Species-Dependent Variations in Erythrocyte Membrane Skeletal Proteins

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Two mammalian species (porcine and murine) have erythrocytes that are being widely used to study membrane protein synthesis and red cell aging. Erythrocytes of these species however, are significantly smaller than those of the human. Before results obtained from study of these red cells can be applied to human cells, the membrane skeleton of these species must be investigated to determine if the skeletal elements are equivalent. Both pig and mouse bands 4.1b were of lower molecular weight than human 4.1b, and the a/b ratio was lower. In each species, 4.1a and b were sequence-related phosphoproteins, and yielded substantially different one-dimensional peptide maps.

ERYTHROCYTES of mice and other small mammals are now being widely used in the study of red cell senescence and red cell membrane protein synthesis during erythropoiesis, problems that are not easily studied in humans, and in the study of hemolytic anemias. The pig is also being used as a model for human hematologic studies, including sideroblastic anemias. Before results obtained from the study of these red cells can be applied to human erythrocytes, the membrane proteins of these species must be thoroughly investigated. The assumption that similar molecular weight polypeptides have identical interactions and functions in various species should not be made without further evidence. It is interesting to note that erythrocytes from most other species of mammals are significantly smaller in volume than those of the human, which suggests that the membrane skeletal elements of these cells may vary.

Erythrocyte shape is maintained by the membrane skeleton, which consists of the polypeptides spectrin, actin, and band 4.1. This fibrous skeletal structure is attached to the membrane through binding of syndeins (bands 2.1–2.6, also called ankyrin), both to spectrin and to the integral membrane protein, band 3. Certain elements of the erythrocyte membrane skeleton seem to be remarkably similar from species to species. For example, both polypeptides of spectrin (bands 1 and 2), band 2.1, and actin (band 5) are always present when erythrocyte ghosts are solubilized in SDS and analyzed by polyacrylamide gel electrophoresis (SDS-PAGE), and have very similar molecular weights, independent of species. Band 3, however, appears to vary with species. Differences in band 3 mobility in continuous buffer SDS-PAGE have been reported for rabbit, camel, and mouse, and are apparent in the gel photographs of Kitao et al. Alterations of band 4.1 (which can be resolved into two peptides, bands 4.1a and 4.1b, with discontinuous buffer SDS-PAGE) and 4.2 have been noted for rabbit, camel, and mouse, and can be seen in the data of Howard et al. for mouse.

Therefore, this work was undertaken to compare the membrane skeleton of human erythrocytes with the commonly used mammalian erythrocyte types of mouse and pig. Comparisons were made on the basis of analysis of polypeptides visualized on discontinuous buffer SDS-PAGE, phosphorylation profiles of ghost and membrane skeletal proteins, and one-dimensional peptide maps of iodinated or phosphorylated polypeptides cleaved with trypsin, chymotrypsin, or thermolysin.

Major points derived from this study are (1) that band 3 one-dimensional peptide maps differ markedly in the three species, but within each species, band 3 that is bound to the membrane skeleton is indistinguishable from the bulk of band 3, and (2) band 4.1 can be resolved into 4.1a and b in all three species, but the ratio of the two polypeptides differs, the apparent molecular weight differs, and one-dimensional peptide maps indicate substantial difference in sequence; within a species, 4.1a and b are sequence-related polypeptides, as has been shown for human 4.1a and b.
VARIATIONS IN RED CELL SKELETAL PROTEINS

MATERIALS AND METHODS

Preparation of Ghosts

Human blood was obtained from normal volunteers; mouse blood was collected after decapitation of Crl:CD-1 adult mice; pig blood was collected at a local slaughter house from freshly killed animals. Erythrocyte ghosts were prepared from freshly drawn blood or 1-day-old blood, anticoagulated with acid citrate/dextrose, by the procedure of Dodge et al. The lysing buffer consisted of 5 mM sodium phosphate, 1 mM EDTA, 0.4 mM diisopropylfluorophosphate (DFP), pH 7.6.

In each experiment, human, pig, and mouse blood was processed in parallel, with the same solutions and always in the presence of the potent protease inhibitor DFP. This protocol was used to prevent artifacts produced by different degrees of proteolysis in the three species.

Preparation of Triton Membrane Skeletons

Triton membrane skeletons were prepared by the method of Yu et al. Ghosts were extracted for 20 min at 4°C in 5-7 volumes of 5 mM sodium phosphate, 1 mM EDTA, 0.4 mM DFP, 25 mM NaCl, 1% Triton X-100, pH 8.0. Membrane skeletons were pelleted at 20,000 rpm, 40 min, 2°C (Beckman J2-21 centrifuge, JA-20 rotor). The membrane skeletons were washed once in lysing buffer and solubilized in 1% SDS, 10 mM Tris, 1 mM EDTA, 32 mM dithiothreitol (DTT), pH 8.0, at 90°C for 10 min.

Preparation of 32P-Labeled Ghosts

Erythrocyte membrane proteins were phosphorylated with 32P by metabolically labeling intact erythrocytes (3 ml packed cells) for 12-15 hr with 32P-orthophosphoric acid (10 mCi, carrier-free in 0.02 M HCl, New England Nuclear, Boston, Mass.) by the method of Bennett and Branton. The labeled phosphate was added to incubation medium containing carrier sodium phosphate. Final concentration of components in the incubation medium were: 130 mM NaCl, 25 mM NaHCO3, 37 mM KCl, 2.4 mM MgCl2, 1.2 mM CaCl2, 1.0 mM adenosine, 12 mM glucose, 0.62 mM Na2PO4 (specific activity 2000 Ci/mmole, New England Nuclear) as described by Goodman et al. One-dimensional peptide maps were then obtained by limited proteolysis in SDS-polyacrylamide gels by the technique of Cleveland et al., as modified by Goodman et al., using 5 μg of enzyme (trypsin, α-chymotrypsin, or thermolysin, obtained from Sigma, St. Louis, Mo.) per gel slice. Maps of band 3 and 4.1 were run on slab gels containing a 3% acrylamide stacking gel portion (6 cm) and separating gels of 12% (band 3) or 15% (band 4.1) acrylamide (10 cm) in the discontinuous buffer system of Laemmli.

Gel Electrophoresis

SDS-PAGE in a continuous buffer system was performed by the method of Fairbanks et al. as modified by Steck and Yu. The discontinuous buffer system of Laemmli was also used for SDS-PAGE. Slab gels were formed with a 13-cm separating gel of 6% acrylamide (acrylamide/bisacrylamide = 37.5) in 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 1 mM EDTA, 0.05% TEMED, 0.05% ammonium persulfate, and a 3-cm stacking gel of 3% acrylamide in 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, 0.05% TEMED, and 0.05% ammonium persulfate.

Molecular weights of bands 4.1a and 4.1b from all species and of bands 3, and 4.2 from mouse and pig were estimated from the relative mobilities of polypeptides from normal human erythrocytes with established molecular weights: 2 band 2, 220,000; band 3, average and leading edge, 95,000 and 89,000; band 4.2, 73,000; band 5, 43,000; and band 6 (where visible on the gel), 33,000. Peak areas were integrated and quantitated by scanning gels on a ZeinZein laser scanning densitometer (LKB instruments), copying the scan onto uniform bond paper, and cutting out and weighing the various peak areas.

Peptide Mapping

Unlabeled or 32P-labeled polypeptides were excised from SDS-polyacrylamide slab gels that had been stained with Coomassie blue and destained for short time periods. If the polypeptides were to be iodinated, the slices were dried and incubated with 125I-Bolton Hunter reagent (200 μCi moniodinated 125I-Bolton Hunter reagent, specific activity 2000 Ci/mmole, New England Nuclear) as described by Goodman et al. One-dimensional peptide maps were then obtained by limited proteolysis in SDS-polyacrylamide gels by the technique of Cleveland et al., as modified by Goodman et al., using 5 μg of enzyme (trypsin, α-chymotrypsin, or thermolysin, obtained from Sigma, St. Louis, Mo.) per gel slice. Maps of band 3 and 4.1 were run on slab gels containing a 3% acrylamide stacking gel portion (6 cm) and separating gels of 12% (band 3) or 15% (band 4.1) acrylamide (10 cm) in the discontinuous buffer system of Laemmli.

Autoradiography

 Autoradiograms were exposed for varying times at –70°C, using Kodak X-Omat XAR-5 film with a DuPont Cronex Lighting Plus intensifying screen.

RESULTS

Coomassie Blue Profiles

Mean cell volumes of pig and mouse erythrocytes were measured with a Coulter Counter (model Z1) with Channelizer and were 54 and 61 cuμ, respectively, when measured by comparison to standard human red cells (87 cuμ). This is in general agreement with other reported values, showing that erythrocytes of these species are significantly smaller than those of humans. Erythrocytes of all three species are biconcave discs.

Polypeptide profiles of human, pig, and mouse erythrocytes separated by SDS-PAGE utilizing a continuous buffer system and stained with Coomassie blue are shown in Fig. 1. The major differences between the species lies in the band 3 and band 4 regions. Where band 3 of the human migrated as a broad, diffuse band with an average apparent molecular weight of 95,000 daltons, band 3 of pig and mouse was more narrow, sharp, and had an average molecular weight of 105,000 and 102,000 daltons, respectively. A difference can also be seen in Fig. 2, where ghost protein was analyzed using the Laemmli discontinuous buffer system. It appears that mouse band 3 has a slightly higher average molecular weight than pig band 3 (98,000 versus 97,000). The reason for the different migrations on the Fairbanks versus the Laemmli gel system is not known, but is a consistent observation.

Band 4.1 migrated as an 80,000 (human), 78,000 (pig), or 76,500 (mouse) dalton polypeptide. Band 4.2 from the pig and mouse (75,000 and 72,500 daltons, respectively) migrated much closer to 4.1 in these systems. This is consistent with the notion that both pig and mouse have a single 4.1 protein. However, it is well known that human 4.1 migrates slightly closer to 4.2 and this may be the basis for the variation in molecular weight observed in human blood. To this end, we have analyzed human erythrocyte ghosts and have observed two closely migrating 4.1 bands, a high molecular weight 4.1a and a low molecular weight 4.1b. We have found that the mobility of these two bands is quite variable and that this may be the basis for the variation in molecular weights observed in human blood.

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SDS-PAGE by the Laemmli discontinuous buffer method resolved pig and mouse 4.1 into two polypeptides, 4.1a and b, as it does for human 4.1 (Fig. 2, lanes A, B, and C). Estimated molecular weights of 4.1a and b were 82,500 ± 900 and 80,500 ± 600 (human); 82,300 ± 800 and 79,200 ± 700 (pig); and 82,200 ± 1000 and 79,000 ± 1000 (mouse) (estimated from the relative mobilities on 3–6 gels of different ghost preparations). The relative amounts of 4.1a and b in the human ghost were approximately equal (a/b = 1.16 ± 0.07), but in pig and mouse ghosts, the ratio of 4.1a/b was 0.66 ± 0.06 and 0.60 ± 0.06, respectively. In each species, 4.1a plus b represents 6% of the total Comassie blue area of total ghost protein run on a 6% polyacrylamide Laemmli gel system. On the Laemmli gel system, although band 4.1a of human ghosts appears to stain slightly more intensely than 4.1b, 4.1b is always slightly broader, with the net result that peak areas are nearly equal. The membrane skeleton of each species contained the same major components, bands 1, 2, syndeins, 3, 4.1, 4.2, and 5. Band 4.1a and b were associated with the membrane skeleton in all species (Fig. 2, lanes D, E, and F). In the membrane skeleton of the human red cell, the ratio of a/b was increased (a/b = 2.01 ± 0.27, n = 5). It also appeared to be increased in the membrane skeleton of pig red cells, but not of mouse. As can be seen in Fig. 2 (G, H, I), two protein bands appear in the electrophoretic profile of the Triton X-100 extract of human and pig ghosts, in the area just below band 3. One-dimensional peptide maps of these bands from the extract of human ghosts (data not shown) indicated close sequence homology to...
bands 4.1 and 4.2. The band above 4.2 is tentatively labeled 4.1b on the basis of its proximity to 4.2. The relative mobility of bands 3, 4.1, and 4.2 were slightly altered, and the band 3 area increased in the gels of the Triton extracts (150 μl layered on each lane). Broadening of band 3 and a small decrease in mobility of band 4.2 was also seen if Triton X-100 was added to ghosts immediately prior to solubilization. This anomaly causes bands 4.1 and 4.2 to migrate closer together. Increased 4.1a/b in the membrane skeleton, and similar bands in the Triton extract, were seen when human blood was first passed through α-cellulose plus microcrystalline cellulose to remove leukocytes more completely, and when 1.0 mM dithiothreitol was included throughout the preparation and extraction of ghosts. In 6% polyacrylamide Laemmli gels, there were no PAS-positive bands exactly comigrating with band 4.1a or b (data not illustrated).

**Phosphorylation of Membrane Skeletal Components**

An autoradiograph of 32P-labeled ghost and membrane skeleton protein separated by SDS-PAGE utilizing the Laemmli discontinuous buffer system is shown in Fig. 3. Band 3 and 4.1a and b were phosphorylated in all three species. Band 4.2 from human and pig erythrocytes was not phosphorylated, but mouse 4.2 was a phosphoprotein. Markedly less phosphate incorporation occurred in the phosphoproteins of pig ghosts. This may be a function of the inability of pig cells to use glucose from the incubation medium as a substrate to maintain high ATP levels, since these cells do not transport glucose effectively. However, since spectrin and band 2.1 were phosphorylated fairly well in this medium, further possibilities remain that bands 3 and 4.1 (and 4.5) from pig erythrocytes contain less phosphate or lack another cofactor for phosphorylation. Phosphate transfer itself is not appreciably slower in pig erythrocytes. Band 2 and the syndeins from pig and mouse ghosts were also phosphoproteins, as they are in human erythrocytes (data not shown).

**Peptide Mapping of Band 3**

Band 3 was excised from SDS-PAGE of whole ghosts, Triton membrane skeletons, and Triton extracts of ghosts. Band 3 remaining with membrane skeletons after Triton extraction of ghosts is designated “bound” band 3, and band 3 solubilized by Triton and found in the supernatant after membrane skeletons were pelleted is designated “free” band 3, in accordance with the accepted definition of a membrane skeleton. A one-dimensional peptide map of iodinated total ghost band 3, bound band 3, and free band 3 from human, pig and mouse cleaved by α-chymotrypsin is presented in Fig. 4. These partial proteolytic maps clearly show that band 3 of each species is quite different, with few common proteolytic fragments produced. However, maps of band 3 bound to the membrane skeleton are identical to maps of free band 3 in each species. Identical conclusions were obtained from maps of phosphorylated band 3 (Fig. 5), and from maps produced by trypsin or thermolysin cleavage (results not illustrated). These conclusions support previous work showing that 90% of the peptides in band 3 behave homogeneously with respect to cleavage products of the bulk of band 3, and several other properties, and results demonstrating that bound band 3 separated by immunoprecipitation of triton extracts with anti-syndein antibodies and free band 3 separated by gel filtration of Triton-solubilized membranes gave nearly identical cyanogen bromide fragments.
Two species studied in this work, pig and mouse, have erythrocytes that are significantly smaller in volume than human erythrocytes. Mobilities of major bands 3 were mapped as described. 

**Peptide Mapping of Band 4.1**

Bands 4.1a and b were individually excised from Laemmli discontinuous buffer SDS-PAGE of whole ghosts. One-dimensional peptide maps of iodinated 4.1a and b, from all three species cleaved with α-chymotrypsin, is presented in Fig. 6. Maps of 4.1a and b were nearly identical within a species (as found by Goodman et al. for human 4.1), but each species was clearly different from the others, although they share some common chymotryptic peptides. Maps produced by chymotrypsin cleavage of phosphorylated 4.1a and b yield the same conclusions, with the exception of minor differences in mouse 4.1a and b (Fig. 7). In the mouse, 4.1a and b are sequence-related polypeptides as suggested by the nearly identical maps of the iodinated polypeptides, but they may have different sites of phosphorylation.

**DISCUSSION**

Two species studied in this work, pig and mouse, have erythrocytes that are significantly smaller in volume than human erythrocytes. Mobilities of major
components of the membrane skeleton, spectrin, bands 2.1, 4.2, and 5, are similar to those of human, but bands 4.1 and 3 differ. In erythrocytes of pig and mouse, band 4.1 has a smaller apparent molecular weight, and 4.1b is 3000 daltons smaller than 4.1a, compared to a difference of 2000 daltons between human 4.1a and b. In each species, 4.1a and b are phosphoproteins, and one-dimensional peptide maps show that they are sequence-related polypeptides and are species specific. In the two smaller erythrocytes, 4.1a/b ratio is 0.6, where the ratio is 1.2 in human erythrocytes. The finding of equal numbers of copies of a and b in humans differs from that of Sauberan et al., who reported a ratio of 2.53 for normal human 4.1a/b. The difference cannot be traced to loss of reducing agent in freezing and reheating samples before SDS-PAGE as those authors suggest, since identical results were found in the present studies when samples were electrophoresed immediately, or after freezing, without boiling in SDS (data not shown). Goodman et al. found a 4.1a/b ratio of 1.0 for normal human erythrocytes, and in figures published by King and Morrison and Mueller and Morrison, 4.1a and b appear to be stained with equal intensity.

The increased ratio of 4.1a/b in membrane skeletons of human and pig red cells could be due to extraction of some 4.1b by Triton X-100, to proteolysis, or to a combination of both. Abundant precautions were taken in these experiments to prevent proteolysis, including use of DFP, EDTA, dithiothreitol, and cold temperatures, and thorough removal of leukocytes. The possibility remains that a portion of band 4.1 is extracted by 1% Triton X-100 in low ionic strength medium.

Band 3 also differs in molecular weight and amino acid sequence in various species. Band 3 from both pig and mouse has a higher apparent molecular weight than human band 3, and all three species have substantially different one-dimensional peptide maps. Higher band 3 molecular weights, in fact, are seen in many species, and in these species, band 3 is much less heterogeneous with respect to its migration in SDS-PAGE, suggesting that the human erythrocyte may be the exception rather than the norm. Abnormal human erythrocytes may also exhibit variations in band 3. In each of the species in the present studies, band 3 bound to the membrane skeleton had a peptide map identical to that of free band 3, after limited proteolytic cleavage by three different enzymes. These results demonstrate that the membrane proteins bands 3, 4.1a, 4.1b, and 4.2 are membrane skeletal elements in all species studied, but that the molecular weights and relative proportions of the membrane skeletal elements are species specific. While the murine and porcine erythrocytes are serving as important models for studies in erythropoiesis, red cell senescence, and membrane structure, our results demonstrating limited sequence homology between murine and porcine bands 3, 4.1a, and 4.1b and their human erythrocyte counterparts, and substantially different band 4.1a/b ratios, underscore the necessity of careful structural and functional studies utilizing purified murine and porcine skeletal proteins before conclusions concerning similarities in protein interactions with the human erythrocyte membrane skeleton can be made.

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