The addition of the tumor promoting phorbol ester 12-O-tetradecanoyl phorbol 13-acetate to intact human red blood cells activates protein kinase C and stimulates the phosphorylation of the membrane skeletal proteins band 4.1 and band 4.9 as well as two proteins of molecular mass 115 and 110 kDa. We show that 12-O-tetradecanoyl phorbol 13-acetate promotes the association of cytosolic protein kinase C with the red cell membrane and that the enzyme is present onghost membranes but is largely absent from inside-out vesicles. We show that micromolar Ca\(^{2+}\) added to ghosts also promotes the phosphorylation of band 4.1 and the \(~100\)-kDa proteins, a reaction which has not been described previously. Digestion and extraction studies show that the 100-kDa proteins are unrelated to band 3 since they are absent from NaOH stripped membranes, but are found in Triton-prepared cytoskeletons. Digestion of intact red cells with chymotrypsin or neuraminidase, which attack principally band 3 and glycophorin, respectively, markedly inhibits protein kinase C phosphorylation of band 4.1 in red cells and ghosts and of the 100-kDa proteins in ghosts. These enzymes have no effect upon the activity of the Ca\(^{2+}\)-activated phosphorylation reaction, suggesting that it does not involve protein kinase C. These results shed light on two phosphorylation reactions which act exclusively on red cell membrane skeletal proteins. Our findings suggest that digestion of the integral membrane proteins band 3 and glycophorin, the principal targets of external protease digestion, affects the activity or specificity of protein kinase C. Finally we have described two apparently novel \(~100\)-kDa phosphorylated proteins which are components of Triton-prepared red cell membrane skeletons.

The membrane skeleton of the human erythrocyte is composed principally of the proteins spectrin, actin, band 4.1, and band 4.9. These proteins form a self-associated membrane-bound network which is held responsible for many of the mechanical properties as well as the shape of the red cell. Although many of the interconnections and associations of these proteins have been extensively studied (see Ref. 1 for review), less attention has been paid to the factors which may regulate these associations.

With the exception of actin, all of the major red cell membrane skeletal proteins are phosphoproteins, including ankyrin, the high affinity membrane attachment site for spectrin (1, 2). Early evidence suggested a correlation between the degree of phosphorylation of membrane skeletal proteins, particularly spectrin, and such parameters as red cell shape (3) and protein-protein associations (4). Although subsequent studies have largely ruled out an effect of spectrin phosphorylation on many of these phenomena (5-7), the possibility exists that phosphorylation of other membrane skeletal proteins, particularly bands 4.1 and 4.9, is important to skeletal organization. One of the major interconnections in the membrane skeleton is the association between spectrin and F-actin, which is mediated or enhanced by band 4.1 (1, 8-10). Recently we have presented evidence that the ability of band 4.1 to promote spectrin binding to actin is sensitive to its overall degree of phosphorylation (11).

Both band 4.1 and proteins in the band 4.9 region are phosphorylated in vitro by a cAMP-stimulated membrane-associated protein kinase (12), and possibly by cytosolic kinases as well (13). They may also be phosphorylated by a cAMP-independent kinase, since membranes from cells metabolically labeled in vitro incorporate \(^{32}\)P in both band 4.1 and band 4.9 (14, see also below). In rabbit erythrocytes, band 4.1 has been reported to be phosphorylated in response to exogenously added phorbol esters, suggesting the presence of protein kinase C (15). However, rabbit erythrocytes evidence several differences from human, both in the protein-staining pattern in the band 4.1 region as well as in the absence of a membrane-associated cAMP-dependent kinase acting on band 4.1 (12, 16). More recently, human erythrocyte bands 4.1 and 4.9 have been shown to be phosphorylated in response to exogenously added phorbol esters, although a characterization of the reaction or of the responsible kinase has not been presented (11, 17).

Here we show that, in human erythrocytes, band 4.1, band 4.9 and two membrane-skeleton-associated \(~100\)-kDa proteins are all phosphorylated in response to TPA.\(^{1}\) We present evidence for TPA-induced translocation of protein kinase C from the cytosol to the red cell membrane and demonstrate a form of transmembrane regulation of this enzyme. We also describe the characteristics of a Ca\(^{2+}\)-dependent kinase which acts on band 4.1 and the 100-kDa proteins. Our studies do not address the phosphorylation of spectrin or ankyrin. These important phosphorylated membrane skeletal proteins co-migrate in the gel system used here and will be the subject of future studies.

**METHODS**

*Metabolic Labeling of Red Blood Cells—20 ml of whole blood was washed three times in 150 mM NaCl. Subsequently, 6-8 ml of packed red blood cells was suspended in 150 ml of a labeling medium containing 150 mM NaCl, 5 mM sodium phosphate, pH 7.6; TFP, trifluoperazine; EGTA, \[(ethylenebis(oxyethylenenitrilo))tetraacetic\] acid; SDS, sodium dodecyl sulfate.*

\(^{1}\) The abbreviations used are: TPA, 12-O-tetradecanoyl phorbol 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline (150 mM NaCl, 5 mM sodium phosphate, pH 7.6); TFP, trifluoperazine; EGTA, \[(ethylenebis(oxyethylenenitrilo))tetraacetic\] acid; SDS, sodium dodecyl sulfate.
Red Cell Membrane Phosphorylation

red cells was suspended in 80 ml of buffer A (135 mM NaCl, 3 mM KCl, 20 mM NaHCO₃, pH 7.5, 2 mM MgCl₂, 1 mM CaCl₂, 200 units/ml penicillin/streptomycin) and incubated for 4 h at 37°C to deplete endogenous ATP stores. Phosphorylation was initiated by transferring 1 ml of packed cells into 4 ml of buffer A plus 1 mM adenosine, 12 mM NaHCO₃, 200 units/ml neuraminidase (Sigma), and washed in this buffer until the ghosts were white.

Additional washes in PBS. The cells were lysed in 20 volumes of ice-cold 5 mM sodium phosphate, pH 7.6, containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride, Sigma) and 1 µg/ml leupeptin. In some cases (see below) or with TPA (Sigma) for 30 min at 37°C (figure legends) followed by two additional washes in PBS. The cells were lysed in 20 volumes of ice-cold 5 mM sodium phosphate, pH 7.6, containing 0.5 mM PMSF and 1 µg/ml leupeptin (Sigma) and washed in this buffer until the ghosts were white.

Phosphorylation of Ghosts and Inside-out Vesicles—Whole blood was washed four times in PBS followed by lysis and four washes in ice-cold 5 mM sodium phosphate pH 7.6 plus 0.5 mM PMSF and 1 µg/ml leupeptin. In some cases (see figures), red cells suspended to 20% v/v in PBS were incubated with TPA at 37°C for 10 min followed by two additional washes in PBS before lysis. Inside-out vesicles (18) were prepared by extraction of ghosts in 30 volumes of 0.1 mM sodium phosphate, pH 8.5, plus 0.5 mM PMSF and 1 µg/ml leupeptin at 37°C for 30 min. The vesicles were washed once in this buffer at 4°C. Phosphorylation of ghosts or vesicles was done by adjusting the membranes to a concentration of 0.5 mg/ml in 5 mM sodium phosphate, pH 6.5, 1 mM EGTA (or various concentrations of CaCl₂, when specified in figure legends), 4 mM NaF, 2 mM PMSF, 100 µg/ml leupeptin, 10 mM MgCl₂, 20 µCi/ml [γ-³²P]ATP (10⁶ Ci/mmol).

Incubation was done as described above. After incubation, 2 mM diisopropyl fluorophosphate was added, and the cells were washed two more times in PBS containing 0.1 mM PMSF and 1 µg/ml leupeptin. In some cases (see figures), the cells were subsequently treated with TPA for 30 min as described above, and in all cases the cells were lysed and washed in 5 mM sodium phosphate, pH 7.6, plus 0.5 mM PMSF and 1 µg/ml leupeptin. Phosphorylation of membranes from normal and digested cells was done as described above.

**RESULTS**

TPA-stimulated Phosphorylation of Membrane Proteins—Incubation of intact red cells with ³²P in physiological saline solution results in the metabolic labeling of numerous membrane proteins. After 18 h in ³²PO₄, label was detected in the spectrin/ankyrin region of the gel, in a pair of proteins of 115 and 110 kDa and in an 86-kDa protein (Fig. 1, lane 1). Phosphorylation in the spectrin/ankyrin region is due to spectrin band 2 and ankyrin which, in this gel system, migrates with or, sometimes, above spectrin band 1. Because of the close proximity of the spectrin and ankyrin phosphorylated bands, we did not attempt any analysis of them in our experiments. They will be the subject of future work.

Addition of TPA to intact cells for 30 min after their equilibration in ³²PO₄ resulted in a dose-dependent increase in labeling of band 4.1 (80 kDa) as well as in the ~100-kDa proteins, band 4.9 and the 86-kDa protein (Fig. 1, lanes 2–7) (see Ref. 22 for nomenclature). The phosphoprotein-labeled band 4.9 (23) was found in all cases to migrate just above actin when autoradiograms were aligned with corresponding Coomassie Blue-stained gels. Phosphorylation of band 4.9 was always increased by TPA, but frequently could be seen on autoradiographs only after prolonged exposure. The response to TPA appeared to be maximal between 0.1 and 0.5 µM of added TPA. The non-tumor-promoting phorbol derivative phorbol 12-myristate 13-acetate-4-O-methyl ether produced no enhancement in phosphorylation (not shown) suggesting that, as in other cell types, the target of TPA action was protein kinase C.

Since the above experiments dealt with phosphorylation of membrane proteins in intact cells, the responsible kinase could have been either cytosolic or membrane-bound. Thus, the action of TPA could have been either to activate the kinase and/or to promote association of the kinase with the membrane. However, it is clear from the above experiments that the phosphoprotein labeled by TPA did not correspond to any of the known membrane proteins of human erythrocytes. This conclusion was derived from experiments which showed that, under the various conditions tested, reducing or lengthening the time of incubation altered the overall level of phosphorylation but did not affect the pattern of labeled proteins.

**Gel Electrophoresis—** Samples were electrophoresed in 10% acrylamide slab gels as described by Hubbard and Lazardes (19), dried between two sheets of dialysis membrane, and autoradiographed using Dupont Cronex X-ray film and an intensifying screen. Autoradiograms were printed as negative images by contact printing onto photographic paper. Molecular masses were determined by calibration of gels using red cell membrane proteins.

**Enzyme Treatment of Red Cells—** Normal or metabolically labeled red cells washed in PBS were suspended to 20% v/v in PBS and incubated with 10 µg/ml trypsin (Sigma, type VII) treatment was done at an enzyme concentration of 200 µg/ml for 2.5 h at 37°C. Neuraminidase treatment was done at a cell concentration of 50% v/v in PBS plus 10 mM CaCl₂ at pH 7.0 with 0.1 unit/ml neuraminidase (Sigma, type V from Clostridium perfringens). In each of the above cases, the cells were washed twice in PBS after incubation, 2 mM diisopropyl fluorophosphate was added, and the cells were washed two more times in PBS containing 0.1 mM PMSF and 1 µg/ml leupeptin. In some cases (see figures), the cells were subsequently treated with TPA for 30 min as described above, and in all cases the cells were lysed and washed in 5 mM sodium phosphate, pH 7.6, plus 0.5 mM PMSF and 1 µg/ml leupeptin. Phosphorylation of membranes from normal and digested cells was done as described above.

**Protein Kinase C Assay—** Red cells were washed three times in PBS and resuspended to 20% v/v. Half of the cells were incubated for 10 min at 37°C with 1 µM TPA, and half received no treatment but were carried through the incubation. After two additional washes in PBS, the cells were lysed in 20 volumes of ice-cold 5 mM sodium phosphate, pH 7.6, 0.1 mM EGTA. The supernatants were diluted on addition 5-fold in the above buffer to a protein concentration of 2.4 mg/ml. Protein kinase C activity was measured as described by Kikkawa et al. (20). Briefly, 55 µl of cytosol was added to (final concentrations given) 20 mM Tris, pH 7.4, 2.0 mM NaF, 10 mM MgCl₂, 200 µg/ml histone (Sigma type III-S), 20 µg/ml phosphatidylserine, 0.1–0.5 mM PIP₃, 1 µM phosphatidylyserine, and 1 mM CaCl₂ or 1 mM EGTA. The lipids were added as a sonicated suspension in 20 mM Tris, pH 7.4. The reaction was allowed to proceed for 10 min at 30°C, after which the mixtures were chilled on ice. Histone-bound ³²P was determined by spotting samples onto phosphocellulose paper which was subsequently washed in water and acetone and counted by liquid scintillation as described by Witt and Roskoski (21).

**FIG. 1. Effect of TPA on the metabolic labeling of red cell membrane proteins by ³²PO₄.** Intact red cells were preincubated with ³²PO₄ as described under "Methods" for 18 h. After incubation, 10 µM [γ-³²P]ATP (1–2 x 10⁶ cpm) was added and the cells were washed, and ghosts were prepared, electrophoresed, and autoradiographed as described under "Methods." The labeled bands were identified by overlaying the autoradiogram on the Coomassie Blue-stained gel (lane 9). G3PD, glyceraldehyde 3-phosphate dehydrogenase.
membrane. To distinguish between these two possibilities, we prepared cytosol-free unsealed ghosts from normal and TPA-treated red cells and examined protein phosphorylation by adding \( \gamma^3\mathrm{P}\)ATP. Fig. 2 shows that ghosts from TPA-treated red cells had a higher degree of phosphorylation in bands 4.1, 4.9, and in a pair of \( \sim 100\)-kDa proteins (compare lanes 1 and 2). The 100-kDa proteins had molecular masses of 115 and 110 kDa and are apparently identical to the 100-kDa proteins labeled in intact cells.

To determine whether the enhanced phosphorylation in ghosts from TPA-treated cells was due to the presence of membrane-bound TPA, we added TPA to ghosts made from normal (non-TPA-treated) cells. Fig. 2, lane 5, shows that this addition had no effect on ghost phosphorylation, with the possible exception of an effect on a 50-kDa membrane-associated protein, whose identity is not known. This and the above-cited evidence demonstrates that TPA must be added to the intact cells for it to be effective, and suggests that TPA promotes the association of protein kinase C with the membrane.

Washing ghosts from TPA-treated cells in isotonic saline had no effect on TPA-stimulated phosphorylation (not shown), suggesting that the kinase can be membrane-associated in the intact cell. However, the kinase is not tightly associated with the membrane, since extraction of ghosts from TPA-treated red cells at low ionic strength (which elutes spectrin and actin and produces inside-out vesicles, 18) results in loss of TPA-dependent phosphorylation (compare lanes 3 and 4, Fig. 2). It should be noted that phosphorylation in the spectrin/ankyrin region of the gel of inside-out vesicles is due principally to ankyrin, since spectrin has been largely removed (Fig. 2, lane 7). Also, Fig. 2 shows that the pattern of phosphorylation in the band 3 region of inside-out vesicles is distinctly different from that of ghosts. This difference was observed consistently, and remains unexplained at this time.

The above experiments suggest that protein kinase C becomes membrane-associated when red cells are treated with TPA. Table I supports this idea by showing that cytosolic red cell protein kinase C activity (measured using histones as a substrate) of TPA-pretreated red cells was less than a third of that of non-TPA-treated cells, suggesting a TPA-induced partitioning of the enzyme onto the membrane.

**Table I**

| Cytosolic protein kinase C activity | pmol/min/mg protein |
|------------------------------------|---------------------|
| Control cells                      | 2.5 ± 0.2           |
| TPA-pretreated cells               | 0.8 ± 0.1           |

Association of Phosphorylated Proteins with the Membrane Skeleton—To further characterize the proteins phosphorylated by protein kinase C we phosphorylated ghosts from normal or TPA-treated cells with \( \gamma^3\mathrm{P}\)ATP, and extracted the ghosts with either 0.1 M NaOH or Triton X-100. These agents extract complimentary sets of proteins from red cell membranes (24, 25). Extraction with cold 0.1 M NaOH removes all peripheral and membrane skeletal proteins, leaving membranes containing principally band 3 and the glycophorins. Extraction with Triton X-100 solubilizes band 3 and the bulk of the glycophorins, leaving behind a sedimentable membrane skeletal "shell" composed principally of spectrin, band 4.1, band 4.9, and actin. Fig. 3 shows that the proteins whose phosphorylation was stimulated by TPA were all present in the Triton pellet (compare lanes 5 and 7), whereas virtually none were found on NaOH-stripped membranes (lane 6). Whereas nearly all of bands 4.1 and 4.9 are recovered in the Triton shells (compare lanes 5 and 7), only a fraction of the 100-kDa proteins and the 50-kDa protein are retained. Nevertheless, the TPA-stimulated kinase appears to have a specificity for the proteins of the membrane skeleton. These include the proteins in the 100-kDa region, which are clearly unrelated to band 3. Band 3 is retained almost quantitatively in the NaOH-stripped membranes (lane 14) and is absent from Triton shells made in either 0.6 or 1 M KCl (lanes 15 and 16). Comparison with Fig. 2, lane 2, suggests that the 110-kDa protein is more tightly associated with the membrane skeleton than the 115-kDa protein, since it resists complete solution from the membrane skeleton even in 1 M KCl (Fig. 3, lane 8).

**Effect of Extracellular Proteases on Protein Kinase C Activity**—In an attempt to characterize further the proteins phosphorylated by protein kinase C, we subjected intact red cells to digestion with chymotrypsin, trypsin, and neuraminidase. Under the conditions used, chymotrypsin cleaves band 3 at the extracellular surface producing \( \sim 55\) and \( \sim 38\)-kDa membrane-associated fragments (26, 27). Extracellular trypsin on the other hand acts only on glycophorins A and C, cleaving them into numerous fragments, but has no effect on band 3 (28, 29). Neuraminidase also acts only on glycoporphin, cleaving terminal sialic acid residues. Fig. 4 shows that chymotrypsin treatment of intact cells resulted in cleavage of band 3 and generation of a 55-kDa membrane-associated band 3 fragment (lane 5). Comparison of the non-TPA-stimulated phosphorylation of these ghosts (done by addition of \( \gamma^3\mathrm{P}\)ATP) with undigested ghosts reveals a reduction of the small amount of labeling in the band 3 region (compare lanes 1 and 2). Neuraminidase had no effect on the phosphorylation in the band 3 region (lane 3).

To examine the effect of digestion on TPA-stimulated phosphorylation, we treated red cells with 1 \( \mu \)M TPA for 30
were washed and lysed in the presence of phosphorylated by the addition of [γ-32P]ATP as described under “Methods.” Some of the phosphorylated ghosts were extracted for 5 min in 20 volumes of ice cold 0.1 N NaOH (lanes 2 and 6) or for 15 min in 20 volumes of ice cold 1% Triton X-100 in 5 mM sodium phosphate, pH 7.6, plus either 0.6 mM KCl (lanes 3 and 7) or 1 mM KCl (lanes 4 and 8). In each case the extracted ghosts were centrifuged at 35,000 × g for 20 min in a Sorvall SS 34 rotor, and the pellets were washed once in 5 mM sodium phosphate, pH 7.6, prior to electrophoresis. Lanes 9–16 show Coomassie Blue-stained gels corresponding to autoradiogram lanes 1–8.

**FIG. 3.** Extraction of phosphorylated ghosts with Triton X-100 and NaOH. Ghosts were prepared from either normal red cells (lanes 1–4) or red cells pretreated as described under “Methods” with 1 μM TPA (lanes 5–8) and were phosphorylated by the addition of [γ-32P]ATP as described under “Methods.” Some of the phosphorylated ghosts were extracted for 5 min in 20 volumes of ice cold 0.1 N NaOH (lanes 2 and 6) or for 15 min in 20 volumes of ice cold 1% Triton X-100 in 5 mM sodium phosphate, pH 7.6, plus either 0.6 mM KCl (lanes 3 and 7) or 1 mM KCl (lanes 4 and 8). In each case the extracted ghosts were centrifuged at 35,000 × g for 20 min in a Sorvall SS 34 rotor, and the pellets were washed once in 5 mM sodium phosphate, pH 7.6, prior to electrophoresis. Lanes 9–16 show Coomassie Blue-stained gels corresponding to autoradiogram lanes 1–8.

**Fig. 4. Effect of extracellular digestion on TPA-stimulated phosphorylation in ghosts.** Red blood cells were washed in saline and some (lanes 7–12) were treated with 1 mM TPA as described under “Methods.” Control cells (lanes 1–6) received no TPA. Some of the control cells were treated with chymotrypsin (Ch, lanes 2 and 5) or neuraminidase (N, lanes 3 and 6) prior to lysis as described under “Methods.” Some of the TPA-treated cells were also treated with chymotrypsin (lanes 8 and 11) or neuraminidase (lanes 9 and 12) subsequent to TPA treatment. The cells were washed and lysed in the presence of protease inhibitors, and ghosts were phosphorylated by the addition of [γ-32P]ATP as described under “Methods.”

Min, digested them with either chymotrypsin or neuraminidase, then phosphorylated the ghosts from these cells by adding [γ-32P]ATP. Fig. 4, lane 7 shows the normal pattern of TPA-stimulated ghost phosphorylation. Lanes 8 and 9 show that TPA-stimulated phosphorylation in ghosts from the digested cells was considerably reduced relative to the undigested cells. This was true of the proteins in the 100-kDa region, band 4.1 as well as band 4.9. (It should be noted that depending upon the gel and how long it was run, the ~100 kDa proteins often appeared as a single broad band as in Fig. 4.) Qualitatively similar results were obtained if the intact cells were digested prior to addition of TPA, rather than after.

The reduction of phosphorylation in the membranes from digested cells could not have been due to proteolysis of the substrates because the Coomassie Blue-stained gels show that the external chymotrypsin treatment did not attack band 4.1 (Fig. 4, lane 11) and the neuraminidase treatment had no effect upon either band 3 or 4.1 staining (Fig. 4, lane 12). To demonstrate further that residual amounts of the enzymes, which might have remained after washing the intact cells and inhibition with diisopropyl fluorophosphate, were not digests band 4.1, we prepared electroblots of gels identical to those in Fig. 4. Staining the electroblots with antisera to band 4.1 showed that there was no diminution in band 4.1 content, and no generation of new bands in the electroblots of digested cells (data not shown).

The effect of enzymatic digestion was studied further by examining TPA-stimulated phosphorylation of proteins in intact red cells metabolically labeled by incubation in 32PO4. Fig. 5 shows that extracellular digestion by trypsin, chymotrypsin and neuraminidase significantly reduced TPA-stimulated phosphorylation of band 4.1 in intact cells (compare lanes 2, 3, and 4 with lane 1). The enzymes had little or no effect on the labeling in intact red cells of proteins in the 100-kDa region by contrast with their effect when membranes were labeled by addition of [32P]ATP to ghosts (Fig. 4). As expected, there is some diminution of labeling in the band 3 region just below the 100-kDa proteins upon chymotryptic digestion. The enzymes also affect the 96-kDa protein which migrates just above band 4.1 in Fig. 5, lane 1.

We propose that the proteolytic enzymes were acting by blocking the action of protein kinase C rather than by attack-
ing the targets of the enzyme. If this is true, then it would be expected that digestion of intact cells equilibrated with $^{32}$PO$_4$ to TPA-induced phosphorylation, rather than before, should have no effect on stimulated phosphorylation of band 4.1. Fig. 5 shows that this is the case (compare lanes 5, 6, and 7 with lanes 2, 3, and 4). The lack of effect of enzymes added after TPA stimulation demonstrates that their inhibitory effect cannot be due to proteolysis of intracellular components by residual enzymes subsequent to lysis.

The above experiments show that digestion of intact red cells with several enzymes which principally affect transmembrane proteins has an inhibitory effect on the action of protein kinase C. The inhibitory effect is seen whether phosphorylation is done by metabolic labeling of intact cells or by addition of $[^{32}$P]ATP to ghosts, although phosphorylation of proteins in the ~100-kDa region is inhibited more in the latter case.

Ca$^{2+}$-dependent Phosphorylation of Membrane Skeletal Proteins—To further investigate the phosphorylation of membrane skeletal proteins, we tested the effect of Ca$^{2+}$ on ghost protein phosphorylation. We used ghosts in all of our Ca$^{2+}$ experiments to ensure access of Ca$^{2+}$ to the cytoplasmic membrane surface. All of our incubations contained leupeptin to inhibit Ca$^{2+}$-activated proteases. Fig. 6A shows that addition of 10 mM Ca$^{2+}$ to ghosts in the presence of $[^{32}$P]ATP resulted in an enhanced phosphorylation of several proteins in the 100-kDa region, as well as of band 4.1 (compare lanes 1 and 2) and the 50-kDa protein. By contrast to the effect of TPA, only a variable and slight enhancement in the phosphorylation of band 4.9 was found. Ca$^{2+}$-dependent phosphorylation of band 4.1 was also observed in inside-out vesicles (compare lanes 4 and 5) but inside-out vesicles showed a different (and unexplained) pattern of phosphorylation in the band 3 region than did ghosts. It appeared that addition of Ca$^{2+}$ to inside-out vesicles enhanced the phosphorylation of a protein which is likely to be band 3, judging by its appearance in the autoradiogram. It was not possible to determine whether Ca$^{2+}$ enhanced the phosphorylation of the ~100-kDa proteins in inside-out vesicles because of the heavy band 3 phosphorylation.

Careful comparison of the ghost proteins whose phosphorylation was enhanced by Ca$^{2+}$ with those whose phosphorylation was enhanced by TPA indicated that the 110-kDa TPA-enhanced protein is affected by Ca$^{2+}$. The 115-kDa protein is also affected, but to a lesser extent. In addition, Ca$^{2+}$-stimulated phosphorylation was observed in proteins of approximately 95, 100 and 150 kDa, which were not noticeably affected by TPA. Fig. 7 shows that the Ca$^{2+}$ dependence of phosphorylation of the ~100-kDa proteins and band 4.1 in ghosts showed a striking dissimilarity. The former was high at 10 mM Ca$^{2+}$ and decreased with added Ca$^{2+}$, whereas the
Phosphorylation—We investigated whether the Ca\(^{2+}\)-dependent
enous calmodulin from red cell membranes (30, 31). These
ence of 0.1 mM EDTA, a condition known to remove endog-
more closely to that of the 100-kDa proteins
proteins was significantly reduced by trifluoperazine (TFP, (lane 3)) and band 4.1 were excised and were analyzed for 32P
content by liquid scintillation counting. Lanes 7–12, Coomassie Blue-stained gel lanes corresponding to autoradiograph, lanes 1–6.

latter increased almost monotonically with added Ca\(^{2+}\). Also, the phosphorylation pattern of the 50-kDa protein appeared to correspond more closely to that of the 100-kDa proteins than to that of band 4.1.

To determine whether calmodulin was involved in the Ca\(^{2+}\)-dependent phosphorylation, we prepared ghosts in the presence of 0.1 mM EDTA, a condition known to remove endog-
ous calmodulin from red cell membranes (30, 31). These ghosts have a normal band 4.1 content (not shown). Fig. 6A (lane 3) shows that such ghosts lacked Ca\(^{2+}\)-stimulated phos-
phorylation of band 4.1 and of the 100-kDa proteins (although the 50-kDa protein still showed enhanced phosphorylation relative to the Ca\(^{2+}\)-free control). Further evidence for the involvement of calmodulin is given by the fact that the Ca\(^{2+}\)-stimulated phosphorylation of band 4.1 and the 100-kDa proteins was significantly reduced by trifluoperazine (TFP, Fig. 6B), which is known to inhibit calmodulin-dependent reactions (32). Paradoxically, phosphorylation of the 50-kDa protein was also reduced by TFP (see “Discussion”).

Effect of Extracellular Proteases on Ca\(^{2+}\)-dependent Phosphorylation—We investigated whether the Ca\(^{2+}\)-dependent phosphorylation reaction had the same sensitivity to extra-

cellular proteases as the TPA-dependent one. Pretreatment of intact cells with chymotrypsin had no effect on the Ca\(^{2+}\)-stimulated phosphorylation of the 100-kDa proteins or of band 4.1 (compare lanes 2 and 3, Fig. 8). This shows that the Ca\(^{2+}\)-dependent phosphorylation reaction is distinct from the TPA-dependent one and is not due to Ca\(^{2+}\) activation of protein kinase C. Fig. 8 also shows that the phosphorylated ~100-kDa proteins are unrelated to band 3, since band 3 is completely eliminated by chymotrypsin digestion (Fig. 8, lane 8), whereas the phosphorylated ~100-kDa proteins are unaf-
fected (lane 3). The 100-kDa proteins phosphorylated in the presence of Ca\(^{2+}\) are largely extracted from ghosts by NaOH (Fig. 8, lane 3) but are present in Triton-prepared membrane
skeletons (Fig. 8, lane 4). Since the ~100-kDa substrates of protein kinase C have a similar behavior, these likely repre-
sent the same membrane-skeleton-associated proteins.

Stoichiometry of Phosphorylation—We have done prelimi-
nary studies to determine the stoichiometry of band 4.1 phos-
phorylation by the Ca\(^{2+}\)- and phorbol ester-stimulated ki-

We have described characteristics of two reactions responsible for phosphorylating membrane skeletal proteins in the human red blood cell. The reaction stimulated by TPA is almost certainly due to red blood cell protein kinase C. In fact, using a standard assay for protein kinase C activity, we have found Ca\(^{2+}\)- and phospholipid-dependent kinase activity in red cell cytosol, as well as in Triton lysates of whole cells (not shown). Our finding that the cytosol of TPA-treated red cells contains less soluble kinase C activity than that of control cells is consistent with a movement or redistribution of the enzyme onto the membrane after stimulation. This hypothesis is supported by the lack of effect of TPA added to ghosts after lysis, suggesting that the enzyme was lost during hemolysis.

The translocation of protein kinase C from the cytosol to membrane fractions has been reported in numerous cell types and occurs in response to TPA (33), thyrotropin-releasing hormone (34), gonadotropin-releasing hormone (35) as well as interleukin-2 and -3 (36, 37). The demonstration of this phenomenon in a cell as simple as the red cell may facilitate the understanding of the significance of this translocation and may lead to the identification of the membrane-binding site for protein kinase C. Our studies show that the major substrates for red cell protein kinase C are all membrane skeletal proteins. Band 4.1 and band 4.9 are known to be membrane skeletal proteins, and we have shown here that two proteins in the 100-kDa region also behave like membrane skeletal proteins and are distinct from band 3, with which they co-migrate on SDS gels.

The identity and function of the 100-kDa proteins remain to be established. Although our digestion and extraction studies rule out the possibility that they are either band 3 or glycoporphin, these latter proteins are phosphorylated during metabolic labeling of intact cells (38–40), and band 3 but not glycoporphin is labeled in ghosts upon addition of [\(\gamma\)-32P]ATP (40–42). Others have also noted the presence of phosphoproteins in the 100-kDa region which are distinct from either glycoporphin or band 3 (38, 39, 41), and rabbit erythrocytes have been reported to contain a 100-kDa protein whose phos-
phorylation was stimulated by TPA (15). In human erythro-
cytes, Johnson et al. (39) described two phosphorylated com-
ponents of molecular mass 105 and 110 kDa which were found with the Triton cytoskeletal fraction, and which were unaf-
fected by protease digestion of intact cells. They showed that

**DISCUSSION**

**Phosphorylation**—We have investigated whether the Ca\(^{2+}\)-dependent phosphorylation reaction had the same sensitivity to extra-
cellular proteases as the TPA-dependent one. Pretreatment of intact cells with chymotrypsin had no effect on the Ca\(^{2+}\)-stimulated phosphorylation of the 100-kDa proteins or of band 4.1 (compare lanes 2 and 3, Fig. 8). This shows that the Ca\(^{2+}\)-dependent phosphorylation reaction is distinct from the TPA-dependent one and is not due to Ca\(^{2+}\) activation of protein kinase C. Fig. 8 also shows that the phosphorylated ~100-kDa proteins are unrelated to band 3, since band 3 is completely eliminated by chymotrypsin digestion (Fig. 8, lane 8), whereas the phosphorylated ~100-kDa proteins are unaffected (lane 3). The 100-kDa proteins phosphorylated in the presence of Ca\(^{2+}\) are largely extracted from ghosts by NaOH (Fig. 8, lane 3) but are present in Triton-prepared membrane

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**DISCUSSION**

We have described characteristics of two reactions responsible for phosphorylating membrane skeletal proteins in the human red blood cell. The reaction stimulated by TPA is almost certainly due to red blood cell protein kinase C. In fact, using a standard assay for protein kinase C activity, we have found Ca\(^{2+}\)- and phospholipid-dependent kinase activity in red cell cytosol, as well as in Triton lysates of whole cells (not shown). Our finding that the cytosol of TPA-treated red cells contains less soluble kinase C activity than that of control cells is consistent with a movement or redistribution of the enzyme onto the membrane after stimulation. This hypothesis is supported by the lack of effect of TPA added to ghosts after lysis, suggesting that the enzyme was lost during hemolysis.

The translocation of protein kinase C from the cytosol to membrane fractions has been reported in numerous cell types and occurs in response to TPA (33), thyrotropin-releasing hormone (34), gonadotropin-releasing hormone (35) as well as interleukin-2 and -3 (36, 37). The demonstration of this phenomenon in a cell as simple as the red cell may facilitate the understanding of the significance of this translocation and may lead to the identification of the membrane-binding site for protein kinase C.

Our studies show that the major substrates for red cell protein kinase C are all membrane skeletal proteins. Band 4.1 and band 4.9 are known to be membrane skeletal proteins, and we have shown here that two proteins in the 100-kDa region also behave like membrane skeletal proteins and are distinct from band 3, with which they co-migrate on SDS gels.

The identity and function of the 100-kDa proteins remain to be established. Although our digestion and extraction studies rule out the possibility that they are either band 3 or glycoporphin, these latter proteins are phosphorylated during metabolic labeling of intact cells (38–40), and band 3 but not glycoporphin is labeled in ghosts upon addition of [\(\gamma\)-32P]ATP (40–42). Others have also noted the presence of phosphoproteins in the 100-kDa region which are distinct from either glycoporphin or band 3 (38, 39, 41), and rabbit erythrocytes have been reported to contain a 100-kDa protein whose phos-
phorylation was stimulated by TPA (15). In human erythro-
cytes, Johnson et al. (39) described two phosphorylated com-
ponents of molecular mass 105 and 110 kDa which were found with the Triton cytoskeletal fraction, and which were unaf-
fected by protease digestion of intact cells. They showed that
proteins in the 100-kDa region must be heavily phosphorylated after Triton X-100 extraction or extracellular protease treatment, was minimal. was found in our studies, Johnson et al. (39) noted that the proteins in the 100-kDa region must be heavily phosphorylated, since the amount of protein staining in that area, particularly after Triton X-100 extraction or extracellular protease treatment, was minimal. At this time we do not know the identity of the 50-kDa membrane-associated protein phosphorylated by [γ-32P]ATP in ghosts. We never observed this phosphoprotein when phosphorylation was done by metabolic labeling. The effect of TPA on 50-kDa phosphorylation was variable (compare Fig. 2, lanes 1 and 2, where no effect is seen and Fig. 3, lanes 1 and 5, where some stimulation is observed). The 50-kDa protein seems to be a substrate for the Ca2+-dependent kinase (Figs. 6A and 7). However, whereas Ca2+-dependent phosphorylation of this protein could be reduced by the calmodulin inhibitor TFP (Fig. 6B), removal of calmodulin from ghosts by a wash in an EGTA-containing buffer did not eliminate its Ca2+-dependent phosphorylation (Fig. 6A). More quantitative analysis of our gels by counting 32P in the 50-kDa band may be needed to resolve this apparent inconsistency. Small amounts of the 50-kDa protein remained with high-salt extracted red cell Triton shells (Fig. 3, lanes 7 and 8) so that it, like the 100-kDa proteins, may represent an as yet unidentified membrane-skeletal component.

The Ca2+-stimulated kinase which acts on band 4.1 and several proteins in the molecular mass range of 100 kDa and higher acts weakly or not at all on band 4.9. Also, the Ca2+-dependence of phosphorylation of band 4.1 and the ~100-kDa proteins is markedly different. It is possible that two separate Ca2+-dependent kinases are acting on the proteins, or that, in addition to activating a kinase, the Ca2+ is having a secondary effect on, for example, the conformation of one or several of the proteins.

The Ca2+-activated phosphorylation was done in the presence of leupeptin to inhibit the action of Ca2+-dependent proteases. Band 4.1, which is very susceptible to proteolysis, appears to be undegraded under these conditions, and in any case the phosphorylation of band 4.1 becomes more intense as Ca2+ is added. The gels do reveal a dependence on Ca2+ of staining in the band 3 region (Fig. 7), which may be related to the change in phosphorylation in this region above 10 μM Ca2+. However, this correlation must be explored further, since Coomassie Blue staining in the band 3 region can be fully eliminated by enzymatic digestion of red cells without affecting Ca2+-dependent phosphorylation of the 100-kDa proteins in ghosts.

A role for calmodulin in the Ca2+-dependent phosphorylation is suggested by the loss of Ca2+-stimulated phosphorylation when ghosts were washed with EGTA, a treatment which is known to elute membrane-associated calmodulin (30, 31), and by the inhibition of phosphorylation by trifluoperazine. It is interesting to note that half-maximal inhibition of red cell membrane Ca2+-calmodulin-dependent ATPase occurs at approximately 40 μM trifluoperazine, with nearly complete inhibition at 150 μM (43). We found substantial inhibition of Ca2+-dependent band 4.1 phosphorylation between 25 and 100 μM trifluoperazine, suggesting a similar target of this agent in these two phenomena.

The phosphorylation of band 4.1 by a Ca2+ (and probably calmodulin-) dependent protein kinase is interesting in light of the recently reported similarity of band 4.1 to the neural protein synapsin (44), which is one of the major substrates for brain Ca2+-calmodulin-dependent protein kinases (45). Also, the action of the Ca2+-dependent kinase on the ~100-kDa proteins is interesting in light of previous work demonstrating enhanced phosphorylation in this region of SDS gels in sickle cells, or red cells loaded with Ca2+ by the ionophore A23187 (46, 47). Our results suggest the possibility that the enhanced phosphorylation was not in band 3 but in the 100-kDa membrane-skeleton-associated proteins, and possibly in band 4.1 as well. The significance of enhanced phosphorylation of these proteins for the properties of the sickle cell membrane skeleton is a matter for future investigation.

It has been reported that protein kinase C in some cells can be activated by Ca2+-dependent proteolysis (20, 48). We must therefore consider the possibility that the effect of Ca2+ in these studies was to activate protein kinase C. There are,
however, three important distinctions between the Ca\(^{2+}\)-dependent reaction and that of protein kinase C. 1) The Ca\(^{2+}\)-dependent reaction has a different specificity than the TPA-dependent one in that it affects band 4.9 little or not at all. By contrast, the TPA-dependent enzyme strongly affects band 4.9, particularly during phosphorylation of ghosts. 2) Pretreatment of intact red cells with chymotrypsin has no effect upon the subsequent ability of Ca\(^{2+}\) to promote phosphorylation of band 4.1 and the 100-kDa proteins. Such treatment greatly reduced or eliminated the action of protein kinase C. 3) Finally, our studies showed that ghosts prepared from non-TPA-treated red cells probably do not contain protein kinase C, since it cannot be stimulated by TPA added to the ghosts. Thus, it seems unlikely that the Ca\(^{2+}\)-stimulated phosphorylation in ghosts could be due to protein kinase C.

At this time, the inhibitory action of extracellular proteases or neuraminidase on the action of protein kinase C remains unexplained. One possibility is that the enzymes affect the ability of protein kinase C to associate with the cell membrane. Extracellular cleavage of band 3 and glycophorin by chymotrypsin and trypsin, respectively, may induce perturbations of the cytoplasmic aspect of the membrane and affect the as yet unidentified binding site for protein kinase C. It is less clear how neuraminidase would have such an effect, but it is not unreasonable to suggest that removal of a high proportion of the charged sialic acid residues from glycophorin would affect its conformation or associations with other proteins.

If extracellular digestion prevented or reversed the association of protein kinase C with the membrane, we would expect that the level of cytosolic protein kinase C, measured as in Table I, would be greater in TPA-treated cells which were digested than in those which were not digested. Experiments done to address this issue (not shown) demonstrated that digestion with chymotrypsin either prior or subsequent to TPA addition had no effect on cytosolic protein kinase C levels, suggesting that digestion does not affect TPA-induced protein kinase C translocation from cytosol to membrane.

A second possibility is that the extracellular enzymes perturb the proteins of the membrane skeleton so that they are no longer susceptible to phosphorylation by membrane-bound protein kinase C. There are numerous precedents for agents which bind to or perturb integral membrane proteins affecting the proteins of the membrane skeleton (49–51). Also, recent studies have established a direct association of band 4.1 with the cytoplasmic domains of band 3 and glycophorin (52, 53). In light of our findings, it is possible that agents which affect red cell shape or mechanical properties via their association with glycophorin or band 3 do so by affecting the activity of protein kinase C.

Band 4.1 in human red cells is known to be phosphorylated by a membrane-associated cAMP-dependent kinase (12). In addition, purified band 4.1 preparations apparently contain a kinase which phosphorylates band 4.1 (54). This latter kinase is independent of Ca\(^{2+}\) and cAMP, and also acts on a variety of substrates including histones.\(^{5}\) With the description of TPA- and Ca\(^{2+}\)-dependent kinases which act on band 4.1, it now seems that band 4.1 is a substrate for four distinct kinases in the red cell. We have already shown that the ability of band 4.1 to promote the binding of spectrin to F-actin is sensitive to the overall degree of band 4.1 phosphorylation (11). Our evidence suggests that the dephosphorylated protein is more effective at promoting spectrin/actin binding than the phosphorylated form. In light of the present findings, additional work is necessary to establish which of the kinases that

\(^{5}\) C. M. Cohen and R. C. Langley, unpublished data.
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