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Erythrocyte membrane skeleton phosphoproteins: identification of two unrelated phosphoproteins in band 4.9

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Human erythrocyte membrane band 4.9 is phosphorylated by several erythrocyte protein kinases. Chromatography of erythrocyte membrane skeleton proteins on DEAE-Sephacel produces two proteins with relative mobilities, on gel electrophoresis, similar to that of band 4.9. The first, with a molecular mass of 49 kDa, is quite basic (p*I* > 8) while the second, 50.5 kDa, is slightly acidic (p*I* = 6.2). Comparative two-dimensional peptide mapping reveals that both proteins are present in band 4.9 on one-dimensional gels of total erythrocyte membrane proteins and membrane skeleton proteins. The 49 kDa protein, but not the 50.5 kDa protein, binds to actin filaments in a sedimentation assay. In intact erythrocytes metabolically labeleld with [^{32}P]orthophosphate, the 49 kDa protein is phosphorylated by protein kinase C, cAMP-dependent protein kinase, and protein kinases which are active in the absence of exogenous kinase activators. In contrast, the 50.5 kDa protein is phosphorylated by protein kinase C but not by the other protein kinases examined. Finally, two-dimensional peptide mapping was employed to compare the 49 kDa protein and a 57 kDa protein which copurifies with, and has many characteristics of, the 49 kDa protein. Significant similarities were found in both ^{125}I-labeled chymotryptic peptide maps and ^{32}P-labeled tryptic peptide maps, suggesting that the 49 kDa and 57 kDa proteins are closely related.

Introduction

All of the major components of the erythrocyte membrane skeleton, except actin, are reversibly phosphorylated by one or more protein kinases, suggesting an important role for phosphorylation in the regulation of erythrocyte membrane properties. Band 4.9 (using Steck's nomenclature [1]), which is consistently present in membrane skeletons prepared under a variety of conditions [2], is phosphorylated by several erythrocyte protein kinases [3–13]. Earlier, we reported that protein kinase C and cAMP-dependent protein kinase phosphorylate different sites in band 4.9 [8]. During subsequent investigation of the phosphorylation of band 4.9, we have separated two proteins with apparent molecular masses similar to band 4.9. Both are present in band 4.9 on one-dimensional gels of whole erythrocyte membranes and membrane skeletons, and both are phosphorylated by protein kinase C. We report here the partial characterization of the two band 4.9 proteins, comparing their interactions with membrane skeleton proteins as well as their phosphorylation by erythrocyte protein kinases. In addition, we

Abbreviations: SDS, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate, Bt_2cAMP, N^6,2′-*O*-dibutyryl cyclic AMP; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid.

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show that one of the proteins is related to a larger phosphoprotein which is also associated with the membrane skeleton. Part of these findings have appeared in abstract form [14].

Materials and Methods

Protein isolation. Proteins from the band 4.9 region were isolated using a method derived from that of Siegel and Branton [7]. Erythrocyte membranes were prepared from fresh human erythrocytes by the method of Dodge et al. [15]. Membrane skeletons were prepared by extracting the ghosts in 1% Triton X-100, 5 mM sodium phosphate, 1 mM EGTA, 2 mM diisopropyl fluorophosphate (DFP) (pH 8.0) for 30 min on ice, and centrifuging for 20 min at 25000 × *g* (4°C). The resulting Triton-insoluble pellet was washed once in the extraction buffer (DFP reduced to 0.1 mM) and twice in the extraction buffer without the detergent (reduced DFP). The final pellet was resuspended in 10 vol. of 0.1 mM EDTA, 0.1 mM DFP (pH 9.4) and incubated at 37°C for 45 min to solubilize the membrane skeleton proteins. After centrifuging for 30 min at 113000 × *g* (4°C) to remove residual insoluble material, the protein solution was brought to 20 mM Tris, 0.5 mM 2-mercaptoethanol (pH 8.3) by the addition of a 10-fold concentrated stock solution, and was loaded onto a 1.6 cm × 90 cm column of DEAE-Sephacel equilibrated with 20 mM KCl, 20 mM Tris, 1 mM EGTA, 0.5 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride (pH 8.3). The bound proteins were eluted with a 500 ml linear gradient of 20–300 mM KCl in the column buffer, and 5.5 ml fractions were collected. Aliquots of the fractions were subjected to SDS-gel electrophoresis and the fractions containing proteins whose relative mobilities were similar to that of band 4.9 were pooled and concentrated by vacuum dialysis against 130 mM KCl, 20 mM NaCl, 10 mM Tris, 1 mM EGTA, 0.5 mM 2-mercaptoethanol, 0.2 mM NaN_3 and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4) (buffer A).

To remove contaminating spectrin and adducin, the concentrated proteins were subjected to rate zonal centrifugation on 5–20% sucrose gradients in buffer A for 24 h at 200000 × *g* (4°C) using an SW 40Ti rotor. Human erythrocyte spectrin and protein 4.1, and rabbit muscle actin were prepared as previously described [16].

Preparation of ^{32}P-labeled proteins. Fresh erythrocytes (2 ml; hematocrit = 50%) were metabolically labeled with [^{32}P]orthophosphate as previously described [8], except that 2 mM dibutyryl cyclic AMP (Bt_2cAMP) or 1 μM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were added simultaneously with the [^{32}P]orthophosphate in order to maximize the relative incorporation of ^{32}P into the sites phosphorylated by the stimulated protein kinases.

Membrane skeleton proteins were prepared as described above and chromatographed on 0.5 cm × 18 cm columns of DEAE-Sephacel. Eluted peak 1 and peak 2 proteins were pooled together, lyophilized and subjected to two-dimensional non-equilibrium pH gradient/SDS-gel electrophoresis.

Gel electrophoresis. Isoelectric focusing and non-equilibrium pH gradient electrophoresis were performed according to the methods of O'Farrell and co-workers [17,18] using 3.8% acrylamide gels containing 2% pH 3–10, 2% pH 4–6, and 2% pH 5–7 ampholytes (LKB).

Non-equilibrium pH gradient electrophoresis was continued until the cytochrome *c* included in samples as a marker reached the bottom of the gel. SDS-gel electrophoresis was performed according to Laemmli [19].

Two-dimensional peptide mapping. Phosphopeptide mapping of ^{32}P-labeled proteins on Kodak cellulose sheets was performed as previously described [8], except that the solvent used for chromatography was *n*-butanol/pyridine/water/acetic acid (6:5:4.5:1) containing 7% 2,5-diaphenyloxazole (w/w). ^{125}I-peptide mapping was performed as described by Speicher et al. [20], using cellulose sheets from EM Science.

Sedimentation assays. All sedimentation experiments were carried out as previously described [16] in isotonic buffer containing 130 mM KCl, 20 mM NaCl, 10 mM Tris, 1 mM EGTA, 2 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 0.2 mM ATP (pH 7.4). Proteins were mixed as described in Results in a final volume of 110 μl and incubated at room temperature for 30 min and then on ice for 60 min. The sample (100 μl) was layered over a 200 μl cushion of 10% sucrose in the same buffer

and centrifuged for 30 min at 150000 × *g* (4°C) in a Beckman SW 50.1 rotor. Equivalent portions of supernatants and pellets were analyzed by SDS-gel electorphoresis.

Materials. Bt_2cAMP and TPA were purchased from Sigma. L-1-Tosylamido-2-phenylethylchloromethylketone-treated trypsin and α-chymotrypsin were purchased from Cooper Biomedical. Carrier-free [^{32}P]orthophosphate in HCl-free aqueous solution (98 mCi/ml) was purchased from Amersham. All other chemicals were of reagent grade or better.

Results

Identification of two approx. 49 kDa membrane skeleton proteins

Membrane skeleton proteins were isolated and chromatographed on DEAE-Sephacel as described in Materials and Methods. Aliquots of fractions were analyzed by SDS-gel electrophoresis. The conductivity of the fractions was measured and the KCl concentration was determined by comparison to standard solutions of known KCl concentration in column buffer. The gels showed that proteins with a relative mobility similar to band 4.9 eluted in two populations (Fig. 1). The first peak eluted in 75–150 mM KCl. The second eluted between 180 and 220 mM KCl. Other proteins present in these fractions included trace amounts of spectrin, adducin, which preceded and overlapped the second peak of approx. 49 kDa and two proteins with apparent molecular masses of between 55 and 65 kDa which eluted with the first peak of approx. 49 kDa. The larger of these two 55–65 kDa proteins largely preceded the 49 kDa peak, and careful selection of fractions reduced its presence in the pooled first peak to a minimum. In contrast, the smaller of these proteins, eluted completely within the peak of the 49 kDa protein. Band 6 eluted well ahead of the first peak.

The apparent molecular masses of the proteins in the two peaks were determined by SDS-gel electrophoresis, using high- and low-molecular-weight standards from Pharmacia. The molecular masses of the two proteins in the first peak were 49 kDa and 57 kDa, while that of the protein in the second peak was 50.5 kDa.

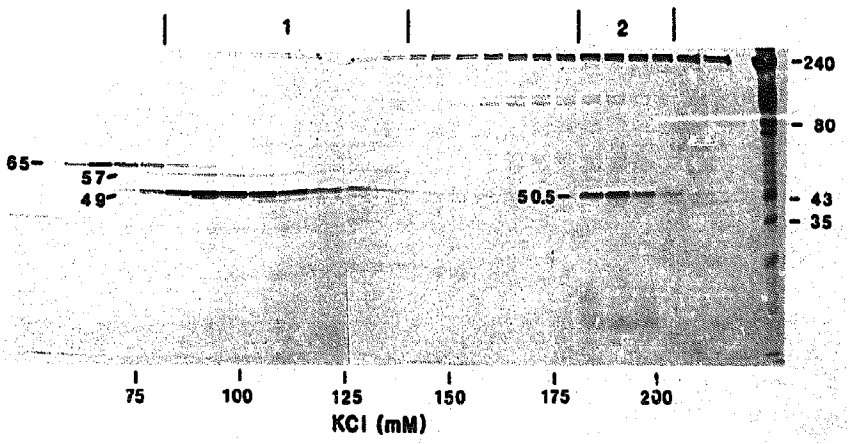

Fig. 1. Separation of two membrane skeleton proteins of approx. 49 kDa. Membrane skeleton proteins were prepared from 200 ml of fresh blood and separated by ion-exchange chromatography on a 1.6 cm × 90 cm column of DEAE-Sephacel equilibrated with 20 mM KCl, 20 mM Tris, 0.5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (pH 8.3) using a 500 ml linear gradient of 20–300 mM KCl. Aliquots of the fractions, as well as total erythrocyte membrane proteins (lane farthest right) were subjected to electrophoresis on 10% polyacrylamide gels with 3.75% stacking gels and the gels stained with Coomassie blue. The KCl concentrations of the fractions were determined by measuring the conductivity of the solution and comparing the values with a standard curve of solutions of known KCl concentration in the column buffer. Molecular masses in kDa are given on the right. The fractions pooled for peak 1 and peak 2 are indicated, as are the positions of the 49 kDa, 50.5 kDa, 57 kDa, and 65 kDa proteins.

The isoelectric points of the proteins were markedly different. When analyzed by standard two-dimensional isoelectric focusing/SDS-gel electrophoresis, with isoelectric focusing proceeding from the basic end of the gel, the 50.5 kDa protein (peak 2) focused at a pH of 6.2, while neither of the proteins in peak 1 entered the gel.

Reversing the direction of isoelectric focusing led to the migration of the peak 1 proteins to the extreme basic end of the gel and a partial loss of the proteins into the basic electrode solution, indicating a p*I* greater than 8. The most satisfactory resolution of the peak 1 proteins was obtained by non-equilibrium pH gradient electrophoresis. Samples were electrophoresed at 1000 V until cytochrome *c* (p*I* = 10.5–10.8), used as a 'tracking dye', migrated to the basic end of the gel, usually after 2–3 h. Under these conditions, both the 49 kDa and the 57 kDa proteins in peak 1 migrated about 75% of the length of the gel while the 50.5 kDa protein (peak 2) migrated only 20–30%.

The 49 kDa and 50.5 kDa proteins excised from two-dimensional polyacrylamide gels, as well as band 4.9 excised from one-dimensional gels of total ghost proteins, were iodinated and subjected to high-resolution two-dimensional peptide mapping. The results, presented in Fig. 2, showed that the two proteins are structurally unrelated. In addition, comparison of the maps of the purified

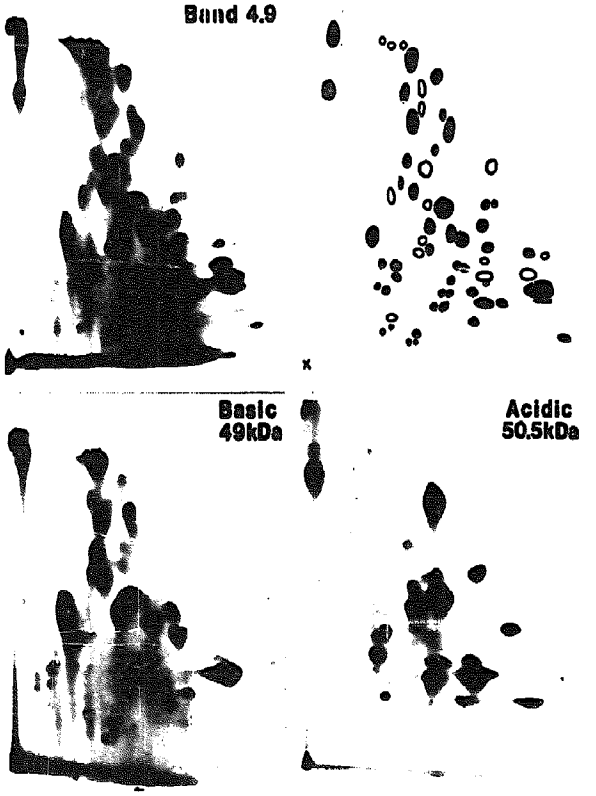

Fig. 2. Autoradiographs of chymotryptic peptide maps of ^{125}I-labeled proteins. Band 4.9 from one-dimensional gels of total erythrocyte membrane proteins and the 49 and 50.5 kDa proteins from two-dimensional non-equilibrium pH gradient/SDS gels were excised, iodinated and mapped as described in the Materials and Methods. Electrophoresis was in the horizontal direction while chromatography was in the vertical direction. The drawing in the upper right panel represents the map of band 4.9 from one-dimensional gels of erythrocyte membrane proteins. Peptides found in the map of the 49 kDa basic protein are represented by the black areas while those found in the map of the 50.5 kDa acidic protein are represented by the stippled areas.

proteins with the map of band 4.9 from ghosts revealed that both proteins were present in that band and together accounted for a majority of the peptides seen in the map of band 4.9.

Protein phosphorylation

Phosphorylation of band 4.9 in intact erythrocytes is stimulated by Bt_2cAMP and TPA, which activate cAMP-dependent protein kinase and protein kinase C, respectively. Experiments were carried out to characterize the phosphorylation of the two band 4.9 proteins by protein kinase C and by cAMP-dependent protein kinase. 2-ml aliquots of erythrocytes (hematocrit = 50%) were metabolically labeled with 2 mCi of [^{32}P]orthophosphate for 2 h at 37°C in the presence of either 2 mM Bt_2cAMP or 1 μM TPA. Control cells were labeled with the same amount of [^{32}P]orthophosphate for 20 h at 37°C. The membrane skeleton proteins were isolated and the band 4.9 proteins were separated from the major membrane skeleton proteins on small DEAE-Sephacel columns as described in Materials and Methods. The fractions containing the 49, 50.5 and 57 kDa protiens were pooled together, dialyzed against 100 mM NH_4HCO_3, lyophilized and subjected to two-dimensional non-equilibrium pH gradient/SDS-gel electrophoresis. The gels were dried and autoradiographed by exposure to Kodak XAR-5 film, using enhancing screens, for 24 h. As seen in Fig. 3, labeling of cells under different conditions led to different patterns of labeling of the band 4.9 proteins. In control cells, the basic 49 kDa and 57 kDa proteins were significantly labeled during the 20-h incubation period. Treatment of the cells with Bt_2cAMP markedly stimulated the incorporation of phosphate into the basic proteins, while the acidic protein was apparently not phosphorylated by the cAMP-dependent protein kinase. On the other hand, activation of protein kinase C led to the phosphorylation of the acidic 50.5 kDa protein as well as the two basic proteins.

The 49 kDa and 50.5 kDa proteins from TPA-treated cells were excised from the gel, digested with trypsin, and the resulting phosphopeptides were analyzed by two-dimensional peptide mapping. The map obtained from the 49 kDa protein (Fig. 4, left panel) was relatively complex, containing several phosphopeptides in a pattern similar to

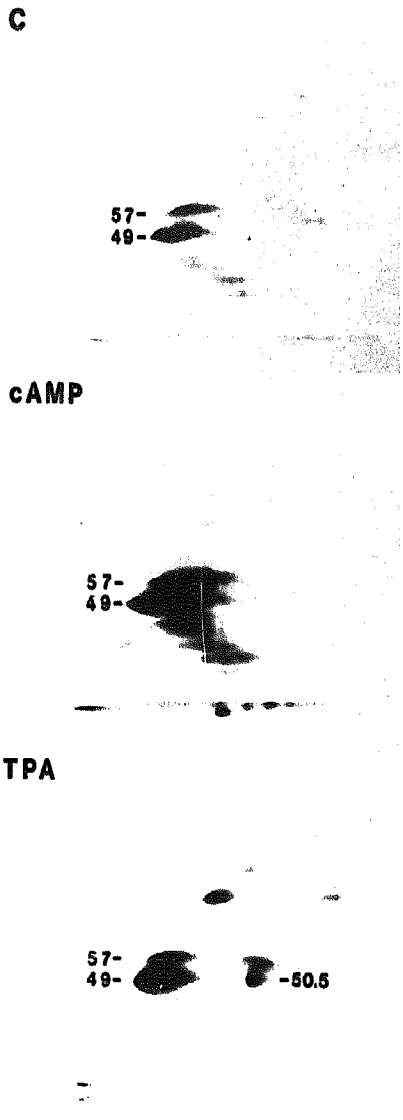

Fig. 3. Autoradiographs of two-dimensional non-equilibrium pH gradient/SDS-polyacrylamide gels of ^{32}P-labeled erythrocyte membrane skeleton proteins. Erythrocytes were labeled with [^{32}P]orthophosphate for 20 h with no added protein kinase activator (C) or for 2 h with 2 mM Bt_2cAMP (cAMP) or 1 μM TPA. Membrane skeleton protein were prepared and separated by two-dimensional non-equilibrium pH gradient/SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The gels were stained, dried and autoradiographed. The positions of the 49, 50.5 and 57 kDa proteins are indicated.

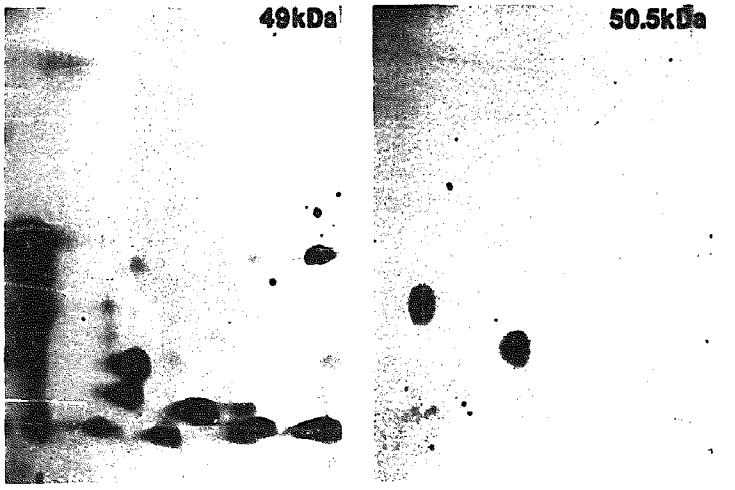

Fig. 4. Autoradiographs of two-dimensional tryptic phosphopeptide maps of the 49 kDa and 50.5 kDa proteins labeled in the presence of TPA. The ^{32}P-labeled 49 kDa and 50.5 kDa proteins from TPA-treated cells were excised from two-dimensional gels (Fig. 3) and mapped using methods described in Materials and Methods. Electrophoresis was in the horizontal direction and chromatography was in the vertical direction.

that previously reported for unfractionated band 4.9 from TPA-treated cells [8]. The map of the phosphopeptides from the 50.5 kDa protein was distinctly different from the map of the 49 kDa protein and contained only two phosphopeptides (Fig. 4, right panel).

Interaction of the band 4.9 proteins with membrane skeleton proteins

The interaction of the proteins with purified components of the membrane skeleton was investigated. The 49 kDa and 50.5 kDa proteins were incubated with muscle actin under polymerizing conditions and the actin filaments separated from soluble material by centrifugation through a layer of 10% sucrose as described previously [16]. The supernatants and pellets were solubilized and analyzed by SDS-gel electrophoresis. The 49 kDa protein and the accompanying 57 kDa protein, but not the 50.5 kDa protein, bound to F-actin (Fig. 5). Addition of spectrin or protein 4.1 to the incubation mixture, singly or together, did not alter the binding of the 49 kDa protein to the sedimented structures, or bring about the binding of the 50.5 kDa protein.

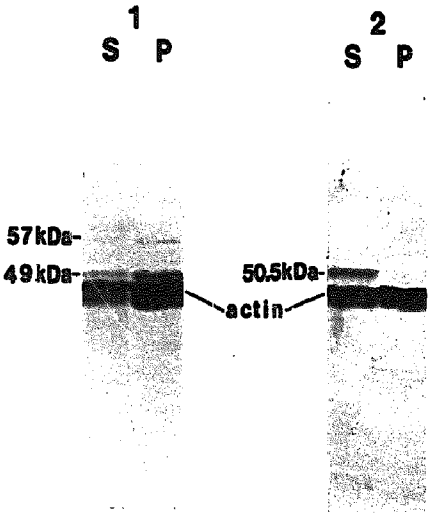

Fig. 5. Interaction of the 49 and 57 kDa protein with F-actin. Partially purified 49 plus 57 kDa and 50.5 kDa proteins were incubated with rabbit skeletal muscle actin under polymerizing conditions. The actin filaments were sedimented through 10% sucrose and the supernatants and pellets were solubilized and electrophoresed on 7–10% gradient SDS-polyacrylamide gels. Panel 1, 49 plus 57 kDa proteins; panel 2, 50.5 kDa proteins.

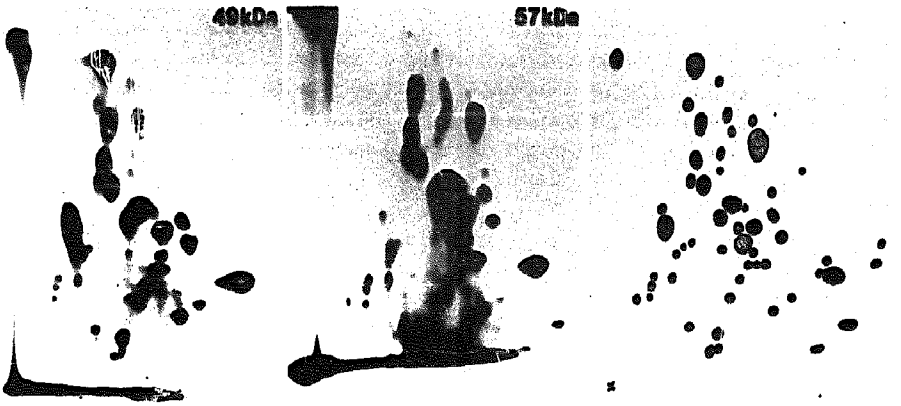

Fig. 6. Autoradiographs of chymotryptic peptide maps of ^{125}I-labeled 49 and 57 kDa proteins. The 49 kDa and 57 kDa proteins from two-dimensional gels were excised, iodinated, and mapped as described in Materials and Methods and shown in Fig. 2. The drawing on the right presents the summation of the maps of the two proteins, with the peptides unique to the 49 kDa protein represented by the cross-hatched areas and those unique to the 57 kDa protein by the stippled areas.

Comparison of the basic 49 and 57 kDa proteins

The behavior of the two proteins in peak 1 was very similar on ion-exchange chromatography and pH gel electrophoresis, as were their phosphorylation by different erythrocyte protein kinases and their ability to bind to F-actin. Therefore, the structural relationship of the two proteins was analyzed by ^{125}I-peptide mapping. The patterns of the two maps showed a high degree of identity (Fig. 6). With the exception of two major peptides from the 49 kDa protein and three or four peptides from the 57 kDa protein, all of the spots were common to the two maps, indicating that the proteins are closely related.

The structural similarity is further endorsed by the two-dimensional maps of tryptic phosphopep-

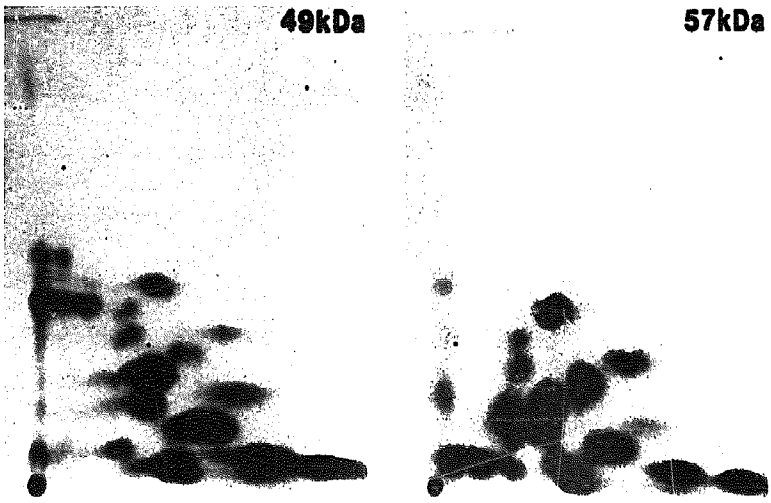

Fig. 7. Autoradiographs of tryptic phosphopeptide maps of the 49 and 57 kDa proteins labeled in the presence of Bt_2cAMP. The ^{32}P-labeled 49 and 57 kDa proteins from Bt_2cAMP-treated cells were excised from two-dimensional gels (Fig. 3) and mapped as described in Fig. 4.

tides obtained from the two proteins following phosphorylation by cAMP-dependent protein kinase (Fig. 7). Nearly all of the phosphopeptides seen in the map of the 49 kDa protein were also present in the map of the 57 kDa protein, although in some cases the relative intensity of the spots differed from one protein to the other.

Discussion

We have found that the erythrocyte membrane skeleton contains two phosphoproteins in the band 4.9 region which can be separated by anion-exchange chromatography. They differ in isoelectric point and, slightly, in apparent molecular mass (49 and 50.5 kDa).

Two-dimensional ^{125}I-peptide mapping indicates that they are not structurally related and that both are present in the band 4.9 observed when erythrocyte membrane proteins are separated on discontinuous SDS-polyacrylamide gels. While both proteins are isolated with the insoluble membrane skeleton when the membrane is extracted with Triton X-100, only the 49 kDa protein binds to F-actin. The absence of binding of the 50.5 kDa protein to actin filaments, even in the presence of spectrin and protein 4.1, indicates that this protein binds to some other component(s) of the membrane skeleton. Identification of the sites to which the 50.5 kDa protein binds awaits further investigation.

The two proteins differ significantly in their phosphorylation in intact cells. The 49 kDa protein is phosphorylated by protein kinase C, cAMP-dependent protein kinase, and protein kinase(s) constitutively active in isolated erythrocytes, while the 50.5 kDa protein is phosphorylated at two sites by protein kinase C, but not by the other protein kinases studied in these experiments. Since the 49 kDa protein is more highly phosphorylated by protein kinase C than is the 50.5 kDa protein, it is the predominant substrate in band 4.9 for all the protein kinases examined in this and in our previous study [8]. The functional effects of these phosphorylation events are not known, although it has been suggested that exposure of erythrocytes to cAMP strengthened the interactions of band 4.9 within the membrane skeleton [7]. If this is true, it could contribute to the mechanism by which β-adrenergic agonists and some prostaglandins, which have been reported to activate erythrocyte adenylate cyclase [22], make the erythrocyte less deformable [23,24].

In their report of the isolation and characterization of an erythrocyte membrane skeleton protein which comigrates with band 4.9 on gel electrophoresis [7], Siegel and Branton gave no indication that a second protein of similar molecular mass was present in their skeleton protein preparations. Comparison of the properties of the 48 kDa protein described by them (co-elution from DEAE-Sephacel with two slightly larger proteins at KCl concentrations less than 170 mM, binding to F-actin, phosphorylation by cAMP-dependent protein kinase) with the results of this study indicates that their 48 kDa protein corresponds to the 49 kDa protein described in this paper. The 50.5 kDa protein has not, to our knowledge, been previously reported, although it could possibly be the tropomyosin-binding protein reported by Fowler [25], which exhibits different mobilities in SDS-gel electrophoresis depending on the buffer conditions. Siegel and Branton also noted similarities in the chromatographic behavior, actin-binding properties, phosphorylation and immunological reactivity of their 48 kDa protein and a 52 kDa protein present in their preparations. Despite the differences in apparent molecular mass, which we cannot explain, the similarities between Siegel's 52 kDa protein and our 57 kDa protein suggest that they are probably the same protein. Here we have confirmed the observations of Siegel and Branton, with the exception of the immunological properties of the two proteins, and obtained further evidence of the relationship of the 57 and the 49 kDa proteins, noting their similar behavior on two-dimensional non-equilibrium pH gradient/SDS-gel electrophoresis and as substrates for protein kinases. Comparative ^{125}I- and ^{32}P-peptide mapping confirmed that the two proteins are closely related. The data obtained are not sufficient to answer the question of whether the 57 kDa protein is a precursor of the 49 kDa protein. Interestingly, Whitfield and colleagues have reported the presence of other proteins in the region between bands 4.2 and 4.9 which are structurally related to one another [26].

The roles of these proteins in erythrocyte membrane function remain to be demonstrated. It is generally accepted that the function of the membrane skeleton is to stabilize the membrane, thus enabling the erythrocyte to withstand the shear stresses encountered during its passage through the microvasculature. Although the formation of the membrane skeleton and its attachment to the membrane can be accounted for by proteins and interactions already described by several investigators (spectrin–spectrin, spectrin–band 4.1–actin, band 4.1–glycophorin, spectrin–ankyrin–band 3) (reviewed in Refs. 27, 28), it is probable that other membrane proteins provide a degree of redundancy within the system or contribute to the regulation of the interaction between various components. Recently described examples of proteins which fill these roles are tropomyosin [29], which is thought to bind to actin filaments and limit the interaction with spectrin to the ends of the filaments [30], and adducin, which promotes the interaction of spectrin and actin [31,32]. The presence within the membrane skeleton of the two band 4.9 proteins described here and the fact that both are substrates for one or more protein kinases suggests that their interactions within the complex are subject to modification as part of the overall regulation of erythrocyte membrane properties. Determination of the specific functions of these proteins and their significance in the erythrocyte membrane awaits further investigation.

Acknowledgments

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