An Analogue of the Erythroid Membrane Skeletal Protein 4.1 in Nonerythroid Cells

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ABSTRACT Protein 4.1 is a crucial component of the erythrocyte membrane skeleton. Responsible for the amplification of the spectrin–actin interaction, its presence is required for the maintenance of erythrocyte integrity. We have demonstrated a 4.1-like protein in nonerythroid cells. An antibody was raised to erythrocyte protein 4.1 purified by KCl extraction (Tyler, J. M., W. R. Hargreaves, and D. Branton, 1979, *Proc. Natl. Acad. Sci. USA*, 76:5192–5196), and used to identify a serologically cross-reactive protein in polymorphonuclear leukocytes, platelets, and lymphoid cells. The cross-reactive protein(s) were localized to various regions of the cells by immunofluorescence microscopy. Quantitative adsorption studies indicated that at least 30–60% of the anti-4.1 antibodies reacted with this protein, demonstrating significant homology between the erythroid and nonerythroid species. A homologous peptide doublet was observed on immunopeptide maps, although there was not complete identity between the two proteins. When compared with erythrocyte protein 4.1, the nonerythroid protein(s) displayed a lower molecular weight—68,000 as compared with 78,000–and did not bind spectrin or the nonerythroid actin-binding protein filamin. There was no detectable cross-reactivity between human acumentin or human tropomyosin-binding protein, which are similarly sized actin-associated proteins, and erythrocyte protein 4.1. The possible origin and significance of 4.1-related protein(s) in nonerythroid cells are discussed.

The erythrocyte provides a unique system for the investigation of the protein interactions required for the maintenance of a membrane skeleton. It is the skeleton, providing a durable yet flexible framework, that determines erythrocyte membrane shape, deformability, and structural integrity (1–5). Operationally defined as the network of proteins remaining after erythrocytes are solubilized in nonionic detergents, the skeleton consists of spectrin; actin; proteins 2.1 (ankyrin), 4.1, and 4.9; and a portion of protein(s) 7 (1, 5, 6). Spectrin heterodimers, the major components of the skeleton, are bound head to head to form tetramers and higher-order oligomers (7). Heterodimers are also bound to ankyrin, protein 4.1, and actin (for a review, see reference 8). Protein 4.1 is a globular protein with a molecular weight of ~78,000 (9–11). It is phosphorylated and carboxymethylated (12, 13), but the function of these posttranslational modifications is not known. When electrophoresed on the discontinuous SDS–polyacrylamide gel system of Laemmli, it can be resolved into two closely spaced bands, 4.1a and 4.1b (13, 14). These bands are structurally similar and functionally indistinguishable (13) and are therefore generally referred to as protein 4.1. Protein 4.1 is required for the binding of F-actin to the cytoplasmic surface of membrane vesicles containing spectrin (15). It binds to spectrin dimers at the same end to which actin filaments bind (16), and this binding amplifies the interaction of spectrin and F-actin in vitro (3, 17–19). Binding of ^{125}I-4.1 to spectrin in vitro is specific and saturable, with a K_D of $1–2 \times 10^{-7}$ M at pH 7.6 (16, 20). At saturation, two molecules of protein 4.1 can be bound to each spectrin dimer. A comparison of the number of molecules of spectrin dimer and protein

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 SEPTEMBER 1984 886–893
 · 0021-9525/84/09/0886/08 $1.00

4.1 in the intact erythrocyte suggests, however, that in vivo the ratio of these proteins is 1:1.

Until recently, some investigators believed the erythrocyte membrane skeleton was an anomaly, the structural elements of which bore little or no resemblance to those of the other cells of the body. It is now clear, however, that several skeletal proteins previously thought to be unique to the erythrocyte are found in a variety of nonerythroid cells. Polyclonal antibodies prepared against ankyrin, the high-affinity membrane attachment site for spectrin, have been used to document the presence of ankyrinlike proteins in neutrophils, platelets, and brain (21), as well as in the microtubule network of cultured cells (22). Spectrinlike proteins have also been identified in many mammalian cells (23–26). Fodrin (brain spectrin) was first described as a high-molecular-weight, axonally transported, spectrinlike protein in mammalian brain, and is also found beneath the plasma membranes of lymphocytes (27–30, 31). The terminal web protein, TW 260/240, another spectrin analogue, has been purified from intestinal brush borders (32, 33).

In an attempt to extend the analogy between the cytoskeletons of erythrocytes and those of other cells, we raised a polyclonal antibody directed specifically against purified erythrocyte protein 4.1. Using this antibody, we identified and characterized a protein 4.1 analogue in polymorphonuclear leukocytes, platelets, and lymphoid cells.

MATERIALS AND METHODS

Cell and Antigen Preparation: Erythrocyte ghosts were prepared according to the method of Fairbanks et al. (11). Spectrin-depleted inside-out erythrocyte vesicles were prepared by centrifugation over dextran (34). Spectrin dimer was prepared according to Harris and Lux (35). Protein 4.1 was purified by salt extraction (9) or by a high-yield detergent extraction (36). The two preparations were used interchangeably, and no differences were noted in antigenicity or binding activity. Acumentin was purified from human polymorphonuclear leukocytes by the method of Southwick et al. (37). Filamin was the gift of Dr. J. Hartwig (Massachusetts General Hospital, Boston, MA).

Human polymorphonuclear leukocytes (PMNs)[1] and a lymphocyte-monocyte fraction were obtained from fresh, anticoagulated human blood by the method of Boyum (38). To inhibit proteolysis they were treated with 1 mM diisopropylfluorophosphate (DFP); 10 mM *N*-ethylmaleimide; or with a mixture of 1 mM DFP, 50 μM [L-*trans*-epoxysuccinylleucylamido(3-methyl)]butane (Ep-475) (39), an inhibitor of cysteine proteases (the gift of Dr. Alfred Goldberg and Dr. Vickie Baracos, Harvard Medical School, Boston, MA), 1 μg/ml each of pepstatin, chymostatin, antipain, and leupeptin (Sigma Chemical Co., St. Louis, MO), and 1 mM Na tetrathionate. To isolate platelets, fresh human blood was withdrawn into citrate-phosphate-dextrose and was centrifuged at 250 *g* for 10 min. The platelet-rich plasma layer was retained, diluted 1:1 with phosphate-buffered saline (PBS: 150 mM NaCl, 7.5 mM sodium phosphate, pH 7.4) made 10 mM in EDTA, and recentrifuged at 250 *g* for 10 min to pellet contaminating PMNs and lymphocytes. This step was repeated twice more. The platelet-rich suspension was then centrifuged at 1,000 *g* for 10–20 min and the platelet pellet was washed in PBS and 10 mM EDTA containing either 1 mM DFP; 10 mM *N*-ethylmaleimide; or 1 mM DFP, 50 μM Ep-475, 1 μg/ml each of pepstatin, chymostatin, antipain, and leupeptin, and 1 mM Na tetrathionate. All steps but the last were performed at room temperature to prevent platelet activation and aggregation. Platelet cytoskeletons were prepared by Triton X-100 extraction (40).

To examine lymphoid cells, Triton X-100-extracted membrane cytoskeletons of the murine tumor cell line P815 were prepared by the method of Lemonnier et al. (41) and were the gift of Dr. M. F. Mescher and Dr. J. Apgar (Harvard Medical School). Protein determinations were by the method of Lowry et al. (42).

Antibody Preparation: To prepare anti-4.1 antibodies, rabbits were injected subcutaneously with 50 μg of sodium dodecyl sulfate (SDS)-denatured protein 4.1 (prepared by dissolving the antigen in 1% SDS and boiling for 1 min), emulsified in an equal volume of Freund's complete adjuvant (Miles Laboratories, Inc., Elkhart, IN). A boosting dose of antigen (50 μg) was administered in incomplete Freund's adjuvant 21 d after the first injection. Blood was collected 7, 9, and 11 d after the second injection. Thereafter the animals were maintained on a bimonthly schedule of boosting with 50 μg of the same protein subcutaneously and bleeding 7 d later. The blood was allowed to clot at 37°C for 1 h, and then placed at 4°C overnight to facilitate clot retraction. After centrifugation at 27,000 *g* for 20 min, the serum was collected and the clot discarded. IgG was purified from immune serum as described by Cebra and Goldstein (43).

Antibody Characterization: Immune sera were characterized by a solid-phase radioimmunoassay (RIA) (44, 45). Polyvinyl chloride (PVC) wells of U-bottomed microtiter plates (Dynatech, Inc., Alexandria, VA, catalog #01-010-2401) were coated with 100 μl of a 5 μg/ml solution of protein 4.1 in PBS or an unrelated control antigen such as BSA. After 2–4 h at room temperature in a humidified atmosphere, the wells were emptied, were washed six to eight times with PBS, and were incubated for 1 h at room temperature with PBS made 10 mg/ml in BSA to block the remaining nonspecific protein-binding sites on the plastic. The wells were again emptied and washed as before, and incubated for 2–4 h at room temperature with various dilutions of anti-4.1 in PBS containing 10 mg/ml BSA. After emptying and further washing, 50,000 cpm (5 ng) of ^{125}I-labeled, affinity-purified, goat anti-rabbit IgG in PBS made 10 mg/ml in BSA were added and the plates were incubated another 2–4 h at room temperature. After emptying and washing as before, the polyvinyl chloride wells were cut apart and each well was counted in a Tracor Analytic gamma counter (Tracor Analytic, Inc., Elk Grove Village, IL).

For affinity chromatography, protein 4.1 was covalently attached to Sepharose 4B (Pharmacia, Inc., Piscataway, NJ), activated with cyanogen bromide, by the method of March et al. (46) except that conjugation was done in 0.2 M Na bicarbonate at pH 8.4 rather than pH 9.5. Concentrations of 0.4–1 mg of protein per ml of packed beads were routinely achieved. Affinity columns were prewashed successively with 10 vol of 0.1 M $NaHCO_3$, 0.4 M NaCl, pH 8; 0.1 M Na acetate, 0.4 M NaCl, pH 4; 0.1 M Na borate, 0.4 M NaCl, pH 8; and 0.1 M $NaHCO_3$, 0.2 M NaCl, pH 8. These washes were followed by 1% Triton X-100 in PBS; by 3 M urea, 10 mM Tris, pH 8; by 3 M potassium thiocyanate and then by PBS. Ammonium sulfate-fractionated or ion exchange-purified antibodies were passed through the column, which was washed with 10–20 vol of PBS; several column volumes of 1% Triton X-100 in PBS; and 3 M urea, 10 mM Tris, pH 8. Antibodies were then eluted with 3 M potassium thiocyanate and immediately dialyzed against several liters of PBS. Protein concentrations were determined by measuring the optical density at 280 nm, using $E^{1\,cm}_{0.1\%} = 1.4$ (43). Goat anti-rabbit IgG serum was purchased from Serasource, Inc. (Berlin, MA), and was affinity-purified as described above on a column of rabbit IgG. To prepare an appropriate control antibody, ammonium sulfate-fractionated anti-4.1 (78 μg) was passed through a column of 4.1-Sepharose containing 45 μg of protein 4.1. A small portion of the nonadherent effluent was saved, and the remainder was passed over a fresh column. This was repeated three more times. The volumes of the saved fractions were normalized to produce equal antibody concentrations (assuming no antibody loss upon adsorption). The fractions were then diluted 1:10 and used in a solid-phase RIA on 4.1-coated polyvinyl chloride wells to monitor removal of anti-4.1 activity by the column. Antidinitrophenyl (anti-DNP) and DNP coupled to BSA (DNP-BSA) were the gift of Dr. L. J. Wysocki (Massachusetts Institute of Technology, Cambridge, MA). Antitropomyosin binding protein (anti-TMBP) was the gift of Dr. S. E. Hitchcock-deGregori (Carnegie-Mellon University, Pittsburgh, PA).

Iodinations: Antibodies were iodinated by the chloramine-T method as described by Hunter and Greenwood (47). Spectrin, filamin, and protein 4.1 were iodinated with Bolton-Hunter reagent (New England Nuclear, Boston, MA) by the method of Bolton and Hunter (48).

Adsorption Assays: Human erythrocyte ghosts, spectrin-depleted inside-out vesicles, PMNs, lymphocytes, or platelets were made 1 mM in DFP and 1 μg/ml each in soybean trypsin inhibitor (Sigma Chemical Co.), pepstatin, chymostatin, antipain, and leupeptin, and were homogenized on ice for 5 min in a Teflon-pestled, Potter-Elvehjem type homogenizer attached to a 3/8-inch drill spun at 1,200 rpm. Increasing quantities (0–200 μg) of total cellular protein were incubated in 5 mM EDTA, 0.1% Triton X-100, 1% BSA, and 7.5 mg/ml nonimmune rabbit IgG in PBS in the presence of 78 μg/ml ammonium sulfate-fractionated anti-4.1 or 0.4 μg/ml anti-DNP. After shaking overnight at 4°C, each sample was pelleted at 12,800 *g* for 15 min and the supernatants were tested for remaining antibody activity. They were applied to the wells of polyvinyl chloride microtiter plates that had previously been incubated with 0.5 μg of protein 4.1 or DNP-BSA and blocked with PBS made 10 mg/ml in BSA. After 4 h at room temperature, the wells were emptied and washed with PBS and 100 μl of ^{125}I-goat anti-rabbit IgG (5 ng of antibody, 50,000 cpm) was added to each well. After 4 h more, the wells were emptied, washed again, and bound radioactivity was measured.

[1] *Abbreviations used in this paper*: DFP, diisopropylfluorophosphate; DNP, dinitrophenyl; PMN, polymorphonuclear leukocyte; RIA, radioimmunoassay; TMBP, tropomyosin binding protein.

Immunofluorescence: Immunofluorescence studies were performed by the method of Pryzwansky et al. (49). Human venous blood was cytocentrifuged onto 12-mm-round coverslips (Carolina Biological Supply Co., Burlington, NC) that had been prewashed with acetone and ethanol. The cells were fixed and permeabilized in a buffered formalin–acetone solution (49). Rabbit antibodies were used at 5–50 μg/ml in PBS, and were followed by a fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Miles Laboratories, Inc.), used at a dilution of 1:40 to 1:500. Coverslips were mounted in 90% glycerol/10% PBS and sealed with nailpolish. The slides were examined with a Zeiss Universal microscope (Carl Zeiss, Inc., New York) equipped for epifluorescence, using a #487710 fluorescein isothiocyanate filter set.

Electrophoretic Transfer Assays: Electrophoretic transfer assays and spectrin binding assays were performed as previously described (36).

Immunopeptide Maps: Whole PMNs (pretreated with 1 mM DFP) and erythrocyte ghosts were boiled for 2 min in 0.125 M Tris-HCl, pH 6.8, 0.5% SDS, 10% glycerol, and 0.0001% bromphenol blue. Digestion was then carried out as described by Cleveland et al. (50) with *Staphylococcus aureus* V8 protease (Miles Laboratories, Inc.), using an enzyme to protein mass ratio of 1:500. The proteolytically digested cell proteins were electrophoresed on adjacent lanes of a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and stained with anti-4.1 as described previously. Although peptides were generated from all the cellular proteins, only those that were derived from protein 4.1 or from a 4.1-like protein and that retained their immunogenicity were resolved on the resulting autoradiogram.

RESULTS

Antibody Activity and Specificity

Immune anti-4.1 was a nonprecipitating antibody. No precipitin lines were observed in double immunodiffusion tests (data not shown); therefore, anti-4.1 was tested for activity in solid-phase RIAs. Ammonium sulfate-fractionated anti-4.1 antibody could be detected at a concentration of <0.5 μg/ml, and binding of anti-4.1 to 4.1-coated wells reached a plateau, demonstrating that the binding to protein 4.1 was saturable (Fig. 1).

To demonstrate the specificity of the anti-4.1 antibody, fresh erythrocyte ghosts and purified protein 4.1 were electrophoresed in adjacent wells of a 10% SDS polyacrylamide gel, transferred to nitrocellulose paper, and stained with Amido Black or with unlabeled, ion-exchange or affinity-purified anti-4.1, followed by ^{125}I-labeled goat anti-rabbit IgG. As shown in Fig. 2*B*, only protein 4.1 (78,000 M_r) was visualized by the antibody when erythrocyte ghosts were stained. No protein bands were seen with the adsorbed anti-4.1 antibody or with ^{125}I-labeled goat anti-rabbit IgG alone (Fig. 2*C*). Thus, the labeling of protein 4.1 was not due to nonspecific adherence of antibodies to the protein. Because the anti-4.1 antibody was highly specific for its respective antigen, it was generally purified only by ion-exchange chromatography.

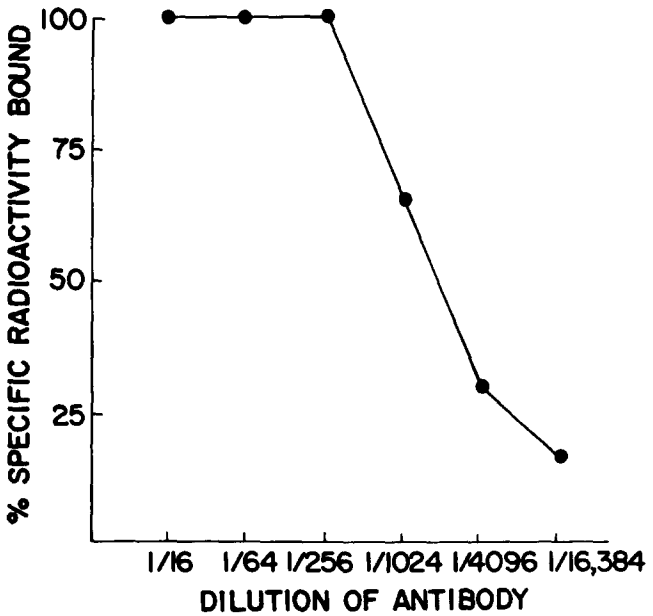

FIGURE 1 Solid-phase RIA showing that the binding of anti-4.1 to 4.1-coated polyvinyl chloride wells is saturable. Ammonium-sulfate fractionated anti-4.1 (8 mg/ml) was diluted as shown. Specific radioactivity bound (percent) was calculated by subtracting the binding of preimmune serum from the binding of immune serum. Percent of specific activity bound represents the maximum number of counts bound.

Identification of a Protein 4.1 Analogue in Nonerythroid Cells

To search for protein 4.1 or a 4.1 analogue in nonerythroid cells, erythrocyte ghosts, total cellular protein from human PMNs, platelets, and a lymphocyte–monocyte fraction were electrophoresed on a 10% SDS–polyacrylamide gel, transferred to nitrocellulose, and stained with anti-4.1 antibody. The results are shown in Fig. 3. Autoradiography showed a single protein band in all of the cells (Fig. 3*B*). However, in the nonerythroid cells, the visualized band had a lower molecular weight (~68,000 M_r) than erythrocyte protein 4.1 (78,000–80,000 M_r). Staining with anti-4.1 previously adsorbed against protein 4.1 did not visualize any protein bands (Fig. 3*C*).

To test the possibility that this lower molecular weight protein could result from the cleavage of a 78,000 M_r nonerythroid 4.1-like protein during cell solubilization, we added an array of protease inhibitors: 10 mM *N*-ethylmaleimide; 1 mM DFP; 50 μM Ep-475 (a sulfhydryl protease inhibitor); 1 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin; and a mixture containing all of these inhibitors. Even when all were present before and during solubilization, we were unable to detect a nonerythroid protein of 78,000 M_r.

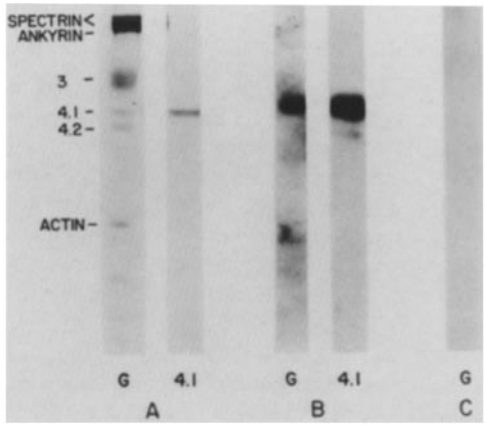

FIGURE 2 Specificity of anti-4.1. Erythrocyte ghosts and protein 4.1 were electrophoresed on a 10% SDS polyacrylamide gel, transferred to nitrocellulose, and stained with ion-exchange purified anti-4.1 or with a 4.1-adsorbed anti-4.1 antibody. (*A*) Coomassie Blue-stained gel of 30 μg of ghosts (*G*) and 10 μg of protein 4.1 (*4.1*). (*B*) An autoradiogram of the electrophoretically-transferred proteins from an identical gel, stained with anti-4.1. (*C*) An autoradiogram of similarly transferred proteins of a ghost preparation stained with the 4.1-adsorbed anti-4.1 antibody. No binding is visible. A similar blank pattern was observed when PBS was substituted for the first antibody, precluding the possibility of nonspecific binding of ^{125}I-goat anti-rabbit IgG to the transferred proteins.

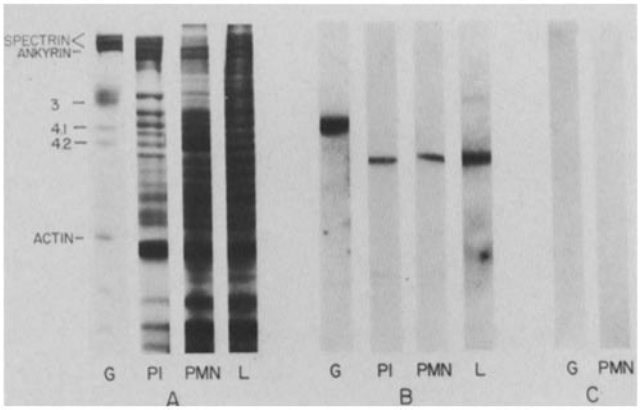

FIGURE 3 Detection of a 4.1-like protein in nonerythroid cells. Total cellular protein (100 μg) from polymorphonuclear leukocytes (*PMN*), platelets (*PI*), and a lymphocyte-monocyte fraction (*L*) was electrophoresed on an SDS polyacrylamide gel, along with 30 μg of erythrocyte ghosts (*G*). After transfer, the nitrocellulose sheets were stained with anti-4.1 or a 4.1-adsorbed anti-4.1 antibody. (*A*) Coomassie Blue-stained gel. (*B*) An autoradiogram of transferred proteins from a duplicate gel stained with anti-4.1. (*C*) An autoradiogram of transferred ghosts and whole PMNs stained with a 4.1-adsorbed anti-4.1 antibody.

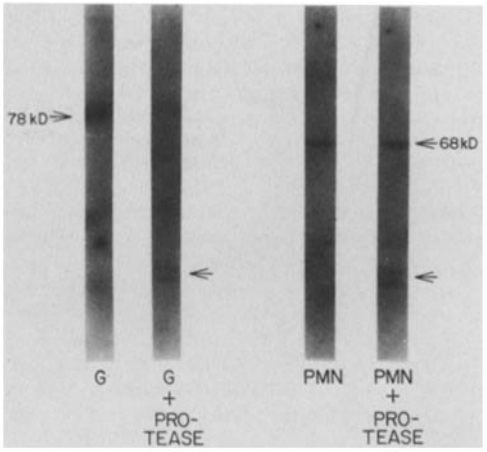

FIGURE 4 Immunopeptide maps. Erythrocyte ghosts (*G*) and polymorphonuclear leukocytes (*PMN*) were digested with *Staphylococcus aureus* V8 protease, run on SDS gels, transferred to nitrocellulose paper, and stained with anti-4.1. On the left are undigested and digested erythrocyte ghosts. On the right, PMNs and digested PMNs. Both digested preparations, when stained with anti-4.1, show an immunologically reactive doublet on the autoradiogram (*arrow*).

In addition, nonerythroid cells were tested for their ability to digest erythroid protein 4.1 to a 68,000 M_r protein. ^{125}I-labeled protein 4.1 was added to 50 μg of platelet protein in the absence of protease inhibitors, solubilized, incubated for periods up to 1 h at 25°C, and analyzed by gel electrophoresis and autoradiography. Even under these provocative conditions no degradation of protein 4.1 was observed.

To demonstrate a structural relationship between 4.1 and the 4.1-like protein, immunopeptide maps were performed (Fig. 4). Erythrocyte ghosts and PMNs digested with *Staphylococcus aureus* V8 protease showed homology between the erythroid and the nonerythroid protein in the form of an immunoreactive doublet visualized at the bottom of the gel.

Adsorption Studies

To further assess the degree of homology between the erythroid and the nonerythroid proteins, an adsorption study was performed to determine what percentage of the antibodies that react with erythroid protein 4.1 also react with the 4.1-analogue(s) in other cell types. Various whole cell homogenates were assayed for the ability to adsorb anti-4.1 antibodies (Fig. 5). Erythrocyte ghosts removed ~75% of the antibody activity, whereas spectrin-depleted membrane vesicles removed ~90% of the antibody activity (presumably removing spectrin from the membrane rendered additional antibody binding sites more accessible). PMNs and platelets removed 60–70% and 20–35% of the anti-4.1 activity, respectively. It is probable that removal of antibody activity plateaued at these lower values for two reasons: (*a*) that not all the sites to which anti-4.1 antibodies bound on the purified protein were accessible in the homogenized cell preparations (and differences in antigen accessibility between platelets and PMNs may explain the different values each attained); and (*b*) the nonerythroid protein is certainly not identical to the erythrocyte protein (and may differ between PMNs and platelets), and probably does not contain some domains to which a subpopulation of the antibodies are directed.

To control for specificity of antibody adsorption, an affin-

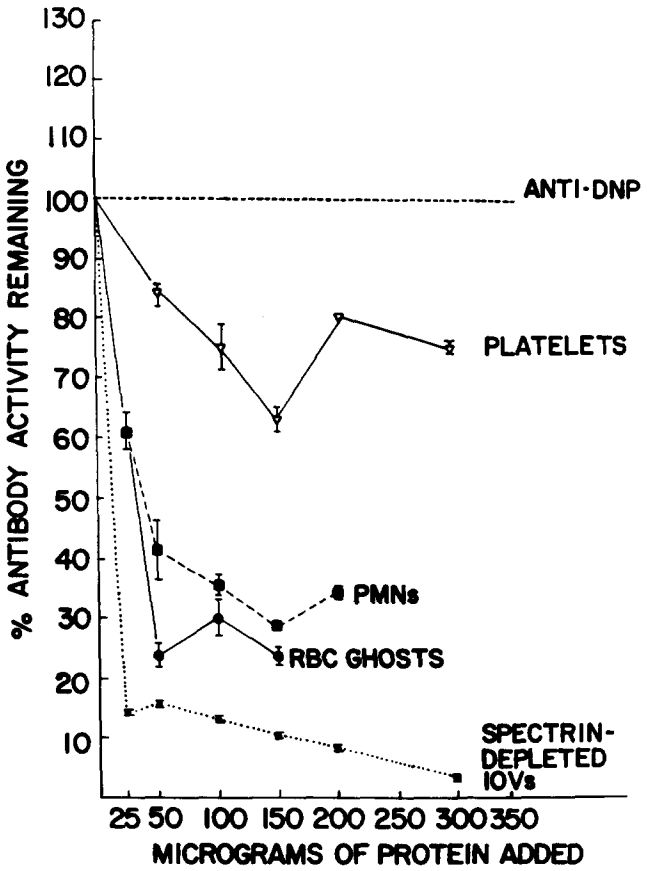

FIGURE 5 Adsorption of antibody activity by cell lysates. Cells were treated as described in the text and incubated with antidinitrophenyl (*ANTI-DNP*) or anti-4.1. In all cell types amd at all levels of cellular protein tested, anti-DNP activity remained at 100%. Shown are polymorphonuclear leukocytes (*PMNs*), platelets, erythrocyte ghosts (*RBC*), and spectrin-depleted erythrocyte membranes (*IOVs*).

ity-purified, anti-DNP antibody was treated in parallel with the above proteins. Because erythrocytes, PMNs, and platelets do not contain DNP, anti-DNP activity should not be removed by the cell homogenates unless (*a*) endogenous proteases are destroying the antibody, yielding falsely positive results; (*b*) antibodies are binding nonspecifically, and/or via Fc receptors; or (*c*) antibodies are "trapped" in membrane vesicles. As shown in Fig. 5, however, anti-DNP activity remained constant (i.e., no anti-DNP activity was removed at all levels of cellular protein tested), indicating that PMNs and platelets did indeed specifically bind anti-4.1 antibodies.

When antibody removal was corrected for differences in cell mass, PMNs and platelets were found to be 50 and 10 times more effective per cell, respectively, at removing anti-4.1 activity than were erythrocytes.

Location of the 4.1-like Protein in Nonerythroid Cells

Immunofluorescent staining of peripheral blood was not routinely performed, due to the high numbers of autofluorescent cells present in the preparations after the fixation step. Difficulties were also encountered with fixatives used to retain cell morphology which increased nonspecific fluorescence. To optimize morphologic integrity and to minimize nonspecific adherence of antibodies, we used the buffered formalin–acetone preparation developed by Pryzwansky et al. (49) rather than the traditional acetone permeabilization and formaldehyde fixation. Even then, however, only a portion of the preparations had low background fluorescence. The reason for this variability was not determined.

In good preparations, staining of PMNs and monocytes (Fig. 6) or lymphocytes (data not shown) with anti-4.1 yielded a diffuse, cytoplasmic fluorescence that excluded the nucleus. Staining of platelets was primarily membrane-associated (Fig. 6). Treatment with similar concentrations of 4.1-adsorbed anti-4.1 (data not shown) or anti-DNP resulted in either faint or nonexistent background staining.

Because protein 4.1 is an important constituent of the erythrocyte skeleton, we searched for the 4.1-like protein in the cytoskeletons of platelets. As shown in Fig. 7, the 4.1-like protein was associated with the Triton X-100-insoluble cytoskeletal fraction of platelets and did not appear in either of the Triton X-100-soluble fractions. In a similar experiment (Fig. 7), the cross-reactive protein was also detected in the cytoskeleton of P815 cells (a murine cell line with lymphoid characteristics [51]).

Reactivity of Cell Homogenates with ^{125}I-Spectrin or ^{125}I-Filamin

Protein 4.1 has been identified as a spectrin-binding protein. We therefore assayed the ability of protein 4.1 and the 4.1-like protein(s) to bind to ^{125}I-spectrin or ^{125}I-filamin (an actin-binding protein) when they were immobilized on nitrocellulose paper. Treatment of the transferred proteins with ^{125}I-spectrin dimer resulted in the staining of protein 4.1 and ankyrin in the erythrocyte ghosts, both previously identified

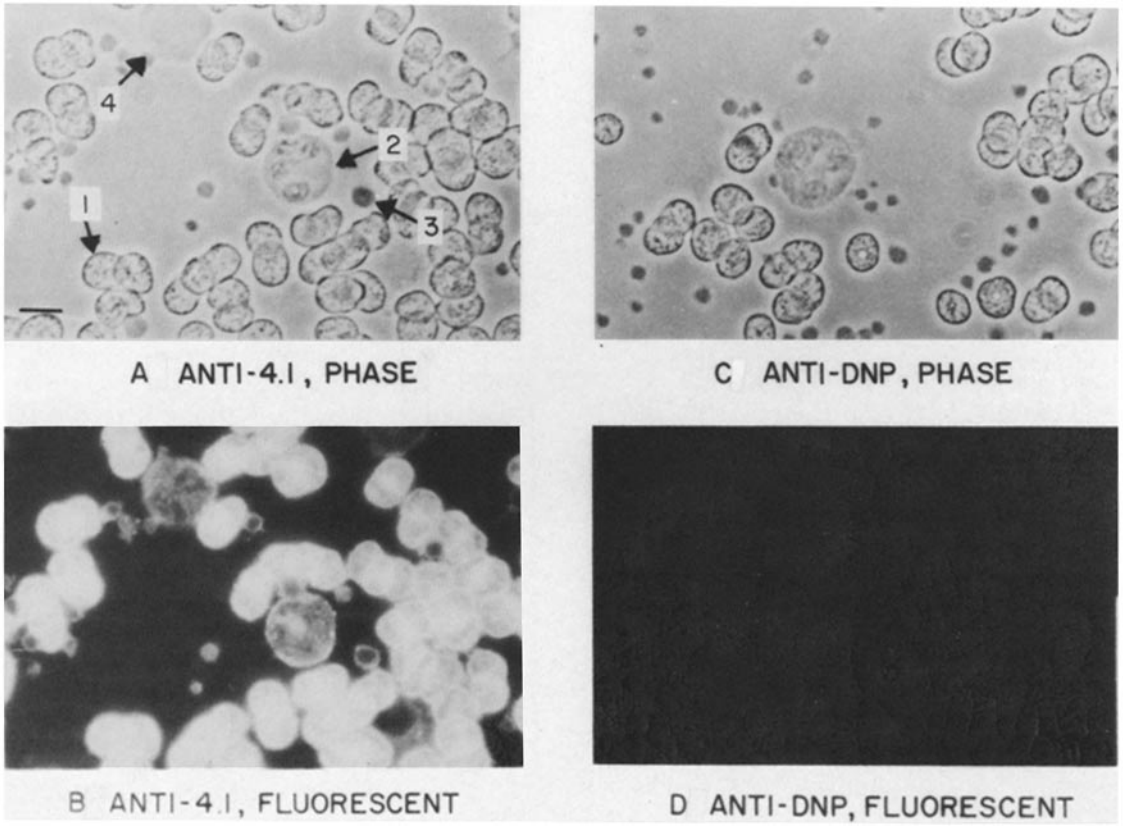

FIGURE 6 Immunofluorescence photomicrographs. Peripheral blood cells were fixed and stained with affinity-purified anti-4.1. Antidinitrophenyl (*ANTI-DNP*) or 4.1-adsorbed anti-4.1 (*ANTI-4.1*) were used as control antibodies. Neither control antibody yielded any specific staining. Final concentrations of antibodies were 25–50 μg/ml. Fluorescein isothiocyanate labeled goat anti-rabbit IgG was used at a dilution of 1:40 to 1:500. Exposures of fluorescence pictures were 1–2 min. *1*, an erythrocyte; *2*, a polymorphonuclear leukocyte; *3*, a platelet; and *4*, a monocyte. Bar, 7 μm.

as spectrin-binding proteins (Fig. 8 *B*). However, no staining was observed in the lanes containing the nonerythroid cells when either ^{125}I-spectrin (Fig. 8 *B*) or ^{125}I-filamin (Fig. 8 *C*) was used, indicating that the cross-reactive, nonerythroid proteins detected by anti-4.1 did not bind either spectrin or filamin under these conditions.

Reactivity with Previously Identified Actin-associated Proteins

Finally, we attempted to determine whether any of the previously identified and isolated actin-associated proteins from leukocytes or platelets was the 4.1-like nonerythroid analogue. The most likely candidate was acumentin, a protein of ~65,000 M_r that caps actin filaments (37). Preliminary studies (52) showed weak cross-reactivity between rabbit 4.1 and rabbit acumentin. To determine if this observation was valid with human proteins, we used purified human acumentin in a solid-phase RIA, and assessed its ability to bind our anti-4.1 antiserum. Essentially no binding was observed, indicating that acumentin was not the nonerythroid analogue (data not shown). We then focused our attention on a 66,000 M_r TMBP isolated from human platelets (53, 54) that inhibits actin polymerization. When anti-TMBP was used in a solid-phase RIA, it did not bind to protein 4.1 (data not shown), and therefore is not the nonerythroid analogue of protein 4.1.

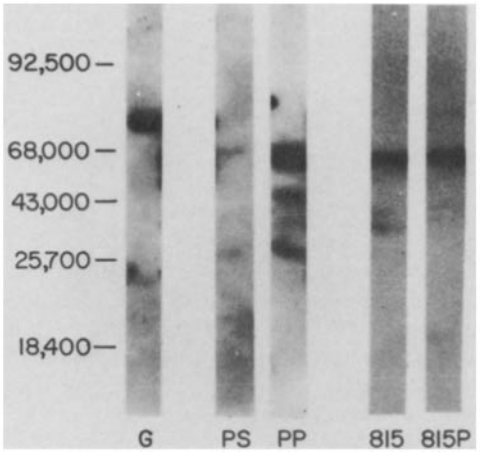

FIGURE 7 Localization of the 4.1-like protein in the cytoskeletons of platelets and P815 cells. Approximately 150 μg of platelet protein obtained at different stages of the extraction procedure, and 10^6 cell equivalents of P815 cells and P815 cytoskeletons were run on an SDS gel, transferred to nitrocellulose paper, and stained with anti-4.1. Shown is an autoradiogram of a transfer of erythrocyte ghosts (*G*), Triton X-100-soluble platelet proteins (*PS*), Triton X-100-insoluble platelet proteins (*PP*), whole P815 cells (*815*), and P815 cytoskeletons (*815P*). The 4.1-like protein appears in the cytoskeletal fraction of both cell types, and, in this preparation of platelet cytoskeletons, shows some proteolytic degradation.

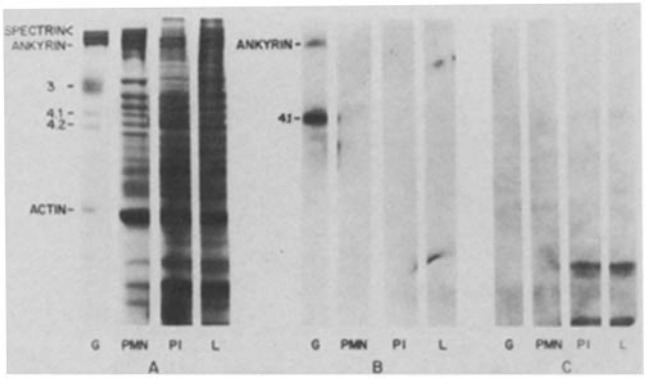

FIGURE 8 Reactivity of electrophoretically transferred cell homogenates with ^{125}I-spectrin or ^{125}I-filamin. Transferred proteins of erythrocytes ghosts (*G*), polymorphonuclear leukocytes (*PMN*), a lymphocyte-monocyte fraction (*L*), and platelets (*PI*) were incubated with ^{125}I-spectrin or ^{125}I-filamin as described in the text. (*A*) Coomassie Blue-stained gel. (*B*) Autoradiogram of transferred proteins from a duplicate gel stained with ^{125}I-spectrin. (*C*) Autoradiogram of similarly transferred proteins stained with ^{125}I-filamin. Notice that ^{125}I-spectrin (*B*) stains both protein 4.1 and ankyrin, ^{125}I-filamin (*C*) stains only unidentified lower molecular weight proteins.

DISCUSSION

Protein 4.1 is an important component of the erythrocyte membrane skeleton. In vitro it functions to enhance the spectrin-actin binding interaction (3, 17–19). In vivo it is required for the maintenance of skeletal integrity: individuals homozygous for a rare form of hereditary elliptocytosis lack all protein 4.1 and display marked red cell fragmentation, poikilocytosis, osmotic fragility, and hemolysis (55, 56).

To facilitate the study of this protein, we raised an antibody to purified protein 4.1 and demonstrated its specificity. Although protein 4.1 was previously thought to be an exclusively erythroid protein, we have used our antibody to show the existence of a protein 4.1 analogue in leukocytes, lymphoid cells, and platelets. This cross-reactive protein has an apparent molecular weight of ~68,000. We cannot absolutely exclude the possibility, however, that a nonerythroid protein of 78,000 M_r actually exists, but is not protected by treatment with a wide battery of protease inhibitors.

Nonerythroid protein 4.1 is associated with the cytoskeletons of platelets and of P815 cells, and it is found in the cytoplasm of leukocytes. We did not attempt to determine whether it is also associated with the cytoskeleton of the latter cells, although this seems a reasonable possibility. In vitro binding assays using the technique of electrophoretic transfer to nitrocellulose paper of proteins separated on SDS polyacrylamide gels, failed to detect binding of spectrin or filamin to the 4.1-like protein. In addition, solid-phase RIAs demonstrated no detectable cross-reactivity between human acumentin or human TMBP and protein 4.1.

If there are two forms of protein 4.1, what is their significance? Actin, also a structural protein, is encoded by a multigene family (57). As many as 17 genes for actin are present in a single organism (e.g., *Dictyostelium*) (58), but the multiple actin genes and proteins are expressed at different times and in different tissues. Investigators have suggested that perhaps an ancestral actin gene has duplicated and diverged. It could be advantageous to be able to activate an actin gene selectively for a specific tissue rather than to modulate one actin gene differentially in different tissues. In the case of protein 4.1, gene duplication at the level of a pluripotent, ancestral stem cell could explain the existence of both erythroid and nonerythroid forms of the protein.

Conversely, it is conceivable that there may be only one gene for protein 4.1, containing multiple exons encoding several domains. Different 4.1-related proteins might then express different but overlapping domains. Different messenger RNA splicing mechanisms may exist in leukocytes and

erythrocytes, allowing a 68,000 M_r protein to be translated in one cell type and a 78,000 M_r protein in the other.

After our initial report of the existence of protein 4.1 in nonerythroid cells (59), Cohen et al. (60, 61) discovered two 4.1 analogues in fibroblasts of apparent molecular weights 78,000 and 90,000. The discrepancy in the sizes of the 4.1 analogues detected in our work and in that of Cohen and his co-workers may be due to the different antibodies used (which may detect different proteins), to the way that we prepared the cells, or to the fact that we examined different cells. The possibility that we are detecting a proteolytic fragment of the proteins they saw must also be considered. In their hands, our antibody detects proteins of both molecular weights 78,000 and 68,000 (62), suggesting (*a*) that our antisera recognize different parts of the 4.1 molecule, and (*b*) that differences in cell preparation contribute to the presence of different molecular weight proteins. Although we went to great lengths to prevent proteolysis and to detect 4.1-proteolytic activity, we will not be able to absolutely exclude proteolysis until the synthesis of the 4.1-like protein(s) has been studied in a cell-free system or its gene(s) has been isolated and characterized.

It is interesting that adsorption studies showed a large amount of apparent homology between erythrocyte 4.1 and the 4.1-like protein. Human platelets and PMNs adsorbed 30–60% of anti-human erythrocyte 4.1 activity. This may indicate extensive homology between the erythroid and nonerythroid proteins or a limited spectrum of epitopes on erythrocyte 4.1 that are seen by the anti-4.1 antibody.

If there is extensive structural homology between the two proteins, they may also exhibit functional homology. In the erythrocyte, protein 4.1 is crucial for the maintenance of skeletal integrity, and in vitro it is required for the amplification of spectrin–actin interactions. The 4.1-like protein is found associated with the cytoskeletons of platelets, P815, and possibly other cells. Other erythrocyte skeletal proteins have been located in nonerythroid cells: both spectrin and ankyrin have been found in brain and liver (21, 25), and brain spectrin retains the ability to bind to erythrocyte ankyrin (31). Recently, brain spectrin has also been shown to form a complex with actin and erythrocyte protein 4.1 (63). As in the erythrocyte, the interaction of brain spectrin with protein 4.1 enhances its attachment to actin. We suspect the 4.1-like protein(s) also function to augment the interactions between spectrinlike proteins and actin in nonerythroid cells. Although we could not detect spectrin-binding to the nonerythroid 4.1, our studies were limited to nitrocellulose transfers. Purification of the 4.1-like protein, the study of its interactions with other skeletal proteins, and a survey of its location in nonerythroid cells containing spectrin and ankyrin analogues are needed.

We thank Dr. L. J. Wysocki for helpful discussions of, and critical comments on, the work and manuscript. We are grateful to P. S. Becker and to Drs. J. Hartwig, A. Goldberg, V. Baracos, M. Mescher, J. Apgar, and S. Hitchcock-deGregori for providing us with purified proteins, cells, and antisera; to Dr. K. B. Pryzwansky for help with immunofluorescence; to Dr. G. Howard for help with electrophoretic transfers; and to P. S. Becker and K. M. John for technical assistance and helpful discussions during the course of this work.

This project was supported by U. S. Public Health Service grants 5R01-AM21836, 5R01-AM27853, and 5P01-27375.

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Received for publication 9 October 1983, and in revised form 9 May 1984.

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