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STUDIES ON ENDOGENOUS PHOSPHORYLATION OF CHICKEN ERYTHROCYTE MEMBRANES

CALCIUM-DEPENDENT PHOSPHORYLATION OF SPECIFIC PROTEINS

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In this work, endogenous phosphorylation of chicken erythrocyte membranes was investigated. The membrane proteins were rapidly phosphorylated endogenously (half maximum time was 30 s) in the presence of millimolar concentration of Mg^{2+} under physiological conditions. As an exogenous substrate, protamine was phosphorylated most rapidly of those tested, and histone, casein and bovine serum albumin were rather poor substrates. Cyclic nucleotides had no effect on the endogenous membrane phosphorylation. EGTA inhibited the phosphorylation of a membrane protein having an approximate molecular weight of 43000, and this inhibition was reversed by the addition of a stoichiometric amount of Ca^{2+}. Furthermore, trifluoperazine, an inhibitor of calmodulin, was found to have the same effect as that of EGTA. The phosphorylated 43 kDa protein could be extracted from the membranes under high salt conditions, and was precipitated specifically with anti-actin antibody. These results suggest that the phosphorylation of a peripheral membrane protein (which has an approximate molecular weight of 43000) of chicken erythrocytes by membranous protein kinase(s) depends on Ca^{2+} and possibly on calmodulin.

Introduction

Protein phosphorylation has been widely observed in prokaryotic and eukaryotic cells and its function has been discussed [1] in relation to enzyme activation, contractile protein regulation and nuclear protein functions.

Recently, regulatory systems of protein phosphorylation have also been found in membrane fractions of various tissues and cells. Specifically, membranes have substrate proteins, protein kinases, phosphoprotein phosphatases [1] and other regulatory enzymes such as adenylate cyclase [2] and cyclic AMP-phosphodiesterase [3]. The phosphorylation has been shown to be controlled by several factors such as cyclic nucleotides, divalent cations [1] and phospholipids [4]. Furthermore, it has been demonstrated that transport of ions [5] and glucose [6], rearrangement of membrane proteins [7] and membrane fluidity [8] may be regulated through membranous protein phosphorylation.

Avian erythrocytes, each of which has a nucleus and some organelles, can be obtained homogeneously on a relatively large scale and have been widely used for studies of β-adrenergic receptor, adenylate cyclase [9] and the response mechanism

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for exogenous stimuli such as that of concanavalin A [10,11].

In this paper, we describe the endogenous phosphorylation of chicken erythrocyte membranes and discuss the calcium-dependent regulation of the reaction.

Materials and Methods

Reagents. [γ-^{32}P]ATP was prepared according to Weiss et al. [12]. $^{32}P_i$ and [γ-^{32}P]GTP were obtained by New England Nuclear (Boston, MA, U.S.A.). Whole histone from calf thymus and protamine sulfate from salmon were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trifluoperazine dimaleate was generously donated by Yoshitomi Seiyaku Co. (Osaka, Japan).

Rabbit anti-chicken gizzard actin antiserum was kindly provided by Dr. I. Yahara (The Metropolitan Institute of Medical Science). Goat anti-rabbit IgG is a product of Miles (Elkhart, Ind., U.S.A.).

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

Membrane preparation. Chicken erythrocyte membranes were prepared from freshly drawn heparinized blood of normal male white leghorn chickens according to the methods of Salesse and Garnier [13], and Snider and Parker [14] with some modifications as follows. The heparinized blood was centrifuged at $500 \times g$ for 5 min, and the plasma and buffy coat were removed. The packed erythrocytes were washed with 5 vol. 10 mM Tris-HCl (pH 7.4)/0.15 M NaCl three times. The washed erythrocytes were then suspended in 2 vol. Buffer I (50 mM Tris-HCl (pH 7.4)/5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.3 mM phenylmethanesulfonyl fluoride (PMSF)/0.1 mM diisopropylfluorophosphate (DFP)) and allowed to swell for 30 min. The swollen erythrocytes were disrupted with a tight-fitting Dounce homogenizer (150 strokes) and centrifuged at $1000 \times g$ for 5 min. The supernatant was centrifuged at $31000 \times g$ for 30 min, and the pellet was suspended homogeneously in 20 ml Buffer I with the Dounce homogenizer (five strokes) and layered on Buffer I containing 50% (w/v) sucrose followed by centrifugation at $12000 \times g$ for 60 min. The interface was collected and suspended in 10 vol. Buffer I. The suspension was centrifuged at $31000 \times g$ for 30 min. The pellet was washed twice with Buffer II (10 mM Tris-HCl (pH 7.4)/0.1 mM dithiothreitol/0.3 mM PMSF/0.1 mM DFP) by centrifugation ($31000 \times g$, 30 min) after Dounce homogenization (five strokes). All the above procedures were carried out at 0–4°C and finished within 12 h. The final pellet was suspended in Buffer II to 4 mg protein/ml and if not used immediately, could be stored at −20°C for 3 months without any loss of enzyme activity. Usually, about 30 mg protein were obtained from 20 ml packed erythrocytes.

Human erythrocyte membranes were prepared from fresh blood of healthy donors according to the method of Dodge et al. [15] using 5 mM Tris-HCl buffer (pH 8.0).

Membrane phosphorylation. A reaction mixture (120 μl) containing membranes (120–240 μg protein), 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 1 mM dithiothreitol was preincubated at 30°C for 5 min and the reaction was started by the addition of 10 μl [γ-^{32}P]ATP (10000–20000 cpm/pmol; final concentration: 20 μM). Incubation was carried out at 30°C for the desired time (usually 30 s) and the reaction was stopped by the addition of 10 μl 20% sodium dodecyl sulfate (SDS) followed by heating at 70°C for 1 min. To the mixture was added 10 μl of 40% sucrose containing a tracking dye (Pronine Y) before electrophoresis.

Measurement of total ^{32}P incorporation. The phosphorylation reaction was carried out in the presence of low specific activity (100–200 cpm/pmol) of [γ-^{32}P]ATP and exogenous proteins (if indicated) under the same conditions as those for membrane phosphorylation. After incubation, ^{32}P incorporation into the trichloroacetic acid-insoluble fraction was measured using a paper disc as described previously [16].

SDS-polyacrylamide gel electrophoresis and autoradiography. A measured amount of phosphorylated membranes was subjected to SDS gel electrophoresis using 5.6% slab gel according to the method of Fairbanks et al. [17]. The gel was then stained with Coomassie brilliant blue and dried at 50°C on a sheet of filter paper (Whatman 3MM) placed on a slab gel dryer. The dried gel was exposed to Kodak X-Omat RP film (Eastman Kodak Co., New York, U.S.A.) for about 50 h. If necessary, autoradiograms were scanned with a

densitometer according to Chaplin et al. [18].

Extraction of proteins from phosphorylated membranes. A reaction mixture (600 μl), which contained chicken erythrocyte membranes (1.6 mg protein), 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 20 μM [γ-^{32}P]ATP ($1.5 \cdot 10^4$ cpm/pmol), was incubated at 30°C for 5 min and added to ice-cold buffer II containing 1 mM NaH_2PO_4 as a phosphatase inhibitor. The mixture was then centrifuged at $31000 \times g$ for 20 min and the pellet suspended in 1 ml of one of the following solutions to extract the phosphorylated proteins: (a) 0.1 mM sodium EDTA (pH 8.0)/0.1 mM dithiothreitol; (b) 0.5 M NaCl in buffer II containing 1 mM NaH_2PO_4; or (c) 0.3% Triton X-100 in buffer II containing 1 mM NaH_2PO_4. The suspension was gently stirred for 60 min and then centrifugated at $31000 \times g$ for 20 min. All extraction procedures were carried out at 0–4°C. To the supernatant, 50 μl 20% SDS was added and the residue was solubilized in 630 μl of the buffer for electrophoresis (40 mM Tris/20 mM sodium acetate/2 mM sodium EDTA, pH 7.4) containing 1% SDS. After dialysis against the buffer for electrophoresis, a measured amount of each sample was subjected to SDS-polyacrylamide gel electrophoresis.

Immunoprecipitation of 43 kDa phosphoprotein. Phosphorylation of chicken erythrocyte membranes (4 mg protein) was carried out in the presence of 200 μM $CaCl_2$ and in the absence of dithiothreitol for 5 min. The phosphorylated proteins were extracted with 0.5 M NaCl/10 mM NaF/1 mM $Na_4P_2O_7$ as a phosphatase inhibitor. Other conditions of phosphorylation and extraction were the same as described above. To the 900 μl of the phosphoprotein extract was added 90 μl of rabbit anti-chicken gizzard actin antiserum and the mixture was incubated for 100 min. Then, goat anti-rabbit IgG antibody solution (200 μl) was added and the mixture was kept for 4 h. Precipitates formed were collected by centrifugation at $11340 \times g$ for 20 min. All procedures were carried out at 0–4°C. The precipitates were solubilized in 500 μl of the buffer for electrophoresis containing 2% SDS and 12 mM dithiothreitol at 70°C, and a 20 μl aliquot was subjected to electrophoresis.

Protein determination. Proteins were determined by the method of Lowry et al. [19] with bovine serum albumin as a standard.

Results

SDS-polyacrylamide gel electrophoresis of chicken erythrocyte membrane proteins

The protein composition of chicken erythrocyte membranes was analyzed by SDS-polyacrylamide gel electrophoresis. For preparation of the membranes, we adopted the combined method of Dounce homogenization and sucrose density separation. The membranes thus obtained showed the same protein pattern upon electrophoresis as that reported by Blanchet [20] and shown in Fig. 1. The most distinct difference in the membranous protein composition between human and chicken erythrocytes was in proteins having an approximate molecular weight (M_r) of 170000 (Fig. 1). Chicken erythrocyte membranes show at least two distinct bands of about 170 kDa, while human erythrocytes lack proteins in this region. The possibility that the 170 kDa membrane proteins are products of degradation by membrane proteases during the preparation of membranes cannot completely be ruled out at present.

Protein phosphorylation of purified plasma membranes by endogenous membrane-associated protein kinases

Endogenous phosphorylation of chicken erythrocyte membrane proteins was investigated. As shown in Fig. 2, many proteins were endogenously phosphorylated in the presence of [γ-^{32}P]ATP and Mg^{2+}. The optimum pH was between 7.0–9.0 and [γ-^{32}P]GTP could not be utilized as a phosphoryl donor instead of [γ-^{32}P]ATP (data not shown).

Exogenous substrates

The phosphorylation of exogenous substrates by chicken erythrocyte membrane-associated protein kinases was studied using histone, protamine, casein and bovine serum albumin as substrates. These proteins have been widely used for investigation of the specificity of various protein kinases. As shown in Fig. 3, protamine was phosphorylated by the membranous protein kinase(s) most rapidly and the other proteins were poor substrates for the kinase.

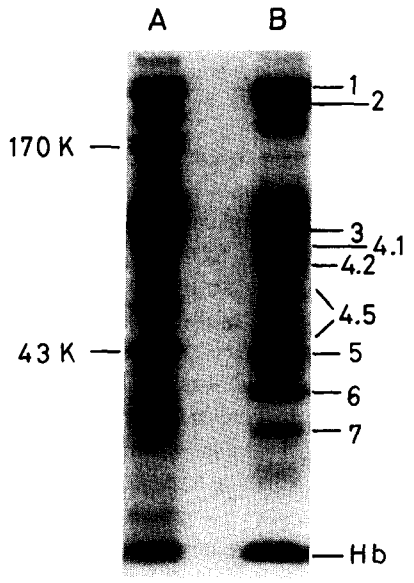

Fig. 1. Sodium dodecyl sulfate electrophoresis of membrane proteins in a 5.6% polyacrylamide slab gel. (A) Chicken erythrocyte membranes, (B) human erythrocyte membranes. Proteins are numbered according to Steck [23].

Effect of EGTA

When 100 μM EGTA was present in the reaction mixture for membrane phosphorylation, the phosphorylation of the protein of approximate molecular weight of 43000 could not be observed and, moreover, the phosphorylated protein bands corresponding to approximate molecular weights of 54000 and 60000 became much fainter, as shown in Fig. 4B. Phosphorylation of these proteins was, however, restored by the addition of $CaCl_2$ (Fig. 4C).

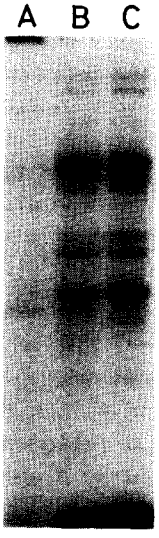

Fig. 2. Autoradiogram of endogenously phosphorylated proteins of chicken erythrocyte membranes in the presence and absence of $MgCl_2$. (A) no $MgCl_2$, (B) 1 mM $MgCl_2$, (C) 10 mM $MgCl_2$. Details are described in Materials and Methods.

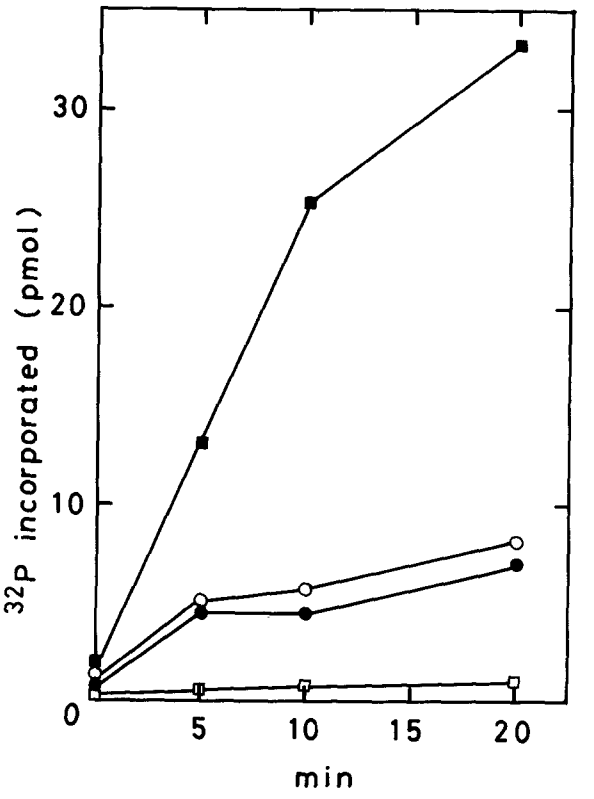

Fig. 3. Phosphorylation of exogenous proteins by chicken erythrocyte membranes. 150 μg of a substrate protein was incubated with membranes (20 μg protein) at 30°C for the indicated times. The net phosphorylation was calculated by substracting the amount of endogenous ^{32}P incorporated into the membranes from the total ^{32}P incorporated into the trichloroacetic acid-insoluble fraction. ●——●, histone; ○——○, casein; ■——■, protamine; □——□, bovine serum albumin.

These results suggest that the phosphorylation of 43 kDa protein, which is one of the most intensively phosphorylated proteins, and possibly the phosphorylation of 54 kDa, and 60 kDa proteins were dependent on Ca^{2+}. On the other hand, human erythrocyte membranes did not show any Ca^{2+}-dependent phosphorylation (Fig. 4D–F). In

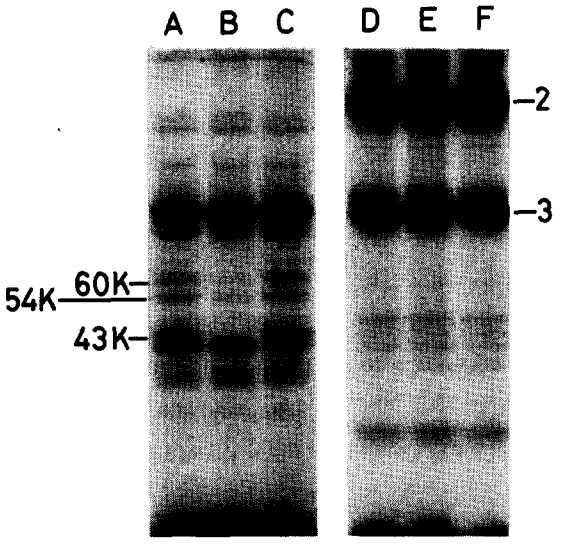

Fig. 4. Effects of EGTA and Ca^{2+} on the phosphorylation of chicken and human erythrocyte membranes. Experimental details are described in Materials and Methods. (A), (B) and (C), chicken erythrocyte membranes; (D), (E) and (F), human erythrocyte membranes. (A) and (D), control; (B) and (E), 100 μM EGTA; (C) and (F), 100 μM EGTA + 100 μM $CaCl_2$.

contrast with the results obtained for turkey erythrocyte membranes [21], cyclic nucleotides (cyclic AMP and cyclic GMP) had no effect on the endogenous phosphorylation of chicken erythrocyte membrane proteins (data not shown).

Time course of phosphorylation

The time course of phosphorylation was studied by determining the total count of ^{32}P incorporated into a trichloroacetic acid-insoluble fraction and by density scanning of the phosphorylated 43 kDa protein on autoradiograms. As shown in Fig. 5, the endogenous phosphorylation was a very rapid reaction, and the half-maximum time of 43 kDa protein phosphorylation was about 30 s. When the incubation was carried out for more than 15 min, the total counts of ^{32}P and ^{32}P incorporated into the 43 kDa protein decreased in a time-dependent manner. This suggests the presence of a phosphoprotein phosphatase in chicken erythrocyte membranes.

Effect of trifluoperazine

We investigated the effect of trifluoperazine,

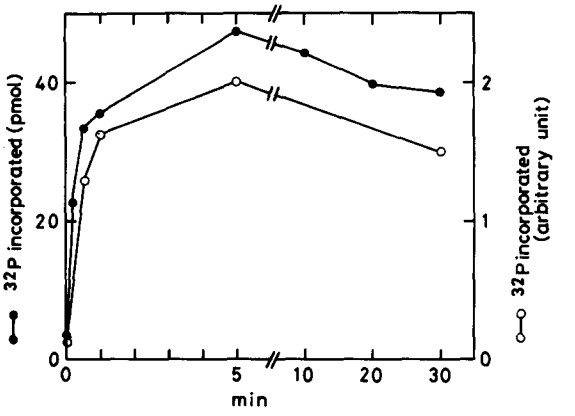

Fig. 5. Time course of endogenous membrane phosphorylation. ●——●, total ^{32}P incorporated into trichloroacetic acid-insoluble fraction; ○——○, ^{32}P incorporated into 43 kDa protein monitored spectrophotometrically on autoradiogram.

which is known as an inhibitor of calmodulin in the presence of Ca^{2+} [22]. As shown in Fig. 6, trifluoperazine completely abolished the phosphorylation of 43 kDa protein, but the effect of the drug on the phosphorylation of both 54 kDa and 60 kDa proteins was less obvious. These results may suggest that calmodulin is involved in the endogenous phosphorylation of the 43 kDa protein in chicken erythrocyte membranes.

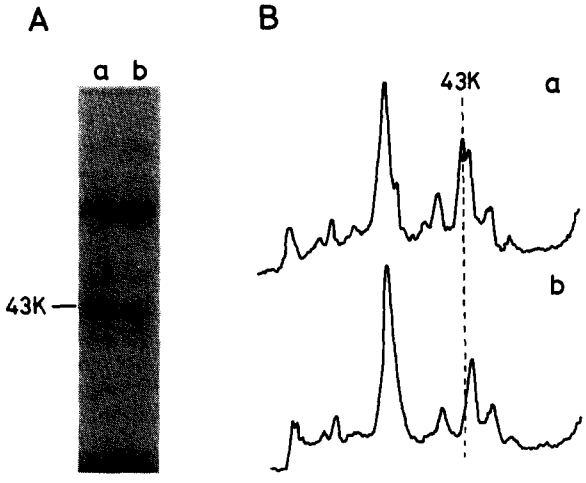

Fig. 6. Effect of trifluoperazine on phosphorylation of chicken erythrocyte membranes, (a) 500 μM maleate, (b) 250 μM trifluoperazine dimaleate. (B) represents densitometric patterns of (A). Experimental details are described in Materials and Methods.

Extraction of the 43 kDa protein

To study the mode of existence of the 43 kDa protein in the chicken erythrocyte membranes, extraction of the protein was attempted under various conditions. As shown in Fig. 7, the 43 kDa protein was solubilized with high salt concentrations most effectively, and extraction with low salt concentrations was found to be ineffective. These low-salt conditions are known to be effective for solubilizing spectrin complexes from human erythrocyte membranes [23]. Triton X-100 solubilized the protein only partially from the membranes.

From these results, the 43 kDa protein seems to be a peripheral protein, which is bound to the membrane by ionic interaction.

Immunoprecipitation of 43 kDa phosphoprotein with an anti-actin antibody

As shown in Fig. 8, the extracted 43 kDa protein was precipitated specifically with rabbit anti-chicken gizzard actin antibody. This result suggests that the 43 kDa protein is an actin-like protein in the membrane.

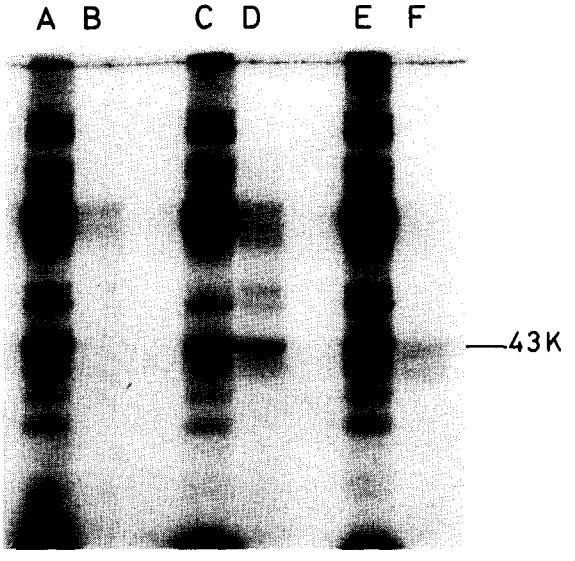

Fig. 7. Autoradiogram showing extraction of phosphorylated 43 kDa protein from chicken erythrocyte membranes. Extraction conditions were as follows. (A) and (B), 0.1 mM EDTA+0.1 mM dithiothreitol (pH 8.0); (C) and (D), 0.5 M NaCl; (E) and (F), 0.3% Triton X-100. After extraction, samples were prepared as described in Materials and Methods. (A), (C), and (E) were the supernatants and (B), (D), and (F) were the residues.

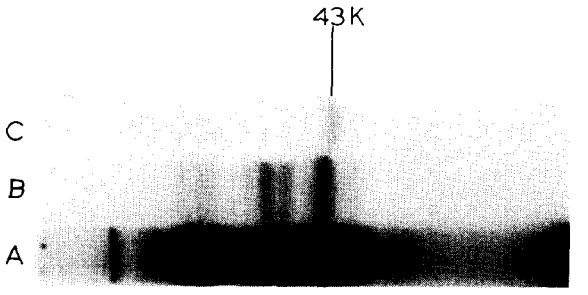

Fig. 8. Autoradiogram of immunoprecipitates of the 43 kDa phosphoprotein with rabbit anti-chicken gizzard actin antibody. Experimental details are described in Materials and Methods. (A), Phosphorylated membranes; (B), Phosphoproteins extracted with 0.5 M NaCl; (C), Immunoprecipitates with rabbit anti-chicken gizzards actin antibody.

Discussion

We have studied the endogenous phosphorylation of chicken erythrocyte membrane proteins and have observed that many of them were phosphorylated under the physiological conditions (Fig. 2). The phosphorylation was a very rapid reaction, as reported for some other membranes [24]. In chicken erythrocyte membranes the phosphorylation of a protein (43 kDa) was observed only in the presence of Ca^{2+}, and this reaction was abolished by EGTA or trifluoperazine, an inhibitor of calmodulin. From these results, calmodulin seems to play an important role in the Ca^{2+}-dependent phosphorylation of a peripheral protein having a molecular weight of 43000 in chicken erythrocyte membranes. When stimulation by concanavalin A was conducted on chicken erythrocytes in the presence of $H_3{}^{32}PO_4$, the phosphorylation of a membrane protein having an approximate molecular weight of 220000 was considerably enhanced. The radioactivity incorporated into this protein in the presence of concanavalin A was about 1.8-times that observed in the experiment without concanavalin A and this enhanced phosphorylation was also found to be Ca^{2+}-dependent and trifluoperazine-sensitive (Nakajima, M. and Osawa, T., unpublished results).

Kakiuchi et al. [25] reported that membrane fractions have intrinsic Ca^{2+} and calmodulin, and previous treatment of membrane with EGTA is essential for investigation of the effect of exoge-

nous calmodulin [26]. Therefore, we prepared 1 mM EGTA-treated membranes by the method of Sobue et al. [26], and the effect of exogenous calmodulin on the phosphorylation of membrane proteins was investigated. However, the treatment of chicken erythrocyte membranes with this concentration of EGTA irreversibly reduced total membranous phosphorylating activity and, therefore, the effect of exogenous calmodulin could not be assessed. Since phenothiazines are known to interact with phospholipids [27], the possibility that trifluoperazine affects the membrane phosphorylating system via Ca^{2+}-activated, phospholipid-dependent protein kinase [28,29] should also be considered. Further investigation is necessary to clarify this point.

To minimize proteolysis during the preparation of human erythrocyte membranes, the presence of a chelating agent in addition to PMSF or DFP is said to be necessary [30,31]. In our present experiments, however, the membranes were prepared only in the presence of PMSF and DFP, because the addition of EDTA at the concentration (1 mM) generally employed for the inhibition of membrane proteases [30–32] was found to reduce irreversibly the phosphorylating activity.

From the result that the phosphorylated 43 kDa protein could be solubilized with 0.5 M NaCl, this protein seems to be a peripheral protein of chicken erythrocyte membranes. Protein phosphorylation in avian red cell membranes was well studied in turkey erythrocyte membranes [21]. In those studies, the addition of either cyclic AMP or Ca^{2+} plus calmodulin to purified turkey erythrocyte membranes increased the incorporation of ^{32}P into goblin, a major protein of M_r 230000 which is tightly associated with plasma membranes of turkey erythrocytes. In chicken erythrocyte membranes, however, a phosphorylated protein band corresponding to goblin could not be detected (Fig. 2).

The phosphorylated 43 kDa protein had the same electrophoretic mobility as actin (band 5) of human erythrocyte membranes on SDS-polyacrylamide gel electrophoresis. Furthermore, the result of immunoprecipitation suggests that this protein is an actin-like protein. Phosphorylation of actin was reported on smooth muscle [33] and liver plasma membranes [34]. Final identification of the 43 kDa protein in this study as actin of chicken erythrocyte membranes awaits the large-scale preparation of this protein.

Recently, a Ca^{2+}-sensitive phosphoprotein was identified as a phosphorylated intermediate of Ca^{2+}-ATPase in rat duodenal epithelium membranes [35]. Since plasma membranes of human erythrocytes are known to contain Ca^{2+}-ATPase [36], there is a possibility that the phosphoproteins observed in the presence of Ca^{2+} in chicken erythrocyte membranes are such an intermediate of Ca^{2+}-ATPase in chicken erythrocytes. However, it should be pointed out that there are some differences between the intermediate and the phosphorylated proteins in this work. The Ca^{2+}-dependent phosphorylation of the proteins in chicken erythrocyte membranes showed an absolute requirement of Mg^{2+} (Fig. 2), while the formation of the intermediate phosphoproteins of Ca^{2+}-ATPase occurred without Mg^{2+} [35]. Therefore, the possibility that the phosphorylated membrane protein in this study is Ca^{2+}-ATPase seems to be remote. Further study is, however, necessary to identify the phosphorylated proteins in chicken erythrocyte membranes.

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References

1 Weller, M. (1979) Protein Phosphorylation, Pion Ltd., London
2 Ross, E.M. and Gilman, A.G. (1980) Annu. Rev. Biochem. 49, 533–564
3 Suzuki, K., Terao, T. and Osawa, T. (1980) Biochim. Biophys. Acta 602, 78–86
4 Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T. and Nishizuka, Y. (1979) J. Biol. Chem. 254, 3692–3695
5 Rudolph, S.A., Beam, K.G. and Greengard, P. (1978) in Membrane Transport Processes (Hoffman, J.F., ed.), Vol. 1, pp. 107–123, Raven Press, New York
6 Chang, K.-J., Marcus, N.A. and Cuatrecasas, P. (1974) J. Biol. Chem. 249, 6854–6865
7 Gazitt, Y., Ohad, I. and Loyter, A. (1976) Biochim. Biophys. Acta 436, 1–14

8 Kury, P.G. and McConnell, H.M. (1975) Biochemistry 14, 2798–2803
9 Øye, I. and Sutherland, E.W. (1966) Biochim. Biophys. Acta 127, 347–354
10 Ito, Y., Yoshimoto, R., Irimura, T., Setaka, M., Shimizu, H. and Osawa, T. (1979) J. Biochem. (Tokyo) 86, 1807–1815
11 Nakajima, M., Tamura, E., Irimura, T.,Toyoshima, S., Hirano, H. and Osawa, T. (1981) J. Biochem. (Tokyo) 89, 665–675
12 Weiss, B., Live, T.R. and Richardson, C.C. (1968) J. Biol. Chem. 243, 4530–4542
13 Salesse, R. and Garnier, J. (1979) Biochim. Biophys. Acta 554, 102–113
14 Snider, D.E., Jr. and Parker, C.W. (1977) Biochem. J. 162, 473–482
15 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
16 Suzuki, K., Terao, T. and Osawa, T. (1981) J. Biochem. (Tokyo) 89, 1–11
17 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
18 Chaplin, D.D., Wedner, H.J. and Parker, C.W. (1979) Biochem. J. 182, 537–546
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
20 Blanchet, J.P. (1974) Exp. Cell Res. 84, 159–166
21 Alper, S.L., Palfrey, H.C., DeRiemer, S.A. and Greengard, P. (1980) J. Biol. Chem. 255, 11029–11039
22 Levin, R.M. and Weiss, B. (1976) Mol. Pharmacol. 12, 581–589
23 Steck, T.L. (1974) J. Cell Biol. 62, 1–19
24 Chaplin, D.D., Wedner, H.J. and Parker, C.W. (1979) Biochem. J. 182, 525–536
25 Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K. Yasuda, S., Kashiba, A., Sobue, K., Ohshima, M. and Nakajima, T. (1978) in Advances in Cyclic Nucleotide Research (George, W.J. and Ignarro, L.J., eds.), Vol. 9, pp. 253–263, Raven Press, New York
26 Sobue, K., Muramoto, Y., Yamazaki, R. and Kakiuchi, S. (1979) FEBS Lett. 105, 105–109
27 Mori, T., Takai, Y., Minakuchi, R., Yu, B. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 8378–8380
28 Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. and Nishizuka, Y. (1979) Biochem. Biophys. Res. Commun. 91, 1218–1224
29 Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 2273–2276
30 Bennett, V. and Stenbuck, P.J. (1980) J. Biol. Chem. 255, 2540–2548
31 Siegel, D.L., Goodman, S.R. and Branton, D. (1980) Biochim. Biophys. Acta 598, 517–527
32 Tyler, J.M., Hargreaves, W.R. and Branton, D. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5192–5196
33 Walsh, M.P., Hinkins, S. and Hartshorne, D.J. (1981) Biochem. Biophys. Res. Commun. 102, 149–157
34 Grazi, E., Ferri, A., Lanzara, V., Magri, E. and Zaccarini, M. (1980) FEBS Lett. 112, 67–69
35 De Jonge, H.R., Ghijsen, W.E.J.M. and Van Os, C.H. (1981) Biochim. Biophys. Acta 647, 140–149
36 Rega, A.F. and Garrahan, P.J. (1980) Biochim. Biophys. Acta 596, 487–489