Proteolytic Analysis of the Topological Arrangement of Red Cell Phosphoproteins†

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ABSTRACT: The topology of human erythrocyte membrane phosphoproteins was determined by using protease digestion and selective solubilization. The distribution of ^{32}P among the membrane polypeptides in intact cells differs from the pattern found in isolated ghosts. The following membrane skeleton polypeptides, the nomenclature of Steck [Steck, T. L. (1972a) *J. Mol. Biol. 66*, 295–305] being used, were phosphorylated: 2, 2.1, 4.1, 4.5b, and 4.9, together with two polypeptides at 105 000 and 110 000 daltons that are heavily phosphorylated. Among integral proteins, band 3 and glycophorins A and B are phosphorylated. Glycophorin C appears to have little turnover of phosphate. Autoradiograms of trypsin-treated membranes permitted identification of the transmembrane proteolytic fragment of glycophorin A as a dimer of 38 000 daltons. The band 5 region contained two phosphoproteins, the peripheral protein 4.9 (48 kdaltons) and PAS-2. Similarly, band 7 resolved into an integral phosphoprotein and a nonphosphorylated protein associated with the membrane skeleton.

Many of the membrane proteins of the red cell are phosphorylated (Shapiro & Marchesi, 1977; Plut et al., 1978; Dzandu & Johnson, 1980). The major phosphoproteins are spectrin band 2 and band 3, although numerous other bands are also phosphorylated, notably the cytoskeletal elements ankyrin and band 4.1. There are no proven functions for the observed phosphorylation, although control of cell shape (Sheetz & Singer, 1977) has been proposed. Since the topological arrangement of membrane polypeptides will place constraints on their function, we have determined which of the phosphoproteins are transmembrane, using protease digestion as a probe. The results show that most of the transmembrane polypeptides are phosphoproteins. Some new polypeptides not detectable by either Coomassie blue or PAS[1] stain can be identified by autoradiography.

Materials and Methods

Erythrocytes were obtained from the Detroit Red Cross Blood Center as day-old packed red cells in CPD (plasma and buffy coat removed). They were washed 3 times in 35 mM Tris-HCl, 118 mM NaCl, 5 mM KCl, 1.7 mg of $MgCl_2$, 1 mM EDTA, and 10 mM glucose, pH 7.4, and suspended at 4×10^9 cells/mL with 2 mM [^{32}P]P_i (0.5 Ci/mmol). Phenylmethanesulfonyl fluoride (PMSF) was added to 20 μg/mL, and the cells were incubated overnight at room temperature. The next day the cells were washed 3 times in the same buffer without Na[^{32}P]P_i and twice in 5 mM Tris-HCl–140 mM NaCl, pH 7.4, and suspended at 2×10^9 cells/mL. For protease digestion, the enzymes were added as 1 mg/mL stock solutions in the same buffer to a final concentration of 100 μg/mL. After 2 h at room temperature, the cells were washed 5 times in 5 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, and 50 μg/mL PMSF, pH 7.4, at 0 °C. Triton residues, or membrane skeletons, were prepared by mixing ghosts with 10 volumes of 1% Triton X-100 (Amersham/Searle, scintillation grade) in the same buffer and pelleting at 22000*g* for 10 min (Yu et al., 1973). Peripheral proteins were solubilized (Steck & Yu, 1973) by suspending ghosts in 15 volumes of 0.1 M NaOH on ice for 10 min, and the integral proteins and lipid were pelleted by centrifugation at 22000*g* for 10 min at 4 °C. The pellets were washed once in the solubilizing solution and directly dissolved in $NaDodSO_4$ sample buffer for electrophoresis.

Electrophoresis was carried out on slab gels with a 4–12% linear acrylamide gradient, with the buffer systems of Fairbanks et al. (1971) or Laemmli (1970). On gradient gels, there is a linear relationship between R_f and log molecular weight, which is not true for isotropic slab gels. Molecular weight standards were phosphorylase, bovine serum albumin (BSA), actin, ribonuclease, and insulin. In addition, the red cell proteins themselves were used as standards, bands 1 and 2 taken to be 240 000 and 220 000 daltons, respectively. For autoradiography, the gels were dried and exposed to Kodak X-omat R film at −40 °C with an intensifying screen. The enzymes were lyophilized trypsin (TRL 3-30D727, Millipore Corp., 203 units/mg), Pronase CB (Calbiochem, Lot 530160), and α-chymotrypsin (type II, Sigma). Other chemicals were reagent grade from Sigma or Mallinckrodt.

Results

The phosphorylated polypeptides exposed on the external surface of the erythrocyte membrane are subject to digestion by proteases, and they can be thereby distinguished from phosphoproteins that do not cross the membrane. It was established that the incubation conditions led to complete hydrolysis of the membrane sites available to the proteases. No appreciable hemolysis occurred during incubation. Kinetic experiments in which protease concentration and time were varied showed no further changes in the polypeptide patterns after 2 h at 25 °C with 100 μg/mL enzyme. In particular, no further cleavages were observed even if the erythrocytes were incubated for 4 h at 37 °C with 200 μg/mL Pronase (Mueller & Morrison, 1974). The Coomassie blue stained gel

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[1] Abbreviations: PAS, periodic acid–Schiff base; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; TPCK, N^{α}-(*p*-tosyl)-L-phenylalanine chloromethyl ketone; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; CPD, citrate–phosphate–dextrose.

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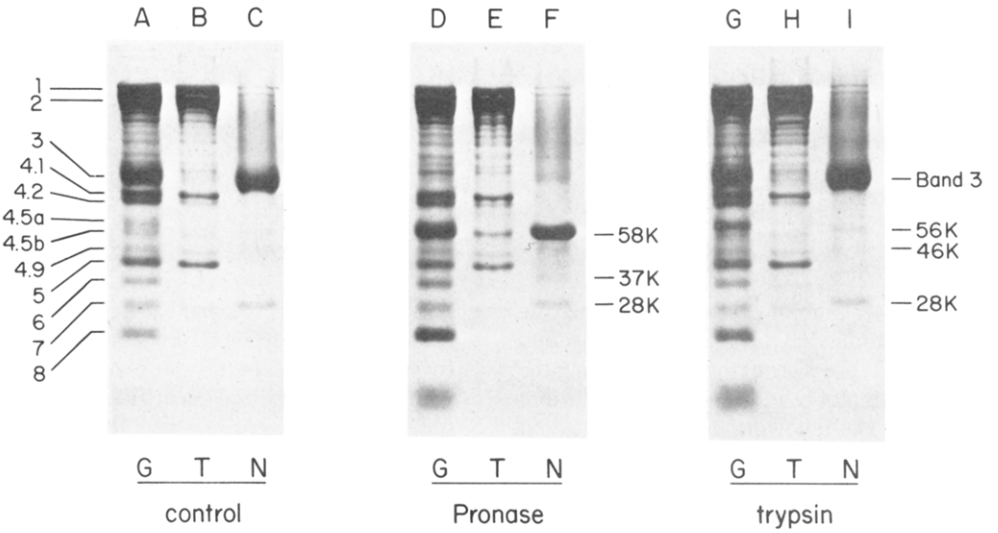

FIGURE 1: $NaDodSO_4$ gels of red cell ghosts, membrane skeletons, and integral proteins from cells incubated with proteases. ^{32}P-Labeled red cells were treated with Pronase or trypsin as described under Materials and Methods. Ghosts (G), Triton residues (T), and NaOH pellets (N) were prepared as described, electrophoresed on a 4–12% acrylamide gradient, and stained with Coomassie blue. The molecular weights of some polypeptide bands are as follows: 1, 240 000; 2, 220 000; 3, 100 000; 4.1, 84 000; 5, 43 000; 6, 36 000; 7, 28 000.

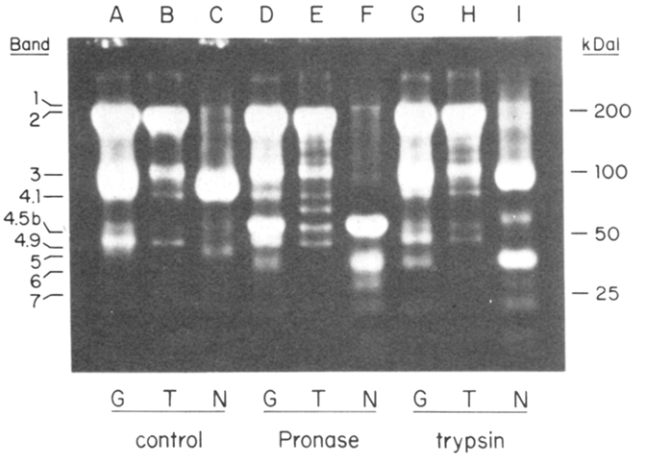

FIGURE 2: Autoradiogram of the slab shown in Figure 1. The position of the Coomassie blue stained polypeptides is indicated.

of a typical experiment is shown in Figure 1, and the autoradiogram of the same gel is shown in Figure 2. Numerous polypeptides are phosphorylated in the intact erythrocyte (Shapiro & Marchesi, 1977; Plut et al., 1978; Dzandu & Johnson, 1980), including bands 2, 3, 4.1, 4.5b, and 4.9.

Integral Phosphoproteins. As first shown by Steck and co-workers, the membrane polypeptides can be fractionated into integral and peripheral classes. Treatment with Triton X-100 solubilizes most of the integral proteins and lipid, yielding an insoluble residue containing the membrane cytoskeleton (Yu et al., 1973). Cold 0.1 N NaOH, on the other hand, solubilizes the peripheral proteins of the membrane, yielding an insoluble pellet of lipid vesicles containing the integral proteins (Steck & Yu, 1973). The pellets resulting from these procedures are therefore complementary. The Coomassie blue stained gels of ghost extracts from phosphorylated erythrocytes (Figure 1A–C) are similar to those reported by Steck's group. The major phosphoproteins in the Triton residue of control cells (Figure 2B) are bands 2 and 2.1, two bands at 110 and 105 kdaltons that are discussed below, and bands 4.1, 4.5b, and 4.9. The NaOH pellet of integral proteins (Figure 2C) has nearly all its ^{32}P in the band 3 region with minor phosphorylated species at 57, 43, 28, and 23 kdaltons.

Membrane Skeleton Phosphoproteins. The polypeptides of the Triton residue were nearly all phosphorylated (Figures 1B and 2B) with the major exceptions of spectrin band 1 and band 5 (actin). Among the heavily phosphorylated species in the Triton residue were two polypeptides in the band 3 region, at 105 and 110 kdaltons. These are not prominent species in the Coomassie blue stained gels, suggesting that they are relatively heavily labeled.

There Are Four Phosphoproteins in the Band 3 Region. The large diffuse band at 80 000–110 000 seen in the autoradiogram of undigested red cells (Figure 2A) partitioned into two bands in the Triton residue (Figure 2B) with molecular weights of 110 000 and 105 000 and an intense ^{32}P band in the NaOH pellet (Figure 2C). However, the alkali-insoluble integral protein band 3 phosphoprotein is itself composed of two polypeptides, the anion channel and PAS-1 (the dimer of glycophorin A). The evidence for this is as follows. Treatment of erythrocytes with Pronase cleaves both glycophorin and the anion channel (Steck, 1972b) and as shown in Figure 2F removes all ^{32}P from the 100-kdalton region of the NaOH pellet. Trypsin, on the other hand, does not cleave the anion channel but does cleave glycophorin A (Steck, 1972b). In accord with the results of Steck, we found no difference in the Coomassie blue stain patterns between the NaOH pellets of undigested and trypsin-treated cells (Figure 1C,I). Examination of the autoradiograms, however, showed ^{32}P remaining in the 100-kdalton region of trypsin-treated erythrocytes (Figure 2I), which can be identified with the anion channel, and a new phosphorylated polypeptide at 38 kdaltons. As discussed below, this new band was most likely the integral portion of the glycophorin dimer. This 38-kdalton phosphoprotein as well as the 60-kdalton proteolytic fragment of the anion channel (Steck, 1972b) is also seen in Pronase-treated cells (Figure 2F).

It is unlikely that either band 2.8 or 2.9 can be identified with the proteolytic product of band 2.1 cleavage, previously called band 3′ (Sheetz & Sawyer, 1978). Band 3′ appears in ghosts during an 8–24-h incubation at 4 °C as the result of the action of endogenous serine protease, which is inhibitable by PMSF (Siegel et al., 1980). In our experiments, the red cells were treated with PMSF before lysis, and the lysis buffers contained PMSF as well as EDTA. In addition, the ghosts were stored at −20 °C and denatured at 100 °C in $NaDodSO_4$

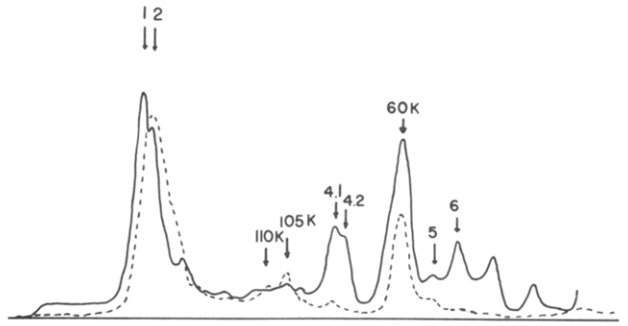

FIGURE 3: Densitometric scans of $NaDodSO_4$ gels of membranes from Pronase-digested human erythrocytes. The Fairbanks et al. (1971) buffers were used on a 4–12% linear acrylamide gradient slab. The gel was stained with Coomassie blue (solid line), dried, and autoradiographed (dashed line). The major ^{32}P peaks are bands 2 and 2.1 and the 60-kdalton fragment of the anion channel. Band 2.8 (10 kdaltons) and band 2.9 (105 kdaltons) have the highest relative specific activities of the polypeptides in this gel.

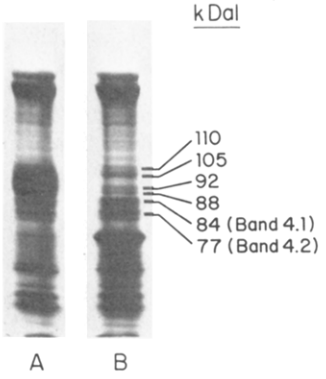

FIGURE 4: Coomassie blue stained electrophoretograms of membranes from control (A) and Pronase (B) erythrocytes, in the Laemmli (1970) buffers on a 4–12% acrylamide gradient. In this system, a number of polypeptides are found in the band 3 region of the gel after exhaustive digestion of the anion channel and PAS-1. Only the 105- and 110-kdalton species are phosphoproteins. In the Laemmli system, band 4.2 splits into two components (Owens et al., 1980).

buffer immediately before electrophoresis. With these procedures, no band 3′ was generated. The amount of phosphorylation in bands 2.8 and 2.9 was inconsistent with the idea that they are proteolytic fragments of band 2.1. Band 3′ is a product of the sequential hydrolysis of band 2.1 through a series of intermediates numbered 2.2–2.6 (Siegel et al., 1980). Densitometric scans of the protein and ^{32}P in electrophoretograms of ghosts from Pronase-digested erythrocytes are shown in Figure 3. Pronase cleaves all of band 3 and PAS-1 (Steck, 1972b; Mueller & Morrison, 1974) and allows quantitation of the underlying bands 2.8 and 2.9. The amount of ^{32}P relative to protein staining intensity is greater in bands 2.8 (110 kdaltons) and 2.9 (105 kdaltons) than in bands 2.3–2.6, which is not consistent with the hypothesis that 2.3–2.6 are precursors of 2.8 and 2.9. Bands 2.8 and 2.9 are likely to be peripheral proteins, since they are not affected by exhaustive Pronase digestion (Figures 1E and 2E) and are found exclusively in the Triton pellet.

More polypeptides are resolved by the Laemmli buffer system than by the Fairbanks system (Owens et al., 1980). There are more polypeptides in the band 3 region than the four phosphoproteins listed above. When ghosts from Pronase-treated cells are electrophoresed in the Laemmli system, the 105- and 110-kdalton polypeptides are evident, and two new bands at 92 and 88 kdaltons are seen (Figure 4). Autoradiograms of these gels showed that the 92- and 88-kdalton bands are not phosphoproteins.

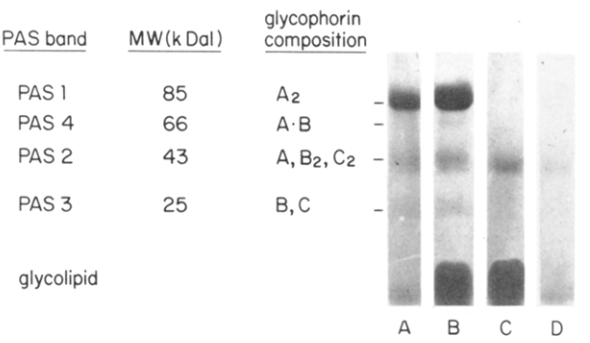

FIGURE 5: PAS-stained 4–12% acrylamide gradient gels, with the Fairbanks buffer system. The PAS band numbering (Steck, 1972a) is indicated, together with the probable composition of the bands (Furthmayr, 1978; Owens et al., 1980): (A) control erythrocytes; (B) NaOH-insoluble membrane proteins from control erythrocytes; (C) NaOH-insoluble proteins from Pronase-treated erythrocytes (the band at approximately 42 kdaltons is the dimer of glycophorin B, which is resistant to proteases); (D) Triton residue of control cell membranes. Glycophorin C is associated with the cytoskeleton (Owens et al., 1980).

There Are Two Phosphoproteins in the Band 4.9–5 Region. The 4.9–5 region of control ghost membranes contained both band 4.9 and the PAS-2 band. The ^{32}P in this region of the autoradiogram of undigested ghosts (Figure 2A) has a diffuse leading edge. The ^{32}P partitioned into a sharp ^{32}P band (48 kdaltons) in the Triton residue (Figure 2B), whereas the diffuse leading edge was found in the NaOH pellet (Figure 2C). PAS staining revealed a band with an apparent molecular weight of 43 000 (Figure 5). The ^{32}P at 43 kdaltons is not phosphorylated actin, which would be found in the Triton residue, but is associated with the PAS-2 band, which includes the monomer of glycophorin A and the dimers of glycophorins C and B (Furthmayr, 1978; Tanner, 1978).

Band 7 Has at Least Two Polypeptides. When ghosts are partitioned into membrane skeleton and integral proteins, Coomassie blue staining material is found at the band 7 region in both fractions (Figure 1B,C). This has been noted earlier (Steck & Yu, 1973; Yu et al., 1973). It is likely that these represent distinct polypeptides: (a) Careful measurement of R_f values suggests that the band 7 material in the Triton residue has a molecular weight of 28 000 while the NaOH pellet band 7 has a molecular weight of 27 000. (b) In membranes, the band 7 region is phosphorylated, but only the NaOH pellet band 7 has ^{32}P label. The Triton residue 28-kdalton species is not a phosphoprotein (Figure 2B,C). As is the case for the band 3 region, membrane phosphorylation revealed new heterogeneity in band 7. Neither of these band 7 polypeptides was cleaved by any protease. Band 7 is not phosphorylated in isolated membranes (Hosey & Tao, 1976).

Phosphorylated Sialoproteins. Three distinct PAS-staining sialoproteins have been described in the red cell membrane. There are more than three PAS-staining bands in $NaDodSO_4$ gel electrophoresis, since the sialoproteins migrate in both monomeric and dimeric forms in the presence of $NaDodSO_4$ [reviewed by Tanner (1978)]. For convenience, we will refer to the individual sialoproteins as glycophorins A, B, and C and use the term PAS only to denote PAS-positive bands on $NaDodSO_4$ gels. The relation between these is summarized (Tanner, 1978; Furthmayr, 1978) in Figure 5. The PAS-2 band at approximately 44 kdaltons is especially complex, containing at least three different sialoprotein species. In our gels, the 20–27-kdalton PAS band sometimes resolved into two bands. These probably represent the monomers of glycophorins B (23 kdaltons) and C (27 kdaltons). This identification is supported by the fact that the 27-kdalton species was

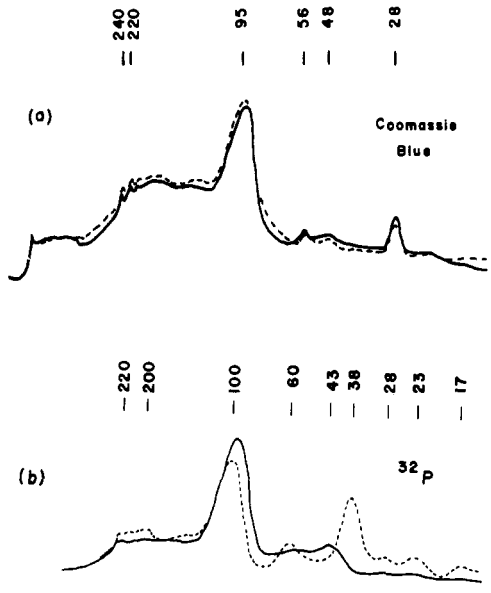

FIGURE 6: Densitometric scans of NaOH-insoluble material of control membranes (solid lines) and membranes from trypsin-digested [^{32}P]P_i-labeled incubated erythrocytes (dashed lines). Equivalent amounts of radioactivity were electrophoresed on 4–12% gradient gels: (a) Coomassie blue stain; (b) autoradiogram. Although trypsin caused no change in the protein pattern detectable by Coomassie blue, the autoradiogram showed a movement of ^{32}P from the 100-kdalton region to the 38-kdalton region, and to a lesser extent to the 60-kdalton region.

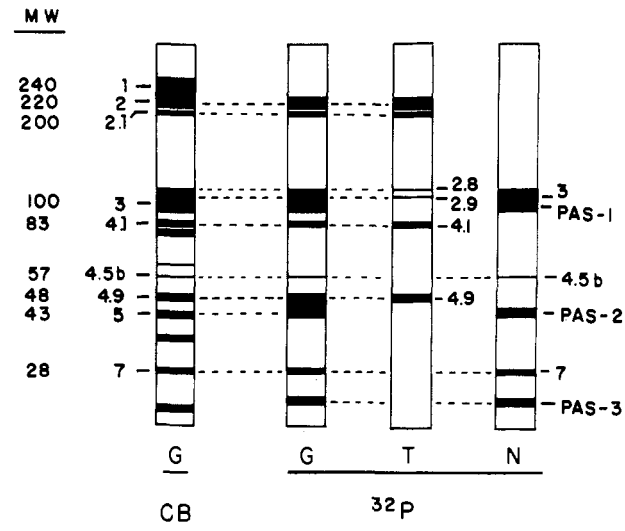

FIGURE 7: Schematic representation of intact cell phosphorylation. The Coomassie blue pattern for ghosts is shown [G (CB)], and the autoradiograms of ghosts (G), Triton-insoluble material (T), and NaOH-insoluble material (N) are shown.

found in Triton shells, which is characteristic of this sialoprotein (Owens et al., 1980). Triton shells also contain a PAS-positive species at an apparent molecular weight of 43 000, which is most likely the dimer of glycophorin C (data not shown).

The major PAS band (PAS-1) is the dimer of glycophorin A, which migrated slightly faster than the anion channel, with an apparent molecular weight of 85 000. Glycophorin A is a phosphoprotein (Shapiro & Marchesi, 1977), and this permitted the tentative identification of its transmembrane moiety after proteolysis, as a 38-kdalton phosphorylated fragment. This was best seen in the integral protein fraction of trypsin-treated erythrocytes (Figure 2I). Steck (1972b) reported that trypsin cleaves glycophorin A but not the anion channel, and in trypsin-digested labeled red cells, a new phosphorylated band was observed at an apparent molecular weight of 38 000. The only larger phosphoproteins present in sufficient amounts to be a precursor of the 38-kdalton band are bands 2, 2.1, and 3 and PAS-1. Since trypsin cleaves only PAS-1 in this group, it seems very likely that the 38-kdalton phosphopeptide represents the membrane-bound fragment of cleaved PAS-1 dimer (glycophorin A). This is supported by densitometric scans (Figure 6). The protein staining pattern of the NaOH-insoluble material of trypsin-digested erythrocytes was indistinguishable from that of the control (Figure 5A). The trypsin autoradiograms, however, had a prominent new phosphoprotein at 38 kdaltons and a striking decrease in radioactivity in the band 3 region (Figure 5B). The decrease was localized at the leading edge of band 3, the position of PAS-1 in this electrophoretic system, as would be expected if the new 38-kdalton species was a product of PAS-1 proteolysis. Comparison of the two autoradiograms also showed the disappearance of radioactivity at 43 kdaltons (PAS-2). The monomer of glycophorin A is one of the components of PAS-2 (Tanner, 1978). Trypsin cleavage of the monomer would therefore be expected to yield a new phosphopeptide at $37/85 \times 43\,000 = 18\,000$, and this was observed (Figures 2E,I and 5B). Phosphorylation therefore allowed the identification of the membrane and cytoplasmic sections of the major sialoprotein. This proteolytic fragment is not otherwise identifiable on $NaDodSO_4$ gels as it does not stain with either Coomassie blue or PAS stain.

Although the specificity of trypsin for cleavage of PAS-1 rather than the anion channel is high, a small amount of the 60-kdalton fragment of the anion channel was consistently seen in autoradiograms of trypsin-digested erythrocyte membranes (Figures 2I and 5B). This was observed with a number of different trypsin preparations, including TPCK–trypsin. This trypsin-mediated cleavage of the anion channel has not been previously observed since the fraction of the anion channel molecules cleaved must be very small. The amount of 60-kdalton fragment was, in fact, not detectable by Coomassie blue staining (Figure 1G,I; Steck, 1972b). Since the anion channel incorporates approximately 100 times more radioactivity than does glycophorin A during incubation with [^{32}P]P_i (Shapiro & Marchesi, 1977), the size of the autoradiographic density of 60 kdaltons is consistent with the cleavage of a very small fraction of the anion channel molecules during trypsin digestion.

Trypsin cleaves glycophorins A and C but not glycophorin B (Furthmayr, 1978; Owens et al., 1980). As reported previously by Bender et al. (1971), we found that heavily loaded gels of Pronase-digested membranes had a PAS-positive band at an apparent molecular weight of 40 000–43 000. This was the expected position of the dimer of glycophorin B and suggests that glycophorin B resisted digestion by Pronase as well as by trypsin. Glycophorin B was phosphorylated since ^{32}P was found in both ghosts and NaOH pellets at 43 000 (glycophorin B dimer position) and 23 000 (glycophorin B monomer position).

Examination of the Triton residue of labeled ghosts suggested that glycophorin C on the other hand is not heavily phosphorylated. Unlike the other sialoproteins, glycophorin C is found in the Triton residue (Owens et al., 1980). Examination of the autoradiogram of Triton residues (Figure 2B) revealed no ^{32}P below the PAS-2 region (23 kdaltons).

Discussion

Figure 7 summarizes our results on the fractionation of proteins phosphorylated in the intact erythrocyte. Comparison of these data with the results of phosphorylation of isolated

ghosts (Avruch et al., 1976; Hosey & Tao, 1977) shows the following differences. Bands 2.8, 4.1 and 4.9 and PAS-2 and PAS-3 are not phosphorylated in the isolated ghost. In the absence of any proteolytic analysis, it is impossible to ascertain if glycophorin A is phosphorylated in the isolated ghost. Hosey & Tao (1977) did report, however, that no anomalously migrating phosphorylated component could be detected in the band 3 regions of autophosphorylated human ghosts, strongly suggesting that glycophorin A was not labeled. It is therefore likely that no sialoproteins are labeled in ghosts during incubation with [γ-^{32}P]ATP, which constitutes a major difference between the situation in intact cells and that in isolated membranes.

It has been occasionally reported that actin (band 5) is phosphorylated in the erythrocyte membrane (Gratzer, 1981). Although radioactivity was found in the band 5 region of $NaDodSO_4$ gels of membranes at an apparent molecular weight of 43 000, the ^{32}P was associated with an integral protein, PAS-2, and no radioactivity was found in actin (Figure 2).

Hosey & Tao (1977) reported that a 100-kdalton polypeptide, which they termed 2.9, was not phosphorylated by human red cell ghosts after addition of [^{32}P]ATP but was labeled if exogenous cAMP-dependent kinase from rabbit erythrocyte cytoplasm was added. This polypeptide was Triton insoluble. Our results have shown that a similar polypeptide of 105 kdaltons is labeled in the intact erythrocyte. This constitutes further evidence of a possible role for cytoplasmic kinases (Plut et al., 1978) in the phosphorylation of membrane components in the intact cell, since the kinases associated with the isolated ghost cannot completely reproduce the phosphorylation seen in the intact cell. Alternatively, however, the intact erythrocyte might contain a phosphatase that is removed during ghost preparation. This phosphatase would permit turnover and labeling of proteins in the intact cell, whereas in the isolated membrane, no such turnover can occur. Resolution of this question will require isolation of the relevant cytoplasmic enzymes.

In addition to band 2.9 (105 kdaltons), there is a second highly phosphorylated peripheral protein in the band 3 region (110 kdaltons) that we have called 2.8. This phosphoprotein was not detected in experiments with isolated ghosts, even after the addition of exogenous kinases. It may be identical with the band 2.7 detected in membranes from human red cells incubated with [^{32}P]P_i and cAMP (Plut et al., 1978), although we have observed it in the absence of cAMP. Plut et al. (1978) did not fractionate the phosphorylated membranes however, and the detection of these 110- and 105-kdalton polypeptides in our experiments depended on the removal of the anion channel and glycophorin A from the band 3 region of the gel by Triton extraction or Pronase digestion.

The possible involvement of cAMP in membrane phosphorylation is of considerable interest. The cAMP-dependent kinase of human erythrocytes specifically labels band 2.1, 4.5, and 4.9; the presence of ^{32}P in these polypeptides in the intact cell would suggest that this enzyme is functional in the human erythrocyte. In fact, Plut et al. (1978) found 8.5 nM cAMP in the human red cell and suggested that the cAMP-dependent kinase was partially activated. This proposal is supported by our results.

The major components of the band 3 region are the anion channel and the dimer of glycophorin A. Chymotrypsin and Pronase cleave the anion channel to a 60- and 36-kdalton fragment, both of which are integral and found in the NaOH pellet (Steck, 1972b). Only the 60-kdalton fragment is phosphorylated (Drickamer, 1976). After Pronase digestion, ^{32}P appears in the 60-kdalton region and in the 38-kdalton region (Figure 2D). The major peak of radioactivity in the 38-kdalton region is also seen after trypsin digestion (Figure 2G), which cleaves only a small fraction of the anion channel. The ^{32}P at 38 kdaltons is therefore attributable to proteolytic cleavage of another polypeptide. The only heavily phosphorylated polypeptides larger than 36 kdaltons are spectrin bands 2, 2.1, 2.8, 2.9, and 3 and the dimer of PAS-1. Since bands 2 through 3 are unaffected by trypsin (Figures 1 and 2G,H), we infer that the 38-kdalton phosphopeptide was a fragment of glycophorin A. Glycophorin A dimerizes through interactions of its hydrophobic segment (Silverberg et al., 1976), which is unlikely to be affected by external proteolysis, and the 38-kdalton fragment is likely to be dimeric.

There is a report that the ^{32}P acceptors in the band 3 region are not identical with the bulk of band 3. In those experiments washed ghosts were phosphorylated with [γ-^{32}P]ATP and endogenous kinases, solubilized in deoxycholate, and chromatographed on Sepharose–Con A (Roses, 1976). Only the unretarded 100-kdalton polypeptide was phosphorylated. The present results with intact erythrocytes suggest, however, that the major phosphorylated species in the band 3 region is homogeneous by a number of criteria. Pronase cleavage converts all the band 3 integral proteins into the 60- and 38-kdalton species, derived from the anion channel and PAS-1, respectively. Similarly, all the phosphorylated band 3 material in trypsin-treated ghosts can be cross-linked by Cu and phenanthroline (data not shown).

The observation that most of the proteins of the red cell appear to be phosphorylated suggests that this posttranslational modification has a role in erythrocyte metabolism. Although the phosphorylation of spectrin does not affect its binding or self-assembly (Anderson & Tyler, 1980; Ungewickell & Gratzer, 1978), possible effects on interactions with ankyrin or actin have not been investigated. Treatment of ghosts with alkaline phosphatase blocks all forms of endocytosis (Hardy et al., 1979) and ATP-induced shape changes (Birchmeier & Singer, 1977). These phenomena were attributed to dephosphorylation of spectrin. The phosphorylation of spectrin relative to other membrane skeletal polypeptides is greater in ghosts than in intact cells, as can be seen by comparison of Figure 2B with any of the reported autoradiograms of ghost autophosphorylation. Examination of the autoradiogram of the Triton residue of ^{32}P-labeled erythrocytes shows, however, that bands 2, 4.1, 4.5b, and 4.9 as well as 2.8 and 2.9 are phosphorylated, and the observed effects could therefore be caused by dephosphorylation of any of these components as well as spectrin band 2. We would suggest that the relationship between the phosphorylation state of these other skeletal polypeptides and the membrane changes induced by alkaline phosphatase might be a valuable line of investigation.

Acknowledgments

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Phospholipid Molecular Species Alterations in Microsomal Membranes as an Initial Key Step during Cellular Acclimation to Low Temperature†

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ABSTRACT: When *Tetrahymena pyriformis* cells were chilled from 39 to 15 °C, fatty acids of the microsomal membrane phospholipids increased significantly in unsaturation over a 15-h acclimation period. During the initial hour following chilling, only a small fraction of the increase in unsaturation had taken place, yet the fluidity of the lipids, as measured earlier in fluorescence depolarization studies [Dickens, B. F., & Thompson, G. A., Jr. (1981) *Biochim. Biophys. Acta 644*, 211], had already increased almost to the level found in cells fully acclimated to 15 °C. The microsomal lipids from 39 and 15 °C grown cells and cells shifted from 39 to 15 °C for 1 h were analyzed in more detail in an effort to discover what compositional changes might be responsible for the rapid increase in fluidity. The three major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and 2-(aminoethyl)phosphonolipid (AEPL), were purified and enzymatically hydrolyzed to diglycerides, which were then converted to *tert*-butyldimethylsilyl (*t*-BDMS) ethers. Coupled gas chromatography–mass spectrometry of the *t*-BDMS derivatives permitted identification of the lipid molecular species and estimation of their relative concentrations under the different temperature conditions. Each phospholipid responded to chilling in a distinctively different way, although there were a few similarities. Thus, PE and PC were alike in showing a marked decrease in combinations of odd chain with even chain fatty acids at low temperature (there were no odd chain fatty acids in AEPL). Likewise, the extent of acyl chain unsaturation increased in combinations of monoalkyl monoacyl species of both PC and AEPL at 15 °C (PE had no alkyl ether side chains). The variety and extent of the changes were in general much greater in PE and PC than in AEPL. The molecular species patterns, particularly in cells exposed to 15 °C for only 1 h, showed very clearly that the extent of fatty acyl group rearrangement by deacylation–reacylation overshadowed acyl chain desaturation as a means of altering lipid structure. In light of these findings, selective phospholipid deacylation–reacylation is indicated as a mechanism which may be of pivotal importance in achieving rapid homeoviscous adaptation.

The protozoan *Tetrahymena pyriformis* has been profitably exploited for studying the mechanism of cellular acclimation to low environmental temperatures (Martin et al., 1976; Fukushima et al., 1977; Dickens & Thompson, 1981). It is widely (Thompson, 1980) but not unanimously (Lands, 1980) believed that a vital response to chilling in *Tetrahymena* and many other organisms is the alteration of membrane lipid composition so as to overcome the rigidifying effect that low temperature invariably has on membrane lipids.

In recent studies (Dickens et al., 1980; Dickens & Thompson, 1980, 1981), our laboratory has begun a detailed correlation of lipid compositional and physical changes in a key *Tetrahymena* membrane system—the endoplasmic reticulum (microsomes). Within as little as 1 h after chilling from 39 to 15 °C (Dickens & Thompson, 1981), we were able to detect (1) a small but significant increase in the unsaturation

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