranes of exogenous substrates such as histone, protamine, casein, and phosvitin was not observed. Furthermore, a purified catalytic subunit of cAMP-dependent protein kinase was rather inactivated by sulphydryl reagents (9). The second explanation is that Hg$^{2+}$ and Cd$^{2+}$ inhibit a phosphoprotein phosphatase of human erythrocyte membranes and enhance the accumulation of phosphoproteins. This possibility was ruled out by the measurement of the rate of dephosphorylation of $^{32}$P-labeled membrane proteins in the presence and absence of Hg$^{2+}$. The results indicated that the rate of removal of $^{32}$P from phosphoproteins was unaffected by the presence of Hg$^{2+}$ (data not shown). The third explanation is that these heavy metal ions bind to proteins probably by covalent linkage through sulphydryl groups of proteins and change the topography of membrane proteins. This facilitates the phosphorylation of several proteins, especially band 4.2 protein. As shown in Table II, band 4.2 protein had the highest number of binding sites for Hg$^{2+}$. Of the various reagents known to react with the sulphydryl group of protein, only pCMB and pCMBS had a similar stimulatory effect on the phosphorylation, and N-ethylmaleimide, 5,5′-dithiobis-(2-nitrobenzoic acid), or iodoacetate did not show any stimulation effect. pCMB and pCMBS were reported to exert a different effect on membrane organization from that of 5,5′-dithio-bis-(2-nitrobenzoic acid) or N-ethylmaleimide (21, 33-35). The results shown in Fig. 6 also supported this assumption. In the membranes treated with Hg$^{2+}$, band 4.2 comes to associate with the spectrin complex and is ready to be phosphorylated by a protein kinase. Among the proteins in the spectrin complex from the Hg$^{2+}$-treated membranes, band 4.2 protein was mainly phosphorylated, while band 2 protein was phosphorylated only very weakly. Furthermore, the phosphorylation of band 4.2 protein was inhibited by the protein kinase inhibitor.

These facts also suggest that band 4.2 protein was phosphorylated by cAMP-dependent protein kinase, because if cAMP-independent protein kinase was present in the spectrin complex, band 2 protein should be heavily phosphorylated.

**TABLE II**

| Protein band | $^{203}$Hg bound/mg protein | nmol |
|--------------|-----------------------------|------|
| 1            | 9.2                         |      |
| 2            | 22.1                        |      |
| 2.1          | 28.1                        |      |
| 3            | 31.5                        |      |
| 4.1          | 34.1                        |      |
| 4.2          | 87.5                        |      |
| 4.5          | 46.7                        |      |
| 5            | 49.3                        |      |
| 6            | 28.7                        |      |
| 7            | 13.6                        |      |

**FIG. 6. Effects of Hg$^{2+}$ treatment on a structure of spectrin complex.** Human erythrocyte membranes (54 mg of protein) were incubated with or without 20 $\mu M$ HgCl$_2$ in 15 ml of 5 mM Tris-HCl (pH 8.0) for 5 min at 37°C. The membranes were then washed three times with 50 ml of ice-cold 5 mM Tris-HCl (pH 8.0) at 0-4°C. Gel filtrations of low ionic strength extracts from untreated (A) and Hg$^{2+}$-treated (B) membranes were carried out on a column of Sepharose 2B. Fractions of 1.1 ml each were collected. C and D, SDS-PAGE of the void volume fractions (Vs) of A and B, respectively. The protein peaks are numbered according to Steck (90).
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FIG. 7. Autoradiogram showing phosphorylation of spectrin complexes. Spectrin complexes (100 μg of protein), which were obtained from the void volume fractions of Fig. 6, were incubated with 25 μM [γ-32P]ATP (18,000 cpm/pmol) in the presence of 50 mM NaCl. Other conditions were the same as membrane phosphorylation. A, membranes; B, spectrin complexes from untreated membranes; C, spectrin complexes from Hg2+-treated membranes.

(11) These results strongly suggest that perturbation of protein-protein interaction of human erythrocyte membranes by the heavy metal ions facilitates the protein phosphorylation reaction and makes cAMP-dependent protein kinase accessible to some membrane proteins.

In this paper, we showed that phosphorylation of several membrane proteins was enhanced by Hg2+ or Cd2+. However, we do not know whether or not the phosphorylation was catalyzed by a single protein kinase. What we can say at present is that at least band 4.2 protein seems to be phosphorylated by cAMP-dependent protein kinase. Further investigations will be necessary to clarify this point.

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