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Differential Phosphorylation of Band 3 and Glycophorin in Intact and Extracted Erythrocyte Membranes

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This report presents an analysis of the phosphorylation of human and rabbit erythrocyte membrane proteins which migrate in $NaDodSO_4$-polyacrylamide gels in the area of the Coomassie Blue-stained proteins generally known as band 3. The phosphorylation of these proteins is of interest as band 3 has been implicated in transport processes. This study shows that there are at least three distinct phosphoproteins associated with the band 3 region of human erythrocyte membranes. These are band 2.9, the major band 3, and PAS-1. The phosphorylation of these proteins is differentially catalyzed by solubilized membrane and cytoplasmic cyclic AMP-dependent and -independent erythrocyte protein kinases. Band 2.9 is present and phosphorylated in unfractionated human and rabbit erythrocyte ghosts but not in NaI- or dimethylmaleic anhydride (DMMA)-extracted membranes. These latter membrane preparations are enriched in band 3 and in sialoglycoproteins. The NaI-extracted ghosts contain residual protein kinase activity which can catalyze the autophosphorylation of band 3 whereas the DMMA-extracted ghosts are usually devoid of any kinase activity. However, both NaI- and DMMA-extracted ghosts, as well as Triton X-100 extracts of the DMMA-extracted ghosts, can be phosphorylated by various erythrocyte protein kinases. The kinases which preferentially phosphorylate the major band 3 protein are inactive towards PAS-1 while the kinases active towards PAS-1 are less active towards band 3. The band 3 protein in the DMMA-extracted ghosts can be cross-linked with the Cu^{2+}-*o*-phenanthroline complex. The cross-linking of band 3 does not affect its capacity to serve as a phosphoryl acceptor nor does phosphorylation affect the capacity of band 3 to form cross-links. In addition to band 2.9, the major band 3 and PAS-1, another minor protein component appears to be present in the band 3 region in human erythrocyte membranes. This protein is specifically phosphorylated by the cyclic AMP-dependent protein kinases isolated from the cytoplasm of rabbit erythrocytes. The rabbit erythrocyte membranes lack PAS-1 and the cyclic AMP-dependent protein kinase substrate.

Key words: erythrocyte membranes, protein phosphorylation, band 2.9, band 3, glycophorin (PAS-1 and PAS-2)

Previous studies from this (1–4) and other laboratories (5–8) have established that human and rabbit erythrocyte membranes contain multiple protein substrates for membrane-bound and soluble cyclic AMP-dependent and -independent erythrocyte pro-

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tein kinases. While erythrocyte membrane phosphorylation occurs in isolated ghosts (1–3, 5, 6) as well as in intact red cells (4, 9, 10), the significance of these reactions remains undocumented. This is largely due to the fact that the identity and functions of many of the membrane proteins which can undergo phosphorylation are unknown.

Of considerable interest in the study of membrane phosphorylation is the reaction involving band 3 (nomenclature of Steck, Ref. 11) as the band 3 protein of human erythrocyte membranes has been implicated in various transport processes (12–18). Band 3 is a lightly glycosylated protein that accounts for approximately 25% of the total Coomassie Blue-stained peptides of the erythrocyte membrane resolved by $NaDodSO_4$-polyacrylamide gel electrophoresis. However, the diffuse region in the gel which is generally designated as band 3 contains one or more substrates for erythrocyte protein kinases (1, 3). That the area stained with Coomassie Blue as band 3 contains more than one peptide species has been suggested for both human (19) and rabbit (20) erythrocytes. In this study we report on an analysis of the phosphorylation of partially purified preparations of human and rabbit band 3 area proteins by erythrocyte protein kinases derived from the membrane and the cytoplasmic fractions. Our results show that particulate and soluble preparations of band 3-enriched membranes of human erythrocytes contain at least 2 distinct peptides which can be differentially phosphorylated by various erythrocyte protein kinases. One of these phosphopeptides is the major band 3 protein while the other appears to be the major erythrocyte sialoglycoprotein, glycophorin (PAS-1). Comparisons are made of the phosphorylation of intact ghosts and the band 3-enriched ghosts.

MATERIALS AND METHODS

Hemoglobin-free ghosts of human and rabbit erythrocytes were prepared from fresh or outdated blood according to the method of Dodge et al. (21) as previously described (22). The cyclic AMP-independent erythrocyte membrane protein kinases were extracted from human and rabbit red cell ghosts with 0.5 M NaCl and partially purified by $(NH_4)_2SO_4$ fractionation and gel filtration (23). These kinases are referred to as HMK, the human erythrocyte membrane kinase, and MK-I and MK-II, the rabbit erythrocyte membrane kinases. The enzymes have been characterized in detail with regards to their activity towards exogenous (23) and membrane substrates (24). In the presence of 0.4 M salt MK-I has a molecular weight (MW) of ~ 100,000 and can catalyze the phosphorylation of casein and membrane proteins using ATP or GTP. In contrast, MK-II and HMK, which appear to be similar in nature, are ~ 30,000 daltons in the presence of salt and can use only ATP to phosphorylate casein and some membrane proteins. Under certain conditions, MK-II and HMK can also use GTP as a phosphoryl donor (23, 24). The cyclic AMP-dependent protein kinases from rabbit erythrocyte lysates, which use only ATP as a phosphoryl donor, were purified according to Tao and Hackett (25). The soluble casein kinases, which can use either ATP or GTP as phosphoryl donor and are independent of cyclic nucleotides, were obtained according to Kumar and Tao (26).

"Purification" of band 3 was achieved by extracting whole ghosts with either 1.0 M NaI (12) or with 2,3-dimethylmaleic anhydride (DMMA) using 1.5–2.0 mg DMMA/mg protein (27). In some instances the latter procedure was repeated a second time. Triton-extraction of the DMMA-extracted ghosts was performed with 0.125% Triton X-100 in 5 mM Tris-HCl, pH 7.5, according to Zala and Kahlenberg (13). The DMMA-, NaI-, or

Triton-extracted ghosts were washed 2–3 times with either 10 mM Tris-HCl, pH 7.5, or with deionized H_2O. The reaction mixture contained (in a final volume of 50 μl): 0.1 M glycine-NaOH, pH 8.5; 10 mM $MgCl_2$; 0.2 mM [γ-^{32}P] ATP or [γ-^{32}P] GTP (150–400 cpm/pmol); ± 10μM cyclic AMP; ± kinases; and 10–30 μg of membrane proteins. Phosphorylation was initiated by the addition of ATP (or GTP), buffer, and Mg^{2+}, and terminated with 20 volumes of a KCl stopping solution (150 mM KCl; 10 mM Tris-HCl, pH 7.5;1 mM EDTA) followed by centrifugation (3). The phosphorylated ghosts were applied to 4% polyacrylamide slab gels (unless otherwise specified) containing 0.2% $NaDodSO_4$ (27, 28), electrophoresed at 75 mA/slab, and stained with Coomassie Brilliant Blue (CBB). The destained gels were dried and radioautograms prepared and scanned using a Zeineh Soft-Laser densitometer (1, 3). The glycoprotein content of membrane extracts was determined in gels stained with periodic acid-Schiff's reagent (PAS) (27, 28).

Cross-linking of DMMA-extracted human erythrocyte membranes was performed before and after phosphorylation. The proteins to be cross-linked before phosphorylation were mixed with an equal volume of 280 μM $CuSO_4$/140 μM *o*-phenanthroline (CuP) and incubated for 20 min at 0°C. The reaction was terminated by 20 volumes of the KCl stopping solution followed by centrifugation at 27,000 × g for 20 min. The pellet was washed once with 20 mM Tris-HCl, pH 7.5. After centrifugation the cross-linked membranes were resuspended and phosphorylated as described above. However, in these studies the phosphorylation reactions (control, CuP treated before and after) were all terminated with 20 volumes of 20 mM Tris-HCl, pH 7.5, and centrifuged. For studies of cross-linking after phosphorylation, the pellets were resuspended to ~20 μl and 20 μl of 280 μM $CuSO_4$/ 140 μM *o*-phenanthroline was added. After a 20 min incubation at 0°C the sample was dissolved in the $NaDodSO_4$ diluent. In these experiments the samples were all dissolved in the $NaDodSO_4$ diluent minus dithiothreitol and applied to $NaDodSO_4$-polyacrylamide gels containing 3.2% acrylamide.

The [γ-^{32}P] ATP and [γ-^{32}P] GTP were obtained from Amhersham/Searle. Electrophoresis supplies were from Bio-Rad. Radioautograms were prepared from Kodak NoScreen Medical X-Ray film. All other reagents were obtained from Sigma Chemical Co. or other commercial sources.

RESULTS

Initial experiments designed to analyze the phosphorylation of band 3-enriched membranes were performed using ghosts which had been extracted with either NaI (1.0 M) or with DMMA (1.5–2.0 mg DMMA/mg protein). As determined by $NaDodSO_4$-polyacrylamide gel electrophoresis, both particulate preparations were enriched with respect to band 3. However, the ghosts extracted with NaI contained a greater number of "other" protein bands than the DMMA pellets (data not shown). Both preparations were analyzed in order to determine if they contained endogenous kinase activity or substrates for the solubilized cyclic AMP-independent human erythrocyte membrane kinase (HMK). Table I shows that autophosphorylation of band 3 occurs in the NaI-extracted but not in the DMMA-extracted ghosts. The autophosphorylation reaction was observed in the presence of ATP but not GTP and was slightly enhanced by cyclic AMP. The results suggest that the NaI-extracted ghosts may contain residual activity of the human erythrocyte membrane cyclic AMP-dependent and -independent kinases. It is evident from Table I that both preparations served equally well as substrates for the solubilized HMK in the presence of ATP. Usage of GTP was poor or nonexistent.

TABLE I. Phosphorylation of Band 3 Protein(s) in NaI- and Dimethylmaleic Anhydride (DMMA)-Extracted Human Erythrocyte Ghosts

Experiment	ATP	ATP + Cyclic AMP	GTP
		(arbitrary densitometric units)	
NaI-Ghosts			
Control	30	44	0.6
+ HMK	170	148	22
DMMA-Ghosts			
Control	0	0	0
+ HMK	150	168	0.8

NaI- or DMMA-extracted ghosts were phosphorylated as described under Methods and electrophoresed in a 4% slab gel. The radioautogram was traced on a desitometer and the areas under the peaks corresponding to band 3 were integrated. The values are expressed as arbitrary densitometric units.

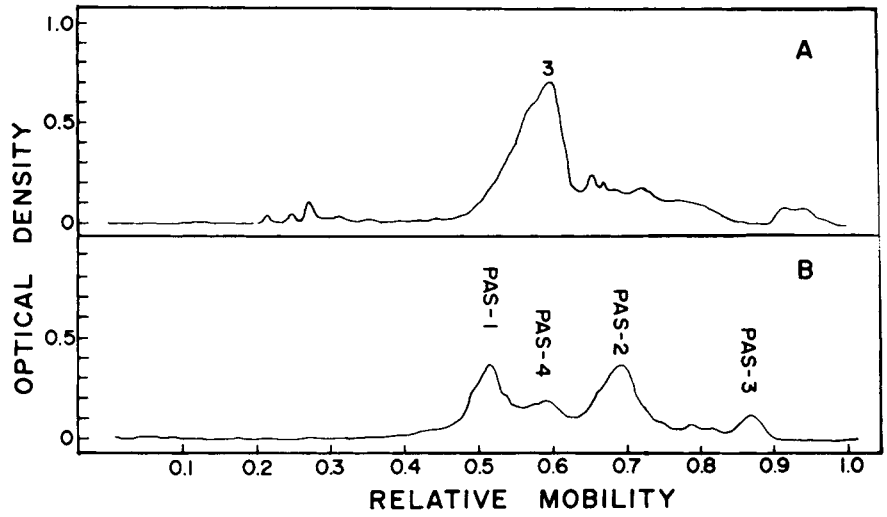

Fig. 1. The proteins and glycoproteins of DMMA-extracted human erythrocyte ghosts (26 μg). The electrophoresis was carried out in $NaDodSO_4$ (0.2%)-polyacrylamide (4%) gels; the gels were stained for protein with Coomassie Blue (A) and for sialoglycoprotein with periodic acid-Schiff's reagent (B).

Since the DMMA-ghosts were considerably more enriched in band 3 and appeared to contain suitable substrate(s) for phosphorylation, further analysis of band 3 phosphorylation was performed using these ghosts. Figure 1 shows the electrophoretic profile of a typical DMMA ghost preparation on a 4% polyacrylamide slab gel. In this preparation, the band 3 protein accounts for greater than 60% of the total CBB-stained protein (Fig. 1A) vs less than 30% in unfractionated human erythrocyte ghosts (data not shown). The DMMA-extracted ghosts appear to retain all the major sialoglycoproteins. The electrophoretic mobilities of the PAS-stained sialoglycoproteins in the 4% gel (Fig. 1B) are slower than in the more commonly used 5 or 5.6% gels (for example, see Refs. 10, 11, 28). This is consistent with the observation that the mobilities of glycoproteins in $NaDodSO_4$

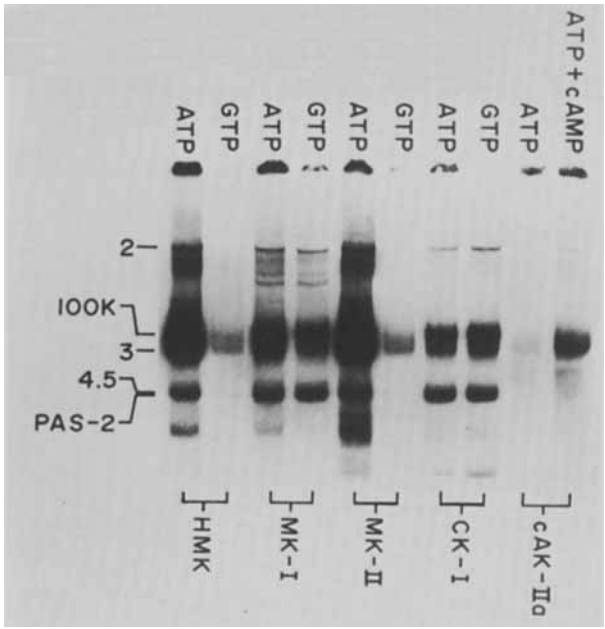

Fig. 2. Radioautogram showing the phosphorylation of DMMA-extracted ghosts by erythrocyte protein kinases. The membranes (13 μg) were phosphorylated in the presence of: HMK (4.8 μg); MK-I (36 μg); MK-II (13 μg); CK-I (0.75 μg); or cAK-IIa (15 μg). The conditions for phosphorylation and electrophoresis were as described in Methods. [γ-^{32}P] ATP:244 cpm/pmol; [γ-^{32}P] GTP:226 cpm/pmol. The radioautogram was exposed for 4 days.

gels are dependent on the percentage of acrylamide cross-linking (29, 30). Analysis of the DMMA pellet of the rabbit erythrocyte membranes gave a CBB-staining profile similar to that of Fig. 1A, although the band 3 peak was more symmetrical. As expected, no PAS-staining component was detected in the rabbit erythrocyte membranes. These membranes do not appear to contain major sialoglycoproteins (1, 20, 31).

Previous studies of membrane phosphorylation by membrane-bound (1) and soluble (3) kinases indicated that more than one peptide migrating in the band 3 area of human and rabbit erythrocyte membranes could serve as substrates for phosphorylation. These proteins were tentatively identified as bands 2.9 and 3, or collectively as area 3 (1, 3). The evidence for the presence of the phosphoproteins in the area of band 3 was based on the study of the effects of pH and/or kinases on their phosphorylation (1, 3). In view of this, it was necessary to determine whether the band 3-enriched ghosts contained multiple protein substrates for phosphorylation. Figure 2 shows a radioautogram depicting the phosphorylation of DMMA-extracted human erythrocyte ghosts by the solubilized membrane kinases HMK, MK-I, and MK-II, by the erythrocyte cytoplasmic casein kinase-I (CK-I), and by cyclic AMP- dependent protein kinase IIa (cAK-IIa). In order to facilitate comparison of the activities of these enzymes toward membrane substrates, the specific activities of the kinases used in these studies were calibrated on the basis of their ability to phosphorylate casein or histone. The amounts of HMK, MK-I, MK-II, and CK-I used would catalyze equivalent amounts of ^{32}P incorporation into casein, while the amount of cAK-IIa used would catalyze twice the amount of ^{32}P incorporation into histone. From Fig. 2 several points are evident. The solubilized membrane kinases HMK and MK-II

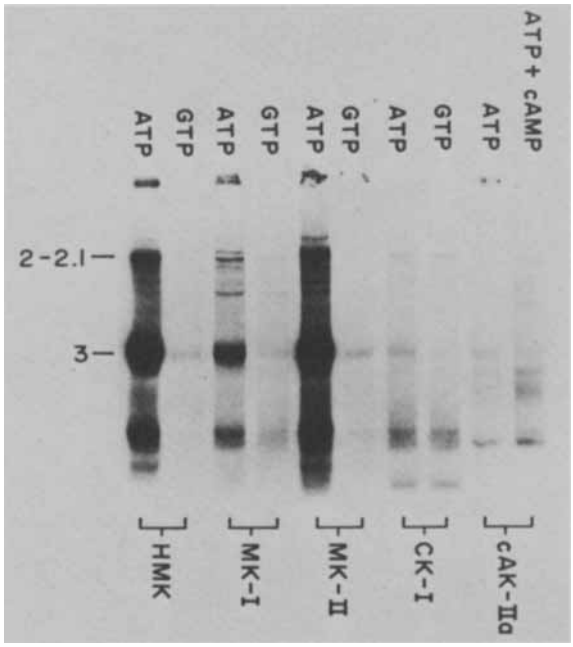

Fig. 3. Radioautogram depicting the phosphorylation of DMMA-extracted rabbit erythrocyte ghosts (6 μg) by erythrocyte protein kinases. Conditions were as described in Fig. 2.

catalyze the phosphorylation of band 3 equally well in the presence of ATP (columns 1 and 5), while neither enzyme uses GTP effectively (columns 2 and 6). However, it is not clear from Fig. 2 whether the phosphorylation in this area is entirely due to band 3 or whether the protein designated as 100K, which migrates in the proximity of band 3, is also phosphorylated by these enzymes. Moreover, MK-I appears to behave differently in that it catalyzes the phosphorylation of 100K and band 3 to the same extent with ATP. However, with GTP, the 100K protein is the preferred substrate. As this enzyme preparation is not homogenous, it is possible that the phosphorylation pattern differences seen with ATP and GTP may reflect the activity of multiple kinases. The cytoplasmic CK-I catalyzes the phosphorylation of the 100K protein and, to a lesser extent, band 3 in the presence of either ATP or GTP. The cyclic AMP-dependent protein kinase IIa (cAK-IIa) catalyzes the phosphorylation of a protein with a mobility similar to band 3 in a reaction that is dependent on cyclic AMP.

Figure 3 shows a similar experiment using DMMA-extracted rabbit erythrocyte membranes. The patterns of phosphorylation obtained with HMK and MK-II are similar to those observed in the DMMA-extracted human erythrocyte ghosts. This appears to be the extent of similarity between the two systems. In the DMMA-extracted rabbit erythrocyte membranes, no comparable phosphorylation of a 100K protein was detected in the presence of MK-I. Furthermore, the phosphorylation of the band 3 area by either CK-I or cAK-IIa is much less prominent in the DMMA-extracted rabbit erythrocyte membranes.

Although the phosphoprotein with a mobility corresponding to 100,000 daltons is not demonstrated in the DMMA-extracted rabbit erythrocyte membranes, we have previously demonstrated that intact rabbit, as well as human, erythrocyte ghosts contain a protein behaving similarly to that designated as 100K in Fig. 2 (1, 3). This protein was tentatively identified as band 2.9 (1, 3). Since the DMMA-extracted human and rabbit

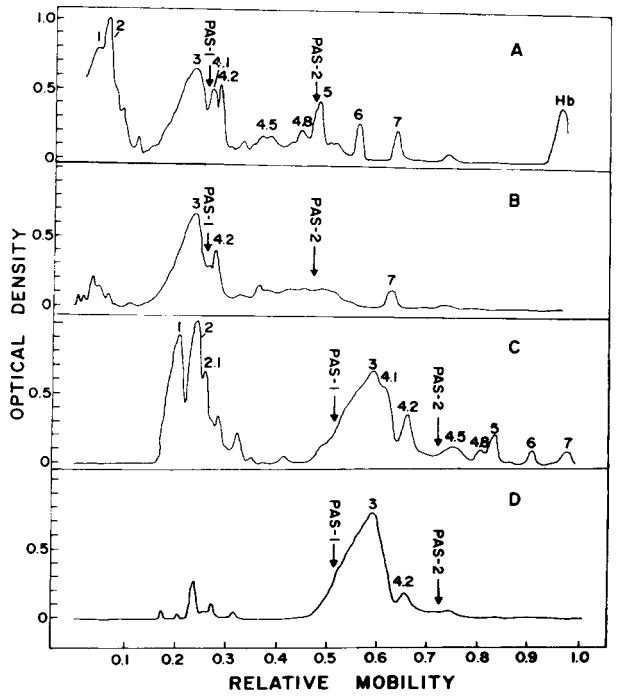

Fig. 4. Mobilities of human erythrocyte membrane proteins and glycoproteins in NaDodSO$_4$ gels containing 7% or 4% polyacrylamide. Duplicate samples of whole ghosts (A and C, 23 μg) or DMMA-extracted ghosts (B and D, 14 μg) were applied to 7% (A and B) or 4% (C and D) gels which were stained with Coomassie Blue or periodic acid-Schiff's reagent.

erythrocyte membranes exhibit a major difference in the phosphorylation in the area of 100K, it becomes necessary to determine whether the human erythrocyte membranes contain, in addition to band 2.9, a distinct protein component with the same mobility, but which is not easily extractable by DMMA. It is of interest to note that PAS-1, which is present in the human but not in the rabbit erythrocyte membranes, also migrates in the area corresponding to 100K. Furthermore, the sialoglycoproteins remain bound to the membranes after extraction with DMMA. In view of these characteristics, the possibility that the 100K phosphoprotein may represent PAS-1 seems likely.

In order to explore the possible relationship of band 2.9 and PAS-1 to the 100K phosphoprotein, the electrophoretic mobilities of these components were examined in gels containing different amounts of acrylamide. The rationale behind this approach is based on the finding that the membrane PAS components behave anomalously during NaDodSO$_4$-polyacrylamide gel electrophoresis when compared to standard proteins (29, 30). This anomalous behavior is due to a decreased binding of NaDodSO$_4$ by the glycoproteins as compared to nonglycosylated, or lightly glycosylated, proteins. As shown by Segrest and Jackson (30), the glycoproteins exhibit a decreased mobility and hence a higher apparent MW in NaDodSO$_4$ gels of low percentage of acrylamide cross-linking. However, in gels of higher acrylamide cross-linking, the electrophoretic mobilities of the glycoproteins reflect more closely their true molecular weight. Figures 4 and 5 compare the CBB- and PAS-staining profiles of human and rabbit erythrocyte membrane proteins in NaDodSO$_4$ gels containing 7% and 4% acrylamide. Figure 4, A and B, shows the electro-

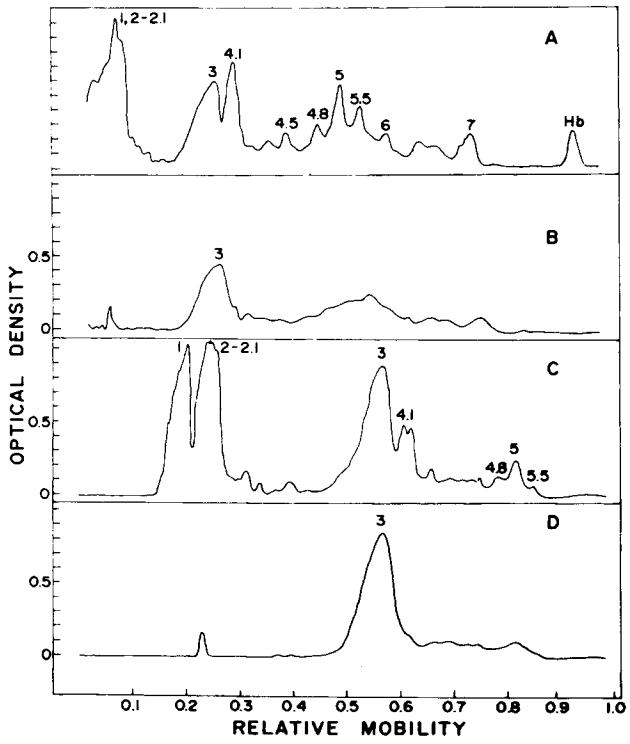

Fig. 5. Mobilities of rabbit erythrocyte membrane proteins in $NaDodSO_4$ gels containing 7% or 4% polyacrylamide. Whole ghosts (A and C, 18 μg) or DMMA-extracted ghosts (B and D, 15 μg) were electrophoresed on 7% (A and B) and 4% (C and D) gels which were stained with Coomassie Blue or periodic acid-Schiff's reagent. No PAS-staining glycopeptides were evident.

phoretic pattern of human erythrocyte membrane proteins and sialoglycoproteins in 7% gels. PAS-1 migrates faster than band 3 but slower than band 4.1, while PAS-2 migrates between 4.8 and 5.0. However, in the 4% gels (Fig. 4, C and D), PAS-1 and PAS-2 exhibit a higher apparent molecular weight and migrate slower than band 3 and band 4.5, respectively. Figure 5 illustrates the mobilities of rabbit erythrocyte membrane proteins in the 7% (Fig. 5, A and B) and 4% (Fig. 5, C and D) gels. No PAS-stained bands are evident.

Figure 6 compares the radioautograms showing the phosphorylation profiles of intact and DMMA-extracted ghosts of human erythrocytes in 4% (top) and 7% (bottom) gels. Several observations can be made. In column 3, which illustrates the phosphorylation of intact ghosts with CK-I and GTP, note that the mobility of the protein designated as 2.9, relative to the other phosphopeptides, is the same in the 2 different gel concentrations; that is, band 2.9 in intact erythrocytes migrates with an apparent MW of approximately 100,000 daltons regardless of the acrylamide concentration. The results suggest that band 2.9 is either lightly glycosylated or not a glycoprotein and is distinct from PAS-1 (see below). On the other hand, the electrophoretic profiles of the phosphoproteins of DMMA-extracted ghosts phosphorylated in the presence of CK-I and ATP (column 4) behave anomalously in $NaDodSO_4$ gels. Of particular interest is the electrophoretic behavior of the phosphopeptide which migrates in the region of 100K in the 4% gel (column 4, top). This phosphopeptide exhibits a different mobility on 7% gel (column 4, bottom). The

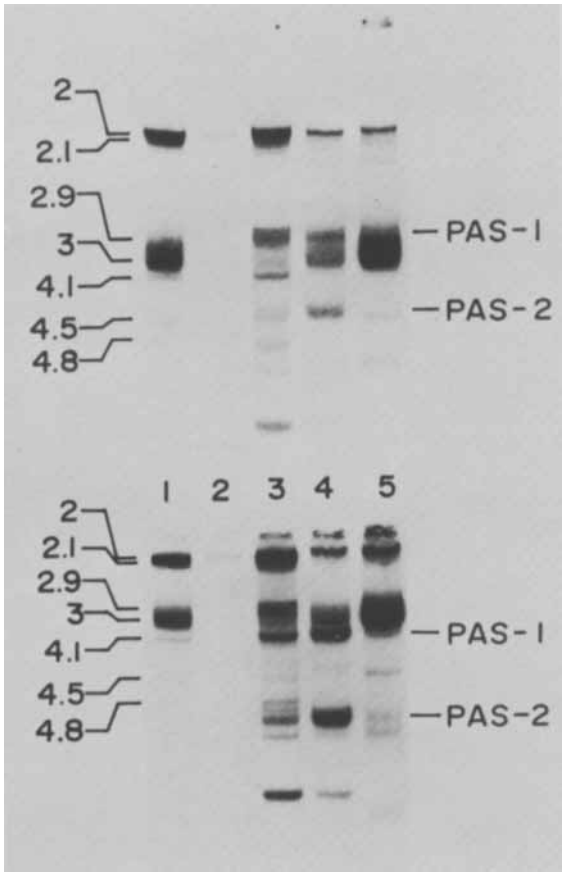

Fig. 6. Differential phosphorylation of bands 2.9, 3, PAS-1, and PAS-2 of human erythrocyte membranes. Phosphorylated proteins were applied to either a 4% (top) or 7% (bottom) gel. (1) whole ghosts + ATP; (2) whole ghosts + GTP; (3) whole ghosts + GTP + CK-I; (4) DMMA-extracted ghosts + ATP + CK-I; (5) DMMA-extracted ghosts + ATP + HMK. Whole ghosts, 23 μg; DMMA-extracted ghosts, 14 μg; CK-I, 1.9 μg; HMK, 4.9 μg; ATP, 350 cpm/pmol; GTP, 390 cpm/ pmol. The radioautograms were exposed for 1 day.

results show that the 100K phosphopeptide of the DMMA-extracted human erythrocyte membranes has the same characteristic anomalous electrophoretic property as the sialoglycoprotein PAS-1 and may be identified with PAS-1 and PAS-2. The two sialoglycoproteins are interconvertible (32, 33). Thus, it appears that PAS-1 and PAS-2 are phosphorylated in the DMMA-extracted ghosts. On the other hand, band 2.9 appears to represent a separate phosphopeptide which is not present in the DMMA-extracted ghosts. It is somewhat more difficult to analyze the phosphorylation of PAS-1 and PAS-2 by CK-I in intact ghosts based on changes in the apparent MW of the PAS-staining components in 4% and 7% gels. The intact ghosts contain many more phosphopeptides than the DMMA-extracted membranes. However, a direct comparison of the phosphopeptides of intact and DMMA-extracted membranes resolved in the 4% and 7% gels (column 3 vs 4) indicates that PAS-1 and PAS-2 in the intact ghosts may be also phosphorylated by CK-I.

The study with HMK reveals a somewhat different phosphorylation pattern; HMK

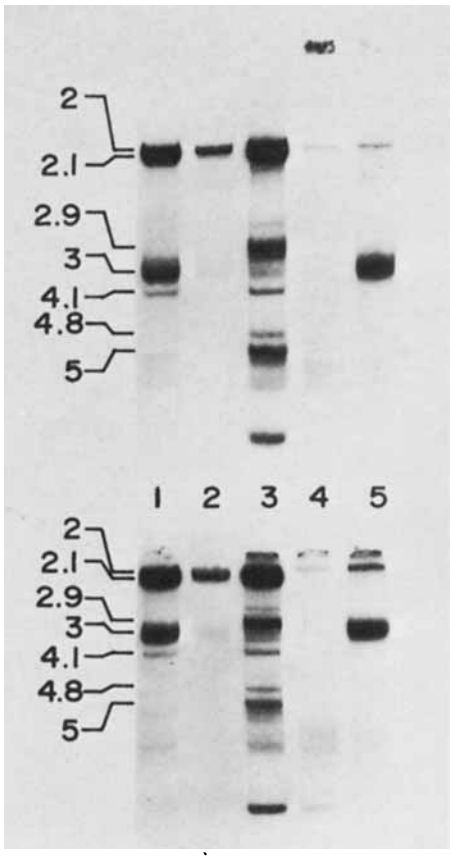

Fig. 7. Phosphorylation of intact (18 μg) and DMMA-extracted (10 μg) rabbit erythrocyte ghosts. Conditions were as described in Fig. 6.

catalyzes the phosphorylation of band 3 and several other minor components in the DMMA-extracted ghosts (column 5). Based on the analysis of the electrophoretic mobilities of the phosphopeptides in 4% and 7% gels (column 5, top vs bottom), it is concluded that the sialoglycoproteins, PAS-1 and PAS-2, in the DMMA-extracted ghosts are not phosphorylated by the solubilized human erythrocyte membrane cyclic AMP-independent protein kinase.

Figure 7 is a similar experiment comparing the phosphorylation patterns of intact and DMMA-extracted rabbit erythrocyte ghosts. As seen in column 3 the unfractionated rabbit ghosts contain a band 2.9 protein that is phosphorylated in the presence of CK-I and GTP. This phosphopeptide is similar to that of the human band 2.9 in that it exhibits no anomalous behavior in $NaDodSO_4$ gels and migrates as a 100,000 dalton protein. In addition, band 2.9 in the rabbit, as in the human, is not retained in the DMMA-extracted ghosts as evidenced by the lack of such a phosphoprotein in these membranes (column 4). No phosphoproteins corresponding to PAS-1 and PAS-2, or to other glycopeptides, are evident in the rabbit erythrocyte membranes; the apparent MWs of the phosphopeptides in these membranes do not vary with the gel concentrations.

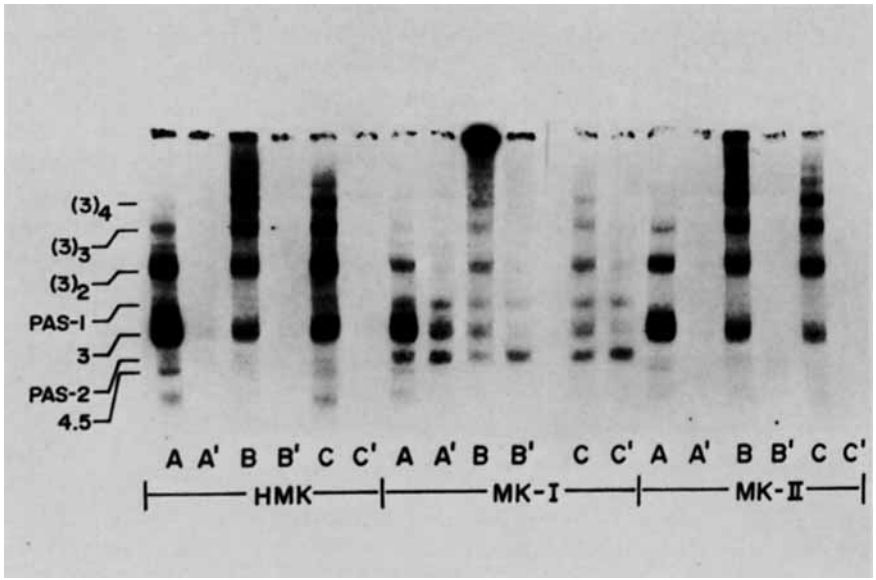

Fig. 8. Phosphorylation of DMMA-extracted human erythrocyte ghosts by HMK, MK-I, and MK-II before and after cross-linking of the membrane proteins with o-phenanthroline-Cu^{2+} complex (CuP). (A) control + ATP; (B) proteins were cross-linked with CuP, then phosphorylated with ATP; (C) proteins were phosphorylated with ATP then cross-linked with CuP. The experimental conditions of the A', B', and C' gels correspond respectively to A, B, and C except with GTP as the phosphoryl donor. DMMA-extracted ghosts, 20 μg; HMK, 7.4 μg; MK-I, 36 μg; MK-II, 13 μg; ATP, 200 cpm/pmol; GTP, 208 cpm/pmol. The radioautogram was exposed for 4 days.

The phosphorylation of the proteins of DMMA-extracted membranes was further examined after extraction with 0.125% Triton X-100 in 5 mM Tris-HCl, pH 7.5 (13). According to Kahlenberg (13), extraction of DMMA-treated human erythrocyte membranes with Triton X-100 yields a membrane preparation containing band 3 and minor components and a supernatant fraction containing both band 3 and the PAS-staining glycoproteins. The residual band 3 associated with the Triton pellets of both the human and rabbit erythrocyte membranes can be phosphorylated by HMK, MK-I, and MK-II in the presence of ATP (data not shown). Similarly, the proteins in the Triton supernatants also can serve as phosphoryl acceptors. The phosphorylation patterns of both the rabbit and human Triton supernatants were identical to those illustrated in Fig. 2.

Recently, Shapiro and Marchesi (10) have examined the labeling of the band 3 proteins with ^{32}P in the intact human erythrocytes. They showed that not all the radioactivity in the band 3 region could be attributed to either band 3 protein or PAS-1 and suggested that the (Na^+, K^+)-ATPase, or other unidentified membrane components, might account for some of the radioactivity. In our present study, it is unlikely that the phosphorylated intermediate of the (Na^+, K^+)-ATPase contributes any labeling to the band 3 region since the acyl phosphate intermediate (34) would not be expected to remain intact during the staining-destaining conditions (1, 5). That the band 3 protein is the major phosphopeptide found in the band 3 area is further substantiated by our cross-linking studies. Figure 8 illustrates the phosphorylation of DMMA-extracted ghosts by

HMK, MK-I, or MK-II before and after treatment with the Cu^{2+}-*o*-phenanthroline complex (CuP) which induces cross-linking of membrane proteins (11, 35). The A gels represent the phosphorylation pattern of band 3 and other components with ATP in the absence of cross-linking. As the samples applied to these gels were not treated with dithiothreitol the formation of the band 3 dimer is evident. Phosphorylation of DMMA-extracted ghosts by HMK, MK-I, and MK-II before (B gels) and after (C gels) cross-linking of proteins with CuP, leads to the formation of trimer, tetramer, and perhaps larger aggregates of the band 3 phosphopeptides. These cross-linked products were further analyzed by 2-dimensional gel electrophoresis (data not shown) in which the cross-links were cleaved by dithiothreitol in the second dimension (35). No significant difference exists between preparations treated with CuP before phosphorylation (B columns) and those treated with CuP after phosphorylation (C columns). The results indicate that phosphorylation of band 3 in the DMMA-extracted ghosts by the solubilized kinases is not inhibited by cross-linking of the band 3 proteins into higher MW aggregates. We also have utilized the 2-dimensional gel electrophoresis procedure to study the phosphorylation of band 3 cross-linked in unfractionated ghosts and found similarly that the major band 3 protein is phosphorylated. However, in contrast to results obtained using the DMMA-extracted ghosts and the isolated kinase, autophosphorylation of band 3 in unfractionated ghosts is inhibited somewhat by the cross-linking of the proteins [(36), and unpublished observations]. The experiments with MK-I show that the phosphorylated peptides corresponding to PAS-1 and PAS-2 do not appear to be cross-linked, an observation consistent with previously reported data (11, 12, 14). The A′, B′, C′ gels show the phosphorylation of DMMA-extracted ghosts in the presence of GTP. We see that HMK and MK-II fail to utilize GTP as the phosphoryl donor. However, MK-I can utilize GTP to phosphorylate band 3, PAS-1 and PAS-2. From the data it is evident that the phosphorylation and the mobility of the PAS components are not affected by treatment with CuP.

DISCUSSION

The results presented in this paper establish the substrate specificity of erhthrocyte protein kinases towards membrane proteins migrating in the CBB-stained area known as band 3. Unfractionated human and rabbit erythrocyte ghosts contain at least 2 proteins, designated as bands 2.9 and 3, which migrate in this area. In addition, the band 3 area of human erythrocyte membranes also contains the sialoglycoprotein PAS-1. Bands 2.9, 3, and PAS-1 are differentially phosphorylated by erythrocyte protein kinases.

Band 2.9, which migrates with the diffusely stained, trailing "tail" of band 3, is phosphorylated in unfractionated human and rabbit erythrocyte ghosts by the cytoplasmic casein kinases, CK-I (Figs. 6 and 7) and CK-II (3). A phosphoprotein with a mobility similar to 2.9 is also detected in the autophosphorylation of rabbit and human erythrocyte membranes (1), and in the phosphorylation of heat-inactivated rabbit erythrocyte membranes by HMK, MK-I, and MK-II (24). Whether this overlap in substrate specificity between MK-I and MK-II or HMK is real or represents an artefact due to the inhomogeneity of the MK-I preparation is not known. Band 2.9 does not remain associated with band 3 after DMMA extraction. Further characterization of band 2.9 with regards to its identity and function as a phosphopeptide awaits isolation of the protein.

The band 3 protein in DMMA-extracted ghosts of human and rabbit erythrocytes is phosphorylated by HMK and MK-II in the presence of ATP but not GTP. That GTP is

not used in the phosphorylation of band 3 in DMMA-extracted ghosts is in contrast to the results obtained for the autophosphorylation of band 3 in unfractionated ghosts [(1, 3) and Figs. 6, 7] and for the phosphorylation of band 3 in heat-inactivated ghosts by HMK and MK-II (24). Under these conditions GTP is used for band 3 phosphorylation, although to a lesser extent than ATP. Band 3 also appears to be a substrate for MK-I and CK-I in the presence of either ATP or GTP; however, these enzymes are much less active towards band 3 in comparison to MK-II or HMK (Fig. 2).

A protein migrating with band 3 in human erythrocyte ghosts is phosphorylated in the presence of cyclic AMP-dependent protein kinase I (data not shown) or IIa (Fig. 2). This reaction requires cyclic AMP and ATP. The fact that this reaction does not occur in DMMA-extracted ghosts of rabbit erythrocytes and that the mobility of the cyclic AMP-dependent substrate appears to differ slightly from the substrate of HMK and MK-II leads us to question whether the cyclic AMP-dependent protein kinase substrate is identical to that of HMK and MK-II. The cyclic AMP-dependent phosphopeptide appears to migrate slightly slower than the leading edge of the HMK or MK-II substrate and does not coincide with PAS-1. These results suggest that another protein may be present in the band 3 of DMMA-extracted membranes which is specifically phosphorylated by cAK-I or cAK-IIa.

The PAS-stained glycopeptides, PAS-1 and PAS-2, are phosphorylated in DMMA-extracted human erythrocyte ghosts by MK-I and CK-I, but not by HMK, MK-II, cAK-IIa (Fig. 2), or cAK-I (data not shown). The phosphorylation of PAS-1 and PAS-2 in the DMMA-extracted ghosts catalyzed by either MK-I or CK-I occurs to the same extent with ATP or GTP. The enzymes which phosphorylate glycophorin are independent of cyclic nucleotides, but can be inhibited by the red cell metabolite 2,3-diphosphoglyceric acid (23, 26). The enzymes MK-I and CK-I are derived from rabbit erythrocyte membranes and cytoplasm, respectively. Salt extracts of human erythrocyte membranes contain very little, if any, kinase activity corresponding to MK-I (23). Whether or not the soluble casein kinases CK-I and CK-II also are present in human erythrocyte lysates has not been investigated. It would seem paradoxical that the human erythrocyte would contain the substrates but not the kinases whereas the rabbit erythrocyte, the kinases but not the glycoprotein substrates. However, that glycophorin appears to be phosphorylated in intact erythrocytes (10) would tend to argue for the presence of kinases similar to MK-I and/or CK-I or CK-II in human erythrocytes.

The physiological role of the phosphorylation of the band 3 area peptides has not been determined but is of great interest in view of the membrane functions attributed to these proteins. Although the identity and function of band 2.9 is unknown, the properties of band 3 and glycophorin, both of which span the membrane asymmetrically, have been extensively studied. Band 3 has been implicated in the transport of anions (14–17) and water (18) across the red cell membrane. That anion transport may be occurring through band 3 has been suggested on the basis of the binding of inhibitors of anion transport to band 3 (14–17). However, Lepke et al. (17) show that binding of the inhibitors also occurs to some degree at sites other than band 3, one of which may correspond to the phosphopeptide we term 2.9. Further evidence suggesting that band 3 contains the anion transport channel comes from reconstitution of the transport by incorporating Triton extracts containing band 3 into lecithin vesicles (16). The possibility that phosphorylation may provide a positive or negative regulation of anion transport has not been determined.

Band 3 is a sulfhydryl-containing protein which is thought to exist in the membrane as a dimer (11, 35). It is of interest that the cross-linking of the band 3 protein by the oxidation of thiol groups with CuP does not inhibit the phosphorylation of band 3 by

the solubilized membrane protein kinases (Fig. 8). Since the phosphorylation of band 3 is not affected by cross-linking of the proteins and vice versa, the data suggest that the cross-linking sites may be remote from the phosphorylation site(s). It has been proposed that the band 3 subunits aggregate to form the aqueous channel for anion transport (15). It seems attractive to postulate that phosphorylation-dephosphorylation of band 3 may play a role in the aggregation-disaggregation of this protein.

Band 3 contains cytoplasmic binding sites for other membrane proteins, including bands 4.2 and 6 (11, 37). Phosphorylation of band 3 has been reported to occur at 2 sites: one within the 10,000 dalton region of the NH_2 terminal (cytoplasmic) and the second site, in an undefined portion of the polypeptide (38). It would be of interest to determine if the binding of the membrane polypeptides to band 3 is dependent on the phosphorylation state of the band 3 protein.

Although band 3 has been implicated previously in glucose transport (12, 13, 39), recent studies in a more defined system indicate that it is not band 3 but a protein migrating in the area designated as 4.5 that may be responsible for the sugar transport (40, 41). It is of interest that a 4.5 area protein in DMMA-extracted human erythrocyte ghosts also is phosphorylated by HMK and MK-II (Fig. 6). The phosphorylation of the protein labelled 4.5 in the DMMA-extracted human erythrocyte ghosts is not catalyzed by the cyclic AMP-dependent protein kinases (Fig. 2). On the other hand, a protein in the intact erythrocyte with similar mobility is phosphorylated in rabbit and human erythrocyte membranes by cyclic AMP-dependent enzymes. That more than one protein may be present in the 4.5 area in intact ghosts is evident in the 7% gel shown in Fig. 4A. The results suggest that the proteins phosphorylated in the 4.5 area in the DMMA-extracted vs the intact erythrocyte membranes may be physically distinct. However, the possibility that there is only one protein substrate for phosphorylation which is modified during the DMMA extraction should not be ruled out.

It has been suggested that glycophorin (PAS-1 and PAS-2), which is phosphorylated in the DMMA-extracted ghosts (Fig. 6) as well as in intact erythrocytes (10), contains receptors or other surface recognition sites of the erythrocyte (42). That PAS-1 and PAS-2 represent the dimer and monomer, respectively, of glycophorin has been adequately demonstrated (32, 33). Under certain conditions used to solubilize membranes for electrophoresis PAS-1 is converted to PAS-2. The results obtained in this paper do not indicate whether the state of aggregation affects the phosphorylation of glycophorin. Although the conformation of glycophorin in the lipid bilayer is not known, it has been suggested that the glycoproteins exist as aggregates in the membrane in order to facilitate receptor function (33). Recently, Shapiro and Marchesi have demonstrated that the site of phosphorylation of glycophorin is located on the cytoplasmic COOH terminal end. It remains to be determined if phosphorylation plays a role in the formation of aggregates of the protein.

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