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Selective Phosphorylation of Erythrocyte Membrane Proteins by the Solubilized Membrane Protein Kinases†

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ABSTRACT: This report describes the substrate and phosphoryl donor specificities of solubilized erythrocyte membrane cyclic adenosine 3′,5′-monophosphate (cAMP)-independent protein kinases toward human and rabbit erythrocyte membrane proteins. Three types of substrate preparations have been utilized: heat-inactivated ghosts, isolated spectrin, and 2,3-dimethylmaleic anhydride (DMMA)-extracted membranes. A 30 000-dalton protein kinase, extracted from either human or rabbit erythrocyte membranes, catalyzes the phosphorylation of heat-inactivated membranes in the presence of ATP. The resulting phosphorylation profile is analogous to that of the autophosphorylation of membranes with ATP (in the absence of cAMP). These kinases also phosphorylate band 2 of isolated spectrin and band 3, but not glycophorin, in the DMMA-extracted ghosts. The ability of the 30 000-dalton kinases to use GTP as a phosphoryl donor appears to be related to the substrate or some other membrane factor. A second kinase, which is 100 000 daltons and derived from rabbit erythrocyte membranes, uses ATP or GTP to phosphorylate membrane proteins 2, 2.1, 2.9–3 in heat-inactivated ghosts, band 2 in isolated spectrin, glycophorin, and to a lesser extent, band 3 in the DMMA-extracted ghosts.

Erythrocyte membrane proteins can be phosphorylated by membrane-bound (Avruch and Fairbanks, 1974; Fairbanks and Avruch, 1974; Hosey and Tao, 1976) and soluble (Hosey and Tao, 1977a) cAMP[1]-dependent and -independent protein kinases. Multiple protein kinase activities are found in rabbit and in human red cell membranes. The autophosphorylation of human erythrocyte membranes is catalyzed by cAMP-dependent and -independent protein kinases. In contrast, the autophosphorylation of rabbit erythrocyte membranes appears to be catalyzed by two protein kinases both of which are independent of cAMP. The cAMP-independent protein kinases from rabbit and human erythrocyte membranes have been solubilized and partially purified (Hosey and Tao, 1977b).

This report deals with the phosphorylation of red cell membrane proteins by these solubilized enzymes. The data suggest that a 30 000-dalton kinase, found in human and in rabbit erythrocyte membranes, can elicit a pattern of phosphorylation in heat-inactivated membranes similar to that obtained in membrane autophosphorylation in the presence of either ATP or GTP. A second kinase of approximately 100 000 daltons, which is extracted from rabbit erythrocyte membranes, uses either ATP or GTP to phosphorylate glycophorin in 2,3-dimethylmaleic anhydride extracted ghosts and to a lesser extent, bands 2–2.1 and 2.9–3 in heated membranes.

Experimental Procedures

Materials. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from either New England Nuclear or Amersham/Searle. ATP and GTP were purchased from P-L Biochemicals. 2,3-Dimethylmaleic anhydride (DMMA) was purchased from Sigma Chemical Co. Electrophoresis supplies were obtained from Bio-Rad Laboratories. Frozen rabbit red blood cells were supplied by Pel-Freez.

Methods. Protein kinases were prepared from membranes of frozen rabbit or outdated human red blood cells as previously described (Hosey and Tao, 1977b). Briefly, the enzymes were extracted from erythrocyte ghosts with 0.5 M NaCl, concen-

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; DMMA, 2,3-dimethylmaleic anhydride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAS, periodic acid–Schiff base; Tris, tris(hydroxymethyl)aminomethane.

trated by ultrafiltration and ammonium sulfate precipitation, and subjected to Sephadex G-100 or G-150 gel filtration in the presence of 0.4 M NaCl. The enzymes obtained from rabbit erythrocyte membranes are designated RMK-I and RMK-II, while that from human is designated HMK. The properties of these enzymes as characterized with regards to casein phosphorylation are discussed elsewhere (Hosey and Tao, 1977b).

Hemoglobin-free red cell ghosts were prepared by the procedure of Dodge et al. (1963) from fresh rabbit or human blood as described (Hosey and Tao, 1975). These ghosts were the source of substrate proteins. Spectrin was extracted from red cell ghosts by incubating membranes in 10 volumes of 0.1 mM EDTA, pH 7.0, for 1 h at 37 °C with gentle agitation (Marchesi, 1974; Gratzer and Beaven, 1975). The solution was centrifuged at 100 000*g* for 60 min. The resulting supernatant was concentrated on a Diaflo PM-10 membrane and was used without further purification.

Band 3 enriched membranes were prepared by extracting human and rabbit erythrocyte ghosts with DMMA using 1.5–2.0 mg of DMMA per mg of protein (Steck and Yu, 1973). In some instances, this procedure was repeated twice. The DMMA-extracted membranes are devoid of protein kinase activity and contain band 3, all the PAS-staining sialoglycoproteins, some residual bands 4.2, 4.5 area proteins, and band 7.

The phosphorylation of membrane proteins was analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis followed by autoradiography (Hosey and Tao, 1976). The phosphorylation reaction contained (in a final volume of 50 μL): 0.1 M buffer (either Tris–acetate, pH 6.0; Tris-HCl, pH 6.5, 7.0, or 7.5; or glycine–NaOH, pH 8.5, as specified in the figure legends); 10 mM $MgCl_2$; 0.2 mM [γ-^{32}P]ATP or 0.2 mM [γ-^{32}P]GTP; 10–40 μg of membrane protein; ± the solubilized kinases. The reactions were carried out at 37 °C for 5 or 10 min and were terminated with 10 volumes of a "KCl-stopping solution" (150 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) followed by centrifugation as described (Hosey and Tao, 1977a). The twice washed membranes were dissolved in $NaDodSO_4$-containing sample buffer, applied to 5% (unless otherwise specified) polyacrylamide slab gels containing 0.2% $NaDodSO_4$ and electrophoresed (Fairbanks et al., 1971). Autoradiograms were prepared from the dried gels and scanned using a Zeineh Soft-Laser densitometer.

Results

Comparison of the Phosphorylation Profiles of Rabbit and Human Erythrocyte Membranes Obtained with RMK-I, RMK-II, and HMK. Extraction of red cell ghosts with 0.5 M NaCl results in the removal of two cAMP-independent protein kinases (RMK-I and RMK-II) from rabbit erythrocyte membranes and of one kinase (HMK) from human erythrocyte membranes (Hosey and Tao, 1977b). RMK-I can utilize either ATP or GTP to phosphorylate casein and has an apparent molecular weight of approximately 100 000 as determined in the presence of salt. RMK-II and HMK prefer ATP as the phosphoryl donor in casein phosphorylation, and both exhibit a molecular weight of approximately 30 000 as determined in the presence of 0.4 M NaCl. HMK is the major casein kinase extracted with salt from human erythrocyte membranes whereas of the two kinases extracted from rabbit erythrocyte membranes, RMK-II accounts for greater than 80% of the total kinase activity. In some instances, a very small amount of a 100 000-dalton kinase was also detected in the human erythrocyte membrane extract. None of the three kinases appear to be derived from or related to cAMP-dependent protein kinases (Hosey and Tao, 1977b).

That the solubilized kinases described above are peripheral membrane proteins as opposed to cytoplasmic contaminants is further supported by several lines of evidence. The ghost preparations can be washed with physiological concentrations of salt (150 mM KCl) with little loss of autophosphorylating activity. If the apparent membrane-bound kinases were translocated cytoplasmic kinases, they should be removed by this treatment (Keely et al., 1975). In addition, the solubilized erythrocyte membrane kinases differ from erythrocyte cytosolic kinases with regards to aggregation, apparent molecular weights, sensitivity to cyclic nucleotides and other modulators, and in other properties (Hosey and Tao, 1977b, unpublished observations). As described herein, the 30 000-dalton membrane kinases also differ in their membrane protein and nucleotide substrate specificities from any protein kinase heretofore isolated from erythrocyte cytosol. The 100 000-dalton protein kinase (RMK-I) from rabbit erythrocyte membranes shows some overlap in phosphoryl donor and acceptor specificities with the cytosolic casein kinases (Kumar and Tao, 1975); however, this does not rule out a membranous origin of the enzyme (see Discussion).

Attempts to characterize the substrate specificities of these solubilized kinases using membrane preparations which contain endogenous kinase activities were unsuccessful. The addition of the solubilized kinases to these membranes failed to elicit an increase in the rate of phosphorylation due to a high content of endogenous kinase activities. Hence, the studies of these solubilized kinases were conducted in heated membranes and in membrane or soluble preparations enriched in specific membrane protein components.

Membrane-bound kinases are rapidly inactivated by heating at 53 °C; after 2 min, less than 35% of the activity remains, and after 20 min, less than 15% (data not shown). The slots labeled "control" in Figure 1 represent autophosphorylation of human and rabbit erythrocyte ghosts which had been heated at 53 °C for 30 min. Under these conditions some residual kinase activity which phosphorylated bands 2–2.1 remained; however, the majority of activity appeared to be inactivated. Figure 1 illustrates the effect of RMK-I, RMK-II, and HMK on the phosphorylation of heat-inactivated human and rabbit erythrocyte membranes (upper and lower panels, respectively). These experiments were repeated many times and each time similar results were obtained. In each pair of samples, the one on the left received ATP, while the sample on the right received GTP. In this experiment, approximately equivalent amounts of activity of RMK-I and RMK-II were used, whereas the activity of HMK was approximately one-third less. The activity of these enzymes was measured using ATP and casein as substrates.

As shown in the upper part of Figure 1, RMK-I catalyzed the phosphorylation of 2–2.1, 2.9–3, and of another protein tentatively designated as 4.5 in heat-inactivated human erythrocyte membranes. The reaction appeared to proceed about as well with GTP as with ATP. These results differed from those obtained under identical conditions using rabbit erythrocyte membranes and RMK-I (Figure 1, lower panel). In rabbit erythrocyte membranes, band 2–2.1 was phosphorylated to a greater extent than band 2.9–3. Although other unidentified minor phosphopeptides were also observed, the phosphorylation of band 4.5 did not appear as evident in the rabbit as in the human erythrocyte ghosts. As will be discussed later (Figure 4), the phosphorylation by RMK-I in human erythrocyte ghosts in the band labeled 4.5 and some of band 3 represents the phosphorylation of glycophorin (PAS-1 and

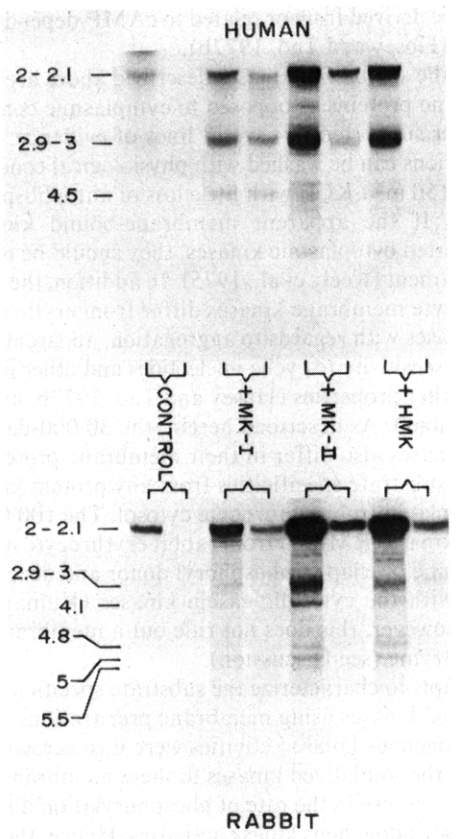

FIGURE 1: Autoradiograms depicting the phosphorylation of human and rabbit erythrocyte membranes by solubilized membrane kinases. Heat-inactivated membranes (22 μg) were phosphorylated at pH 7.5 alone (control) or in the presence of RMK-I (36 μg), RMK-II (11 μg), or HMK (10 μg). In each pair, the sample on the left received [γ-^{32}P]ATP (185 cpm/pmol) while that on the right, [γ-^{32}P]GTP (170 cpm/pmol). The autoradiogram was exposed for 3 days.

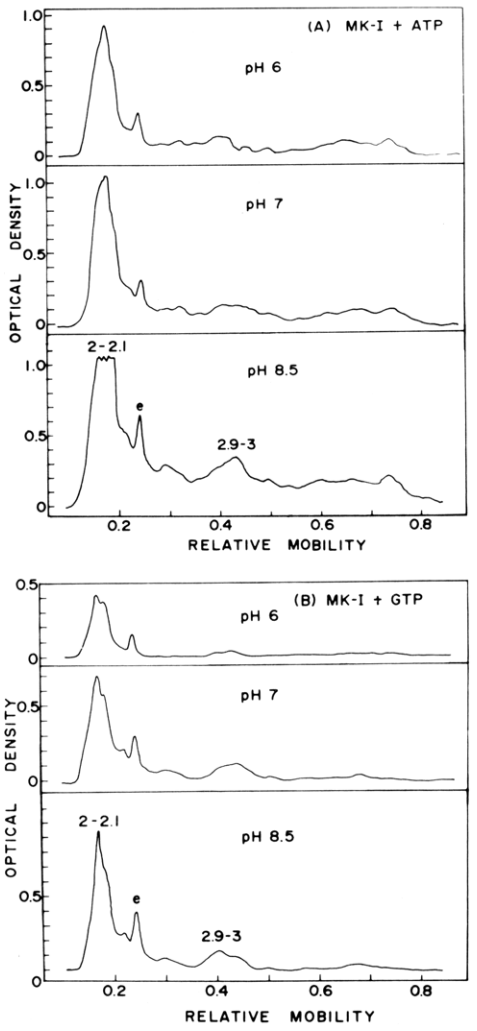

FIGURE 2: Densitometric tracing of an autoradiogram depicting the effect of pH on the phosphorylation of rabbit erythrocyte membranes by RMK-I. Membranes (22 μg) were heat-inactivated and then phosphorylated at the pH specified with RMK-I (72 μg) in the presence of (A) [γ-^{32}P]ATP (320 cpm/pmol) or (B) [γ-^{32}P]GTP (350 cpm/pmol). The autoradiogram represents a 4-day exposure of the film to the gel.

2). This explains the lack of similar phosphorylation in the rabbit as glycophorin is not a component of the rabbit erythrocyte membranes (Hamaguchi and Cleve, 1972; Vimr and Carter, 1976).

The results obtained with RMK-II and HMK shown in Figure 1 indicate that these two enzymes have similar substrate specificities toward membrane proteins. In heated human erythrocyte ghosts, RMK-II and HMK both catalyzed the phosphorylation of bands 2–2.1, 2.9–3 and several minor proteins in the area below band 3 in the presence of ATP. When GTP was used as the phosphoryl donor for either RMK-II or HMK, radioactivity was observed in bands 2 and 2.9–3. The phosphorylation of rabbit erythrocyte membranes by RMK-II and HMK in the presence of ATP occurred in bands 2–2.1, 2.9–3, 4.1, 4.8, 5, and other minor proteins. Some differences in substrate specificity between the two enzymes were apparent with regards to the minor phosphoproteins. Although it appeared that band 4.1 was not phosphorylated as well by HMK as by RMK-II, this difference was not as marked in other experiments. In the presence of GTP, band 2 was the major radioactive species present with little labeling apparent in area 3. From the results presented in Figure 1, it was obvious that more radioactivity was incorporated from either ATP or GTP with any kinase into area 3 of human erythrocyte membranes than into area 3 of rabbit erythrocyte membranes. The opposite appeared to be true of bands 2–2.1. Similar results have been observed for the autophosphorylation of rabbit and human erythrocyte membranes (Hosey and Tao, 1976).

The heat-inactivated erythrocyte ghosts were phosphorylated to a greater extent by RMK-II and HMK than by RMK-I, in the presence of either ATP or GTP. The phosphorylation profiles obtained with RMK-II and HMK were similar to the patterns of autophosphorylation of intact ghosts (Hosey and Tao, 1976). The most surprising result of this study was that RMK-II and HMK could effectively utilize GTP to phosphorylate membrane proteins. This is in contrast to the phosphorylation of casein where these enzyme preparations specifically utilized ATP as the phosphoryl donor (Hosey and Tao, 1977b). Because of this unusual behavior, it was necessary to determine whether [γ-^{32}P]GTP directly served as the phosphoryl donor or whether a transferase was present in the membrane preparations which might transfer the terminal phosphate of GTP to membrane-bound ADP to form [γ-^{32}P]ATP. We therefore incubated [γ-^{32}P]GTP $\pm$ 2 mM ADP with the membranes and the kinase preparations under kinase assay conditions at 37 °C for 5 min and separated the nucleotides by cellulose thin-layer chromatography (Anthony and Spector, 1972). In no instance did the membranes or any of the kinase preparations convert [γ-^{32}P]GTP to [γ-^{32}P]ATP. Hence, it appears that [γ-^{32}P]GTP is a true donor for our kinase preparations.

Bylund and Krebs (1975) have shown that some proteins

TABLE I: Phosphorylation of Band 2 of Isolated Rabbit Erythrocyte Spectrin by Solubilized Erythrocyte Membrane Kinases.

Addition	ATP[b]	GTP[b]	ATP/GTP[b]
Spectrin[a]	14	2	7
+ RMK-I	31	10	3.1
+ RMK-II	83	26	3.1
+ HMK	52	13	4.0
+ RMK-I and RMK-II	81	28	2.9

[a] Spectrin (18 μg) was phosphorylated as described under Methods at pH 7.5 using either [γ-^{32}P]ATP (200 cpm/pmol) or [γ-^{32}P]GTP (230 cpm/pmol) as the phosphoryl donor. The concentrations of RMK-I, RMK-II, and HMK were 31, 0.85, and 0.96 μg of protein, respectively. The incubation was conducted at 37 °C for 10 min. [b] Arbitrary densitometric units.

can serve as substrates for phosphorylation in the denatured but not in the native state. Conceivably, heat denaturation of membrane proteins could also create a similar situation. However, the data shown in Figure 1 indicate that the phosphorylation profiles obtained using the heated membranes and the solubilized kinases are similar to those obtained from autophosphorylation of untreated membranes (Hosey and Tao, 1976); no new major substrates are apparent as a result of heating. Hence, the heated membrane preparations can serve as substrates and, in spite of any possible limitation created by denaturation, provide certain information regarding the properties of the solubilized kinases.

Effect of pH on the Phosphorylation of Membrane Proteins by RMK-I and RMK-II. We have previously established that the autophosphorylation of erythrocyte membrane polypeptides varies with the pH of the reaction mixture (Hosey and Tao, 1976). Hence, the phosphorylation of heat-inactivated rabbit erythrocyte membranes by RMK-I and RMK-II was analyzed at various pH values. Figure 2 illustrates the effect of pH on the phosphorylation of heated membranes by RMK-I in the presence of ATP (Figure 2A) or GTP (Figure 2B). With either phosphoryl donor, phosphorylation occurred to the greatest extent at pH 8.5. The identifiable substrates of RMK-I were in the areas of 2–2.1 and 2.9–3. The phosphopeptide designated as "e" appeared to be derived from the enzyme preparation that was not removed from the membranes after two washes with the KCl-stopping solution. Figures 3A and 3B illustrate the results of similar experiments performed using RMK-II. While all reactions catalyzed by RMK-II occurred to the greatest extent at pH 8.5, certain autophosphorylation reactions, such as of bands 4.1 and 5.5, occur to a greater extent at pH 6.0 than at any other pH value tested (Hosey and Tao, 1976). The phosphorylation of spectrin band 2 is affected only slightly by changes in pH, both in autophosphorylation (Hosey and Tao, 1976) and in phosphorylation of heated ghosts by RMK-II (Figure 3).

Although the pH–activity profiles exhibit certain differences between autophosphorylation and phosphorylation of heated ghosts by the solubilized kinases, these experiments provide useful information regarding the substrate and phosphoryl donor specificities of the membrane kinases. At all pH values studied (including data not shown for pH 6.5 and 7.5), the amount of ^{32}P incorporated into membrane proteins was greater using RMK-II than RMK-I. In the experiments shown in Figures 2 and 3, the amount of RMK-I used was twice that of RMK-II as calculated on the basis of their ability to phosphorylate casein. Based on calculations of the area under the peaks of the densitometric tracings of autoradiograms, ATP was two to four times more effective than GTP as a phosphoryl donor for either kinase at all pH values studied (data not shown).

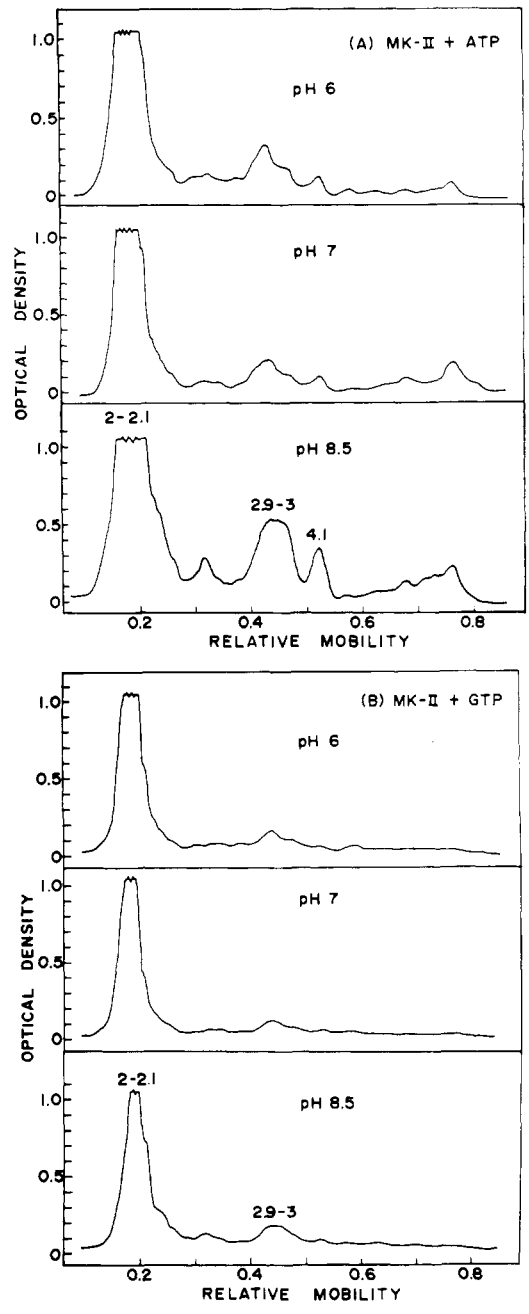

FIGURE 3: Densitometric tracing of an autoradiogram depicting the effect of pH on the phosphorylation of rabbit erythrocyte membranes by RMK-II. Conditions were as described under Figure 2 except that RMK-I was replaced by RMK-II (11 μg).

Phosphorylation of Spectrin by Solubilized Rabbit and Human Erythrocyte Membrane Kinases. Since band 2 of spectrin appeared to be a substrate for all three solubilized kinases in heat-inactivated membranes, it was of interest to study the phosphorylation of spectrin extracted from rabbit erythrocyte membranes. Spectrin was isolated as described in Methods. Under these conditions, some kinase activity which phosphorylated band 2 of spectrin was also extracted. The nature of this kinase is unknown. In any event, band 2, but not band 1, of the isolated spectrin was a substrate for RMK-I, RMK-II, and HMK using either ATP or GTP as the phosphoryl donor (Table I). However, ATP was at least three times more effective than GTP as a phosphoryl donor. The phosphorylation of band 2 by both RMK-I and RMK-II was not

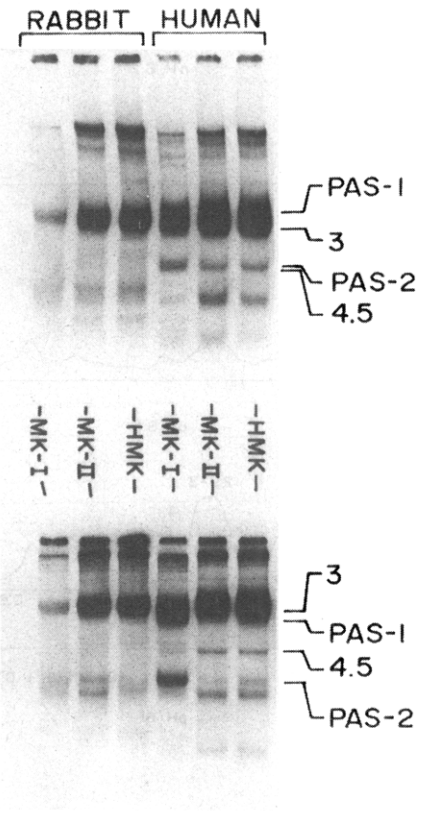

FIGURE 4: Autoradiograms depicting the differential phosphorylation of glycophorin and band 3 in DMMA-extracted human and rabbit erythrocyte ghosts by the solubilized membrane kinases. The top and bottom autoradiograms are from 4% and 7% acrylamide gels, respectively. DMMA-extracted rabbit (20 μg) and human (19 μg) erythrocyte membranes were phosphorylated at pH 8.5 in the presence of [γ-^{32}P]ATP (152 cpm/pmol) and either RMK-I (36 μg), RMK-II (13 μg), or HMK (5 μg). The autoradiogram was exposed for 5 days. The sialoglycoproteins, PAS-1 and PAS-2, were identified by staining with periodic acid–Schiff (PAS) reagent (Fairbanks et al., 1971).

additive, but equal to that observed with RMK-II alone. These results suggest that the sites phosphorylated by RMK-II may include those acted upon by RMK-I. An alternative explanation of the results would be that band 2 contains multiple phosphoryl acceptor sites which cannot be simultaneously phosphorylated. Whether both kinases play a role in the phosphorylation of spectrin in erythrocytes remains to be determined.

Differential Phosphorylation of Band 3 and Glycophorin in DMMA-Extracted Ghosts by the Solubilized Membrane Kinases. The band 3 area appears as a diffusely stained component and may contain more than one substrate for phosphorylation (Hosey and Tao, 1976, 1977a). The possibility that the solubilized kinases may selectively phosphorylate these substrates is investigated in DMMA-extracted membranes. These membranes are enriched in bands 3, 4.5, and 7 and in the case of human erythrocyte membranes, the entire array of sialoglycoproteins (Steck and Yu, 1973). Band 2.9 is not present in these membranes (Hosey and Tao, 1977c). The major sialoglycoprotein, glycophorin, appears on $NaDodSO_4$ gels as PAS-1 (the dimer) and PAS-2 (the monomer). In the 5% gel system used in the previous studies, PAS-1 comigrated with the major Coomassie blue stained band 3 protein. In this study, we varied the conditions of electrophoresis in order to separate these components. The PAS-staining sialoglycoproteins do not bind $NaDodSO_4$ to the same extent as the standard proteins. As a result, their relative mobilities (Segrest et al., 1971) in $NaDodSO_4$–polyacrylamide gels vary with acrylamide concentration. In the experiments illustrated in Figure 4, the electrophoreses were carried out in two different acrylamide gel concentrations, 4% (top) and 7% (bottom), both in the presence of 0.2% $NaDodSO_4$. In the 4% gels, PAS-1 and PAS-2 traveled slightly slower than band 3 and band 4.5, respectively. In the 7% gels, PAS-I migrated faster than band 3, near band 4.1, while PAS-2 migrated between band 4.8 and 5. Figure 4 shows the effect of RMK-I, RMK-II, and HMK on the phosphorylation of DMMA-extracted rabbit and human erythrocyte membranes. In the rabbit preparation, the phosphorylation of band 3 was catalyzed by HMK and RMK-II to a greater extent than by RMK-I. There appeared to be only one phosphoryl acceptor substrate in the band 3 area in the DMMA-extracted rabbit erythrocyte membranes. The rabbit erythrocyte membranes do not appear to contain glycophorin (Hamaguchi and Cleve, 1972; Vimr and Carter, 1976). In contrast, two phosphopeptides were evident in the band 3 area in the human preparation phosphorylated by RMK-I. When the mobilities of the peptides phosphorylated by RMK-I were compared in the 4% (top) and the 7% (bottom) gels, it became evident that one of the phosphopeptides in the band 3 area exhibited the anomalous electrophoretic mobilities characteristic of PAS-1; i.e., it migrated slower than band 3 in the 4% gels and faster in the 7% gels. Furthermore, the phosphorylation of a peptide with the electrophoretic characteristic of PAS-2 was also apparent in the presence of RMK-I. In contrast, the peptides phosphorylated by RMK-II and HMK in the DMMA-extracted human erythrocyte membranes ran "true"; i.e., they exhibited the same apparent molecular weights regardless of the acrylamide cross-linking. These experiments show that RMK-I, but not RMK-II or HMK, catalyzes the phosphorylation of glycophorin (PAS-1 and 2). On the other hand, RMK-II and HMK are more active than RMK-I toward band 3. It is of interest that RMK-I can use ATP and GTP equally well to phosphorylate glycophorin or band 3 whereas MK-II and HMK preferentially utilize ATP to phosphorylate band 3 in the DMMA-extracted membranes (data not shown).

Discussion

The results presented in this communication indicate that heat-inactivated rabbit and human erythrocyte membranes, isolated spectrin, and DMMA-extracted ghosts contain polypeptide substrates for the solubilized erythrocyte membrane protein kinases. Despite the fact that heating membranes at 53 °C most likely led to denaturation of most, if not all, of the proteins, it was surprising to find that the phosphopeptide profile of these membranes closely resembled that of the intact membranes. However, subtle differences do exist between the reconstituted and the intact membrane phosphorylation systems. The polypeptides of the heated membranes are phosphorylated by the solubilized kinases to a greater extent at alkaline pH. On the other hand, in the autophosphorylation of intact ghosts, several polypeptides are phosphorylated better at acidic pH and others at basic pH.

The data presented in this report contain two sets of observations concerning the characteristics of the solubilized erythrocyte membrane protein kinases. One concerns their substrate specificities, the other their phosphoryl donor specificities. Interestingly, there appears to be somewhat of a relationship between these two properties.

Although the solubilized kinases have overlapping substrate specificities, there are membrane polypeptides which appear to be differentially phosphorylated by RMK-I or RMK-II and HMK. RMK-I phosphorylates glycophorin whereas RMK-II

and HMK do not. However, RMK-II and HMK phosphorylate band 4.1 and other lower molecular weight proteins that do not seem to be phosphorylated by RMK-I. Furthermore, RMK-II and HMK catalyze the phosphorylation of bands 2 and 3 to a much greater extent than does RMK-I.

It is tempting to designate RMK-I as "glycophorin kinase" since the sialoglycoprotein is phosphorylated by this membrane enzyme. However, a cytoplasmic cAMP-independent protein kinase found in rabbit erythrocytes (Kumar and Tao, 1975) can also catalyze the phosphorylation of glycophorin (Hosey and Tao, 1977c). The cytoplasmic kinase and RMK-I have several similar properties. Whether they represent two distinct enzymes or are membrane-bound and soluble forms of the same enzyme remains to be determined. Furthermore, RMK-I is a rabbit enzyme whereas glycophorin is a protein found in human but not in rabbit erythrocyte membranes. Thus it seems inappropriate to designate RMK-I as glycophorin kinase as it lacks the substrate in a homologous system. However, in human erythrocyte membranes, it appears that an enzyme similar to RMK-I may be present (unpublished data). This enzyme constitutes a very minor component and can be separated from HMK by gel filtration. In addition, a protein kinase that appears to be similar to the rabbit cytoplasmic kinase which phosphorylates glycophorin has been obtained from human erythrocyte cytosol (R. Kumar, personal communication). This kinase has not been tested for activity toward glycophorin. It has been recently reported that glycophorin is phosphorylated in intact cells (Shapiro and Marchesi, 1977).

The overlap in substrate specificities of RMK-I vs. RMK-II and HMK toward bands 2, 2.1, 2.9, and 3 needs to be further clarified. It is not clear whether these enzymes are actually phosphorylating the same substrates or different proteins with similar electrophoretic mobilities. If there is indeed an overlap in substrate specificities, it remains to be determined whether these enzymes phosphorylate the same or different acceptor sites of the substrate.

The second interesting aspect of the present study is the finding that both RMK-II and HMK can, under certain conditions, utilize GTP to phosphorylate some of the membrane proteins. These enzymes have previously been shown to specifically utilize ATP but not GTP to phosphorylate casein (Hosey and Tao, 1977b). The effectiveness of GTP as a substrate in the phosphorylation of membrane proteins is about two to four times less than that of ATP. However, the two phosphoryl donors do not produce the same phosphorylation patterns. In the presence of ATP, RMK-II and HMK catalyze the phosphorylation of bands 2–2.1, 2.9–3, 4.1, and other minor components in heated ghosts, whereas in the presence of GTP only bands 2–2.1 and 2.9–3 are phosphorylated to any significant degree (Figures 1 and 3). In addition, HMK and RMK-II use GTP with the same efficiency as does RMK-I to phosphorylate band 2 of isolated spectrin. In DMMA-extracted ghosts, RMK-II and HMK utilize GTP very poorly, if at all, to catalyze band 3 phosphorylation, whereas RMK-I uses GTP and ATP equally well. It is interesting that RMK-II and HMK only use GTP to phosphorylate those membrane proteins which can also be phosphorylated with GTP by RMK-I. The results indicate that phosphorylation profiles characteristic of ATP or GTP-driven autophosphorylation can be achieved by either RMK-II or HMK alone. These observations are amenable to several interpretations. Conceivably, the RMK-II and HMK preparations may be heterogeneous and may contain a separate kinase which can utilize GTP to phosphorylate certain membrane proteins but not others or casein. Alternatively, HMK and RMK-II may be multifunctional proteins that can catalyze at least two independent reactions. The occurrence of multifunctional enzymes is well-documented by Kirschner and Bisswanger (1976). Lastly, it is also possible that the catalytic activity of HMK and RMK-II may be modified by the substrates or other membrane factors. For example, in the presence of band 2, the enzyme may be modified such that it can utilize GTP in addition to ATP in the reaction. Which among these possibilities can best explain the above observations can be resolved when homogeneous enzyme preparations are available. Unfortunately, due to the instability of these enzymes, we have had some difficulties in further purifiying these enzymes beyond the degree that we have achieved.

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