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PHOSPHORYLATION OF MEMBRANE PROTEINS FROM *PLASMODIUM BERGHEI*-INFECTED RED CELLS

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SUMMARY

Membrane from *Plasmodium berghei*-infected mouse red cells has a different pattern of phosphorylation by (γ-^{32}P)ATP from normal membrane. A phosphorylated membrane protein of apparent molecular weight 42,000, absent in membrane from normal cells, can be detected in membrane from infected cells. The new phosphorylated protein can be extracted by 0.1 mM EDTA but not by triton X-100, indicating that it may be red cell actin.

Some proteins of the red cell membrane undergo phosphorylation by ATP, as shown by autoradiography of dodecylsulphate polyacrylamide gel separation of the membrane following incubation with (γ-^{32}P)ATP (1-3). It has been shown that membrane from sickle red cells (4) and cells of patients with hereditary spherocytosis (5,6), hereditary stomatocytosis (7) and myotonic and Duchenne muscular dystrophy (8) show abnormal patterns of phosphorylation. Although the pattern of phosphorylation is dependent on assay methods (9,10) and its relationship to pathophysiology is still unclear, under defined conditions it can still serve a useful function as indicator of membrane abnormality. Malaria infection produces various changes in red cell membrane structure (11-13), and might therefore be accompanied by an abnormal phosphorylation pattern. This paper reports striking changes in the phosphorylation pattern of membrane fraction isolated from mouse red cells infected with *Plasmodium berghei*. Phosphorylation of intact red cells reveals similar alterations due to infection, therefore suggesting that they may have some physiological significance.

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Prior to each experiment 30S subunits were activated by incubation at 40° for 30 min in 200 mM NH_4Cl, 20 mM Tris-HCl pH 7.8, 20 mM magnesium acetate, 2 mM dithiothreitol. 50S subunits and 70S ribosomes were activated at 30°for 30 min in 60 mM NH_4Cl, 40 mM KCl, 10 mM Tris-HCl pH 7.8, 20 mM magnesium acetate, 1 mM dithiothreitol. Treatment of ribosomal particles with antibiotics was performed in mixtures (95-125 µl) containing either 2.7 µM 30S subunits, 2.3 µM 50S subunits or 1.3 µM 70S ribosomes and antibiotics as specified, in the same ionic environment as the activation mixtures, excepting magnesium acetate which was lowered to 12 mM. After incubation at 30° for 10 min, excess unbound antibiotic was removed by Sepharose 6B filtration at room temperature in a column (0.6 x 15 cm) equilibrated with 65 mM NH_4Cl, 20 mM Tris-HCl pH 7.8, 12 mM magnesium acetate and 6 mM 2-mercaptoethanol. Other methods are specified in the legends to Tables and Figure.

Results. *Stabilization of GDP·EF-G·50S subunit·fusidic acid complex.* Our recent work has shown that aminoglycoside antibiotics stabilize the guanosine nucleotide·EF-G·70S ribosome complexes (8). Table I shows that many aminoglycosides also stabilized the [^{3}H]GDP·EF-G·50S-subunit·fusidic acid complex, and as with complexes containing 70S ribosomes (8), neomycin B, gentamicin C1a, kanamycin B and tobramycin were the most active drugs. In

Table I. Effect of aminoglycoside antibiotics (0.1 mM) on the dissociation of [^{3}H]GDP·EF-G·50S-subunit·fusidic acid complexes

Antibiotic	[^{3}H]GDP released (%)
None	100
Neamine	84
Ribostamycin	83
Paromomycin	74
Neomycin C	60
Neomycin B	22
Gentamicin C1	83
Gentamicin C1a	30
Sisomicin	58
Verdamicin	48
Kanamycin A	79
Kanamycin B	29
Tobramycin	42
Streptomycin	113
Dihidrostreptomycin	105
Bluensomycin	96

[^{3}H]GDP·EF-G·50S-subunit·fusidic acid complexes were formed in mixtures containing: 12 mM NH_4Cl, 4 mM KCl, 10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 1 mM dithiothreitol, 2.5 mM fusidic acid, 7.5 A_{260} units/ml 50S subunits, 45 µg/ml EF-G and 1 µM [^{3}H]GDP (2050 cpm/pmol). After 30 min at 30°, complexed [^{3}H]GDP (0.73-0.96 molecules/50S subunit) was determined by diluting 10 µl samples with 4 ml of dilution buffer (10 mM NH_4Cl, 10 mM Tris-HCl pH 7.8, 10 mM magnesium acetate, 2 mM fusidic acid and 0.1 mg/ml bovine serum albumin) and filtration through nitrocellulose membranes (8,9). Other 10 µl portions were diluted with 4 ml of dilution buffer prewarmed to 30° and containing the specified antibiotics at 0.1 mM. After 2 min of incubation at 30°, the amount of [^{3}H]GDP remaining in complex was determined. Released [^{3}H]GDP was calculated by difference and, in controls without antibiotic, was 0.25-0.44 molecules/50S subunit.

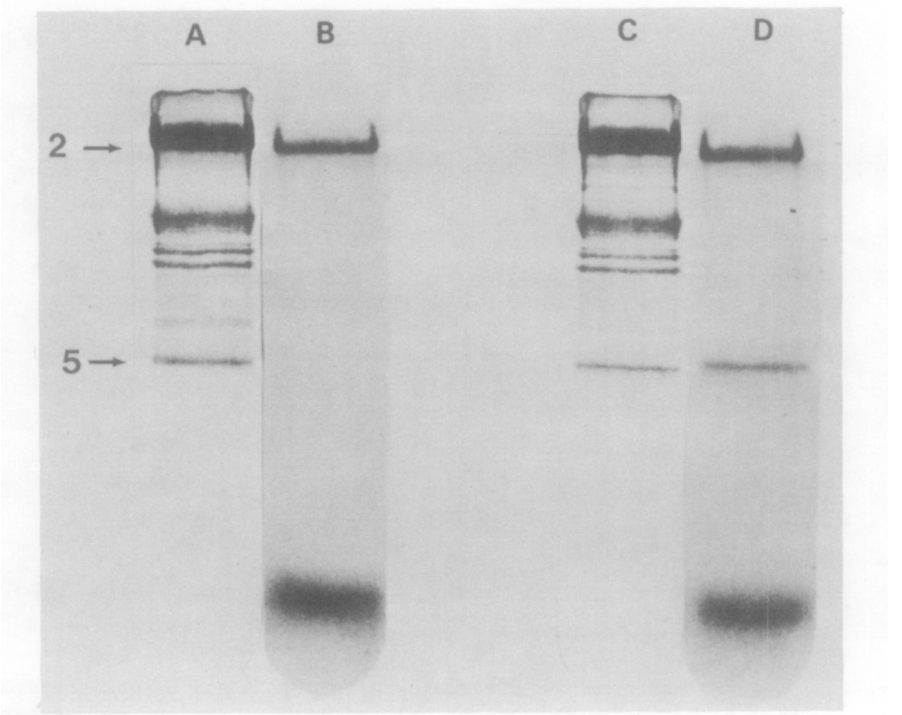

Figure 1. A, staining pattern of dodecylsulphate-acrylamide gel from normal mouse red cell membrane; B, autoradiograph of the same gel as in A; C, staining pattern of the gel from *P. berghei*-infected red cell membrane; D, autoradiograph of the same gel as in C.

Membrane was extracted with 0.1 mM EDTA as described by Marchesi *et al.*(16) and with 1% triton X-100 as described by Yu *et al.* (17). Protein was determined by the Lowry method (18).

Results

Alterations can be observed in the dodecylsulphate-polyacrylamide gel pattern of the membrane fractions of *P. berghei*-infected cells prepared by hypotonic lysis (Fig. 1). When membrane from infected cells is compared with normal membrane by means of autoradiography following incubation with (γ-^{32}P)ATP and electrophoresis, a distinct new phosphorylated band can be seen at an apparent molecular weight of 42000 (Fig. 1). In addition, some minor new phosphorylated bands can also be observed. The protein staining densities of these new bands show only a minor variation from normal, hence

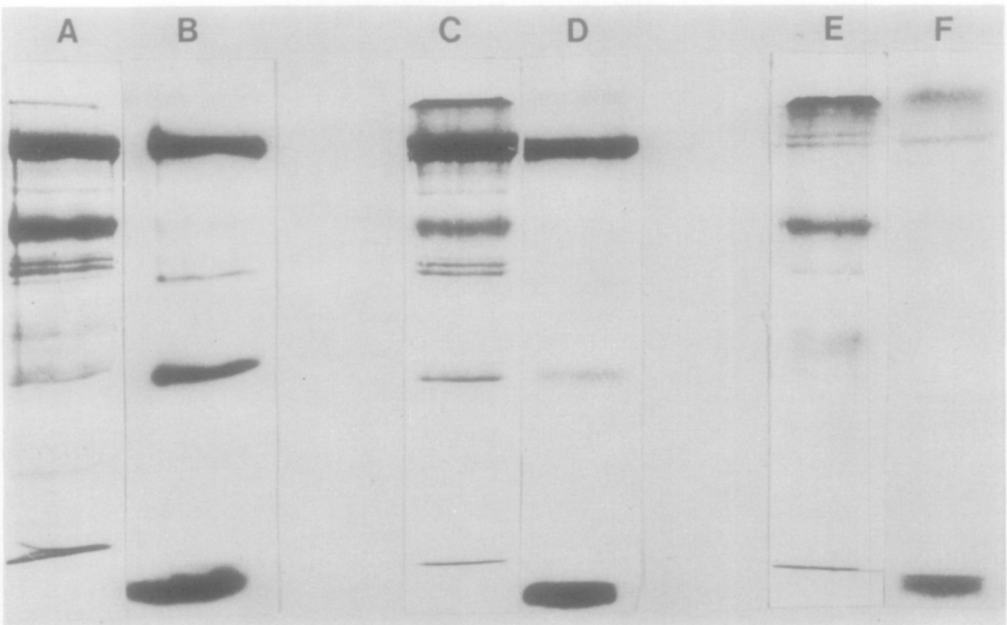

Figure 2. Staining patterns and autoradiographs of: A and B, gel prepared from *P. berghei*-infected red cell membrane; C and D, gel prepared from infected cell membrane after extraction with 1% triton X-100; E and F, gel prepared from infected cell membrane after extraction with 0.1 mM EDTA.

suggesting that the alterations is not simply due to relative increase in the amount of proteins. The major new band has the same mobility as red cell actin (band 5), and can be completely extracted by 0.1 mM EDTA, pH 8, but not with 1% triton X-100 (Fig. 2).

The phosphorylation of red cell membrane proteins is catalysed by endogenous protein kinases, some of which are activated by Ca^{2+} and c-AMP. No appreciable effect of either Ca^{2+} or c-AMP can be observed in the phosphorylation of the new bands from infected cells (data not shown).

Membrane protein phosphorylation is also measured in intact *P. berghei* infected erythrocyte. The pattern of phosphorylation in intact cells is similar to the isolated membrane fraction after 2 h incubation, especially with respect to the appearance of the 42000 M.W. band (Fig. 3).

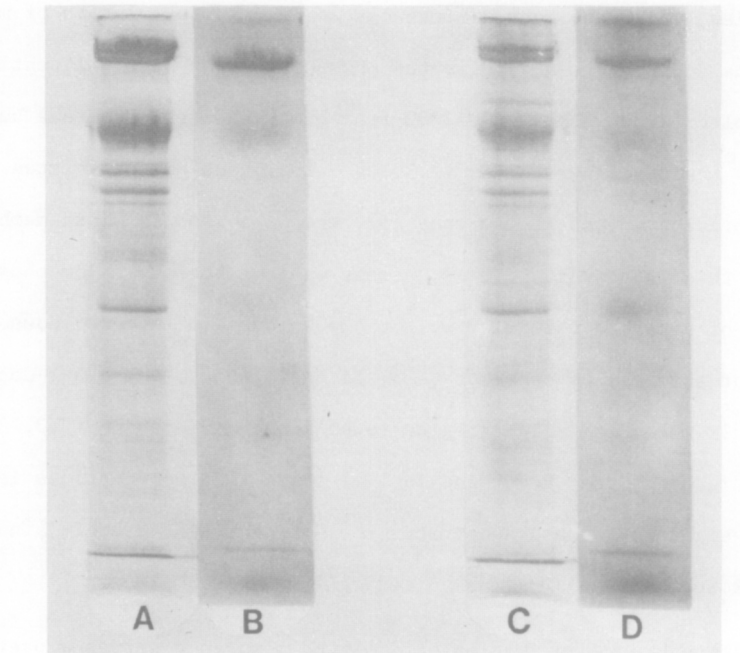

Figure 3. Staining patterns and autoradiographs of gel from membrane prepared after intact cell phosphorylation: A and B, normal cells; C and D, *P. berghei*-infected cells.

Discussion

The alterations in the phosphorylation pattern of membrane proteins prepared from *P. berghei*-infected mouse red cells (Fig. 1) could either originate from red cell membrane components or from new parasite material present in the membrane fraction. It is notable however that the new phosphorylated bands are already present in the early stage of infection, when there is still relatively little parasite material (data not shown). The major new band coincides and may be identical with band 5 (apparent molecular weight 42000) of normal red cell membrane. The fact that it can be extracted with 0.1 mM EDTA but not with triton X-100 suggests further that it is probably actin (see e.g., ref. 19). Rigorous proof of its origin, however must await further studies.

While phosphorylation of bands 2 and 2.1 is a common feature found in both infected and normal mouse red cells as well as in red cells from

other species, there are many differences in autophosphorylation of membranes from various sources. c-AMP-activated phosphorylation of a polypeptide of apparent molecular weight 50000-52000 from human red cell membrane has been reported by various laboratories (2,3,20). Analogous phosphorylated peptide is barely present in rabbit membrane (3), and is absent in mouse membrane studied in this laboratory. It is unclear whether the new major phosphorylated band of apparent molecular weight 42000 found in infected mouse cells, which is not affected by c-AMP or Ca^{2+}, is analogous to the c-AMP-dependent bands in this molecular weight region found in human membrane (2,3,20). It is also of interest that Hosey and Tao (4) found that polypeptides in this molecular weight region are substrates for GTP-dependent protein kinases in sickle-cell but not in normal human red cell membrane.

Although phosphorylation of isolated membranes serves a useful diagnostic function, as has been shown for some pathological systems (4-8), it may have little physiological significance if changes in phosphorylation pattern cannot be observed in intact cells (10). In hereditary spherocytosis, for example, where conflicting results were reported on phosphorylation of isolated red cell membrane (8,9), no change could be observed in intact cells (10). In contrast we observed similar changes in membrane phosphorylation pattern on incubation of intact normal and malaria-infected cells with $^{32}P_i$, as on incubation of isolated membranes with (γ-^{32}P)ATP. These changes may therefore have physiological significance.

It has been shown (21-23) that membrane phosphorylation exerts control over red cell shape and deformability. Furthermore, interaction between spectrin and actin, which is likely to regulate the mechanical properties of the membrane, has been shown to depend on phosphorylation of band 2 of spectrin. Phosphorylation of actin, however, has never been clearly observed before. The relationship of abnormal phosphorylation reported here with changes in red cell shape (11,24), cation transport (25) and other membrane lesions known to occur in malarial infection remains to be elucidated.

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