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Research Article

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Isolation of Proteins Related to the Rh Polypeptides from Nonhuman Erythrocytes

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Abstract

It is thought that the Rh antigens may be important in maintaining normal erythocyte membrane integrity. Despite their name, Rh antigens are serologically present only on human erythrocytes. Rh structural polymorphisms are known to reside within a family of nonglycosylated M_r 32,000 integral membrane proteins that can be purified by hydroxylapatite chromatography. M_r 32,000 integral membrane proteins were purified similarly from erythrocyte membrane vesicles prepared from rhesus monkeys, cows, cats, and rats, but could not be purified from human Rh_{mod} erythrocytes, a rare syndrome lacking Rh antigens. The purified M_r 32,000 polypeptides were labeled with ^{125}I, digested with chymotrypsin, and found to be 30–60% identical to human Rh polypeptides when compared by two-dimensional iodopeptide mapping. The physiologic function of the Rh polypeptides remains to be identified; however, the existence of related proteins in nonhuman erythrocytes supports the concept that the Rh polypeptides are erythrocyte membrane components of fundamental significance.

Introduction

The complex Rh blood group system is of great clinical importance and has recently been characterized on a molecular level (see reviews 1–3). A family of M_r 32,000 erythrocyte integral membrane proteins (referred to as the Rh polypeptides) can be surface ^{125}I-labeled on intact erythrocytes and immunoprecipitated with antibodies specific for Rh (D), (c), and (E) (4, 5). No surface carbohydrate has been identified on the Rh polypeptides (6), and the Rh polypeptides are associated with the erythrocyte membrane skeleton (7, 8). Erythrocytes from Rh(D) positive and negative individuals bear Rh polypeptides that are similar but nonidentical (9) due to partial divergence among the D, c, and E polypeptides (10, 11). Purification of the Rh polypeptides has very recently been achieved by immunological (12) and nonimmunological methods (9, 13).

While it has been proposed that the Rh polypeptides play an essential role in erythrocyte membrane physiology, no primary role has yet been identified. Individuals with the rare Rh_{null} and Rh_{mod} syndromes (14) lack virtually all D, C, c, E, and e antigens. Rh_{null} erythrocytes are misshapen and fragile, and suffer from a variety of membrane defects including partial loss of phospholipid bilayer asymmetry (15) and abnormal function of multiple membrane transporters (16, 17).

The Rh antigens are found only on human erythrocytes. The Rh system was mistakenly named after the rhesus monkey due to confusion with the LW antigen (named after Landsteiner and Wiener), but erythrocytes from neither the rhesus monkey, other nonhuman primates, nor any other mammalian species contain the Rh antigens (18). Chimpanzees have a blood group system with similarities to Rh (19), but the serologically defined surface Rh epitope has undoubtedly diverged extensively between species, for considerable divergence exists even between Rh positive and negative humans. Nevertheless, if the core Rh polypeptides are of general importance to erythrocyte membranes, existence in diverse species should be expected.

Identification of proteins related to the Rh polypeptides in nonhuman erythrocytes and nonerythroid tissues is not possible with current immunological or molecular genetic methods. Rh-immune globulin reacts strongly with the Rh antigens only on intact membranes but fails to react on immunoblots. Likewise, no cDNA probe yet exists for identification of mRNA for the Rh polypeptides. An alternate approach would be to isolate putative nonhuman forms of the Rh polypeptides by the nonimmunological method that exploits the unique behavior of the Rh polypeptides on hydroxylapatite chromatography. Similarities between isolated proteins and the human Rh polypeptides may be confirmed by two-dimensional iodopeptide mapping. This report describes successful application of these methods to erythrocytes from four nonhuman species and demonstrates that proteins related to the Rh polypeptides are general components of mammalian erythrocytes.

Methods

Human blood anticoagulated with citrate-phosphate-dextrose-adenine solution was obtained from the American Red Cross (Baltimore, MD). Whole human blood from a donor with Rh_{mod} phenotype was provided by Dr. Peter Issitt, South Florida Regional Blood Center (Miami, FL). Whole blood was obtained from animal sources by the Johns Hopkins Comparative Medicine Department (Baltimore, MD) and anticoagulated with acid-citrate-dextrose or EDTA. Erythrocyte membrane vesicles were prepared and depleted of all peripheral proteins with 1 M KI, as described by Bennett (20).

Human Rh polypeptides were purified essentially as described (9). KI-stripped membrane vesicles were prepared from 100–200 ml of human erythrocytes and solubilized in 1.2% (wt/vol) SDS, 7.5 mM sodium phosphate (pH 7.4), and 1 mM DTT. A tracer of immunoprecipitated surface ^{125}I-labeled Rh protein was prepared (13) and ~ 20,000 cpm (< 1 μg protein) was added to the solubilized vesicles. The material was adsorbed onto and eluted from a 1.6 × 30-cm column packed with high resolution hydroxylapatite (Calbiochem-Beh-

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ring Corp., La Jolla, CA) and eluted isocratically with 0.2% (wt/vol) SDS, 0.3 M sodium phosphate (pH 7.4), 1 mM DTT, and 1 mM NaN_3 followed by a 500-ml gradient of 0.3–0.8 M sodium phosphate in the same buffer. More than 99% of all protein eluted before the peak of ^{125}I-Rh polypeptides, which eluted late in the gradient. The fractions containing the peak of ^{125}I-labeled tracer were combined, dialyzed against the same buffer with 7.5 mM sodium phosphate, and concentrated to 20 ml. The Rh polypeptides were further purified by preparative SDS-PAGE. Identical methods were used for isolation of proteins from 50–200 ml of blood from other species.

Two-dimensional iodopeptide maps were prepared from the isolated proteins using a method (9) adapted from that of Elder and colleagues (21). Basically, 10 μg of purified protein was precipitated in chilled acetone, solubilized in 5% SDS, and labeled with 1 mCi of ^{125}I after chloramine T oxidation. The ^{125}I-labeled proteins were isolated by preparative SDS-PAGE, and the desiccated gel slices were digested extensively with alpha chymotrypsin (Cooper Biomedical, Malvern, PA). The released peptides were analyzed in two dimensions on Merck cellulose TLC sheets: (*a*) electrophoresis, and (*b*) TLC. Iodopeptides were visualized by autoradiography.

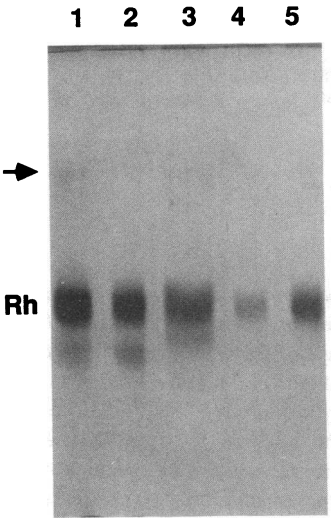

Figure 1. Silver-stained 12% SDS-PAGE gel (22) of human Rh polypeptides and M_r 32,000 proteins isolated from erythrocytes of four nonhuman species (see Methods): human (lane *1*), rhesus monkey (lane *2*), cow (lane *3*), cat (lane *4*), and rat (lane *5*). Differences in staining were due to unequal protein loading. The human Rh polypeptides are labeled "Rh" and the dimer is denoted with an arrow. The material of slightly faster electrophoretic mobilities in lanes *1*, *2*, and *3* is probably contaminant.

Results

Isolation of M_r 32,000 proteins. Hydroxylapatite chromatography of SDS-solubilized membrane vesicles permitted isolation of M_r 32,000 erythrocyte integral membrane proteins from each of several nonhuman species. Erythrocytes from the nonhuman species lack serologically detectable Rh antigens, but hydroxylapatite elutions of the M_r 32,000 proteins from each species coincided exactly with the peak of human ^{125}I-labeled Rh polypeptide tracer. Like the Rh polypeptides, the M_r 32,000 proteins eluted only at 0.6–0.8 M sodium phosphate which is above the concentration at which 99% of all erythrocyte integral membrane proteins are eluted (9).

The M_r 32,000 proteins were further purified by preparative SDS-PAGE and were compared with purified human Rh polypeptides (Fig. 1). All of the M_r 32,000 proteins migrated with electrophoretic mobilities identical to the human Rh polypeptides, and all formed electrophoretic bands with hazy, indistinct margins. Faint bands were also noted in the lanes of the cow and rat preparations (lanes *3* and *5*) that corresponded to the human Rh polypeptide dimers. The human and monkey preparations (lanes *1* and *2*) were contaminated with a small amount of a protein of M_r 28,000 that was determined by immunoblotting (not shown) to be identical to that recently described (23). This contaminant was removed during the SDS-PAGE step of two-dimensional iodopeptide analysis (below). The cow preparation (lane *3*) was contaminated by a small amount of a protein of M_r 31,000 that may represent degradation of the M_r 32,000 protein. Precise determination of the concentrations of the M_r 32,000 proteins in nonhuman erythrocytes was not attempted since this would require assumptions based on recovery of the human ^{125}I-labeled tracer. Nevertheless, ~ 100–200 μg of protein was isolated from each species, and analysis of SDS-PAGE gels indicated that the overall abundance of the nonhuman M_r 32,000 proteins is similar to the abundance of human Rh polypeptides, ~ 0.5% of the total membrane protein (9, 13).

Structural similarities. The nonhuman proteins were directly compared with human Rh polypeptides to assess the overall degree of similarity between the proteins. The purified proteins were denatured in SDS, labeled to high specific activity with ^{125}I, digested with chymotrypsin, and analyzed in two dimensions. Such analyses of human Rh polypeptides have previously demonstrated highly reproducible patterns. The iodopeptides are generally well separated on the two-dimensional maps, although some of the asymmetric spots probably represent partial overlap of two or more different iodopeptides (9). Consistent differences were previously noted between iodopeptides prepared from Rh polypeptides isolated from Rh(D)-positive and -negative humans (9). Two-dimensional iodopeptide maps of human Rh polypeptides have been shown to represent a composite of D, c, and E, which are related but nonidentical polypeptides (11).

Two-dimensional iodopeptide maps prepared from the M_r 32,000 proteins isolated from each of the nonhuman species demonstrated a different pattern for each species, but similarities to human Rh polypeptides were always apparent. Two iodopeptides were consistently identified in the digests of all species (denoted by arrows in Fig. 2, *top right*). Three iodopeptides were found in the human preparation and three of the four nonhuman preparations. At least three iodopeptides were found only in the human preparation. While the preparations from the different nonhuman species were distinct from one another, several iodopeptides from each nonhuman preparation comigrated with some of the human iodopeptides. Moreover, the shapes of the comigrating iodopeptides were also generally similar. The least homology was noted for the cow preparation from which 5 of 17 iodopeptides directly overlay human iodopeptides. The greatest homology was noted for the rat preparation from which 8 of 14 iodopeptides directly overlay human iodopeptides. Intermediate degrees of homology were noted for the rhesus monkey and cat preparations from which 7 of 18 and 7 of 14 iodopeptides overlay human iodopeptides, respectively. The similarities were highly reproducible when assessed by repeated analyses of the same or newly prepared iodopeptides. Digestions with trypsin were technically less satisfactory due to incomplete digestion and smearing (not shown), yet the overall similarities between human and nonhuman proteins were notable.

Discussion

This report describes isolation from nonhuman erythrocytes of proteins that are related to the human Rh polypeptides. The

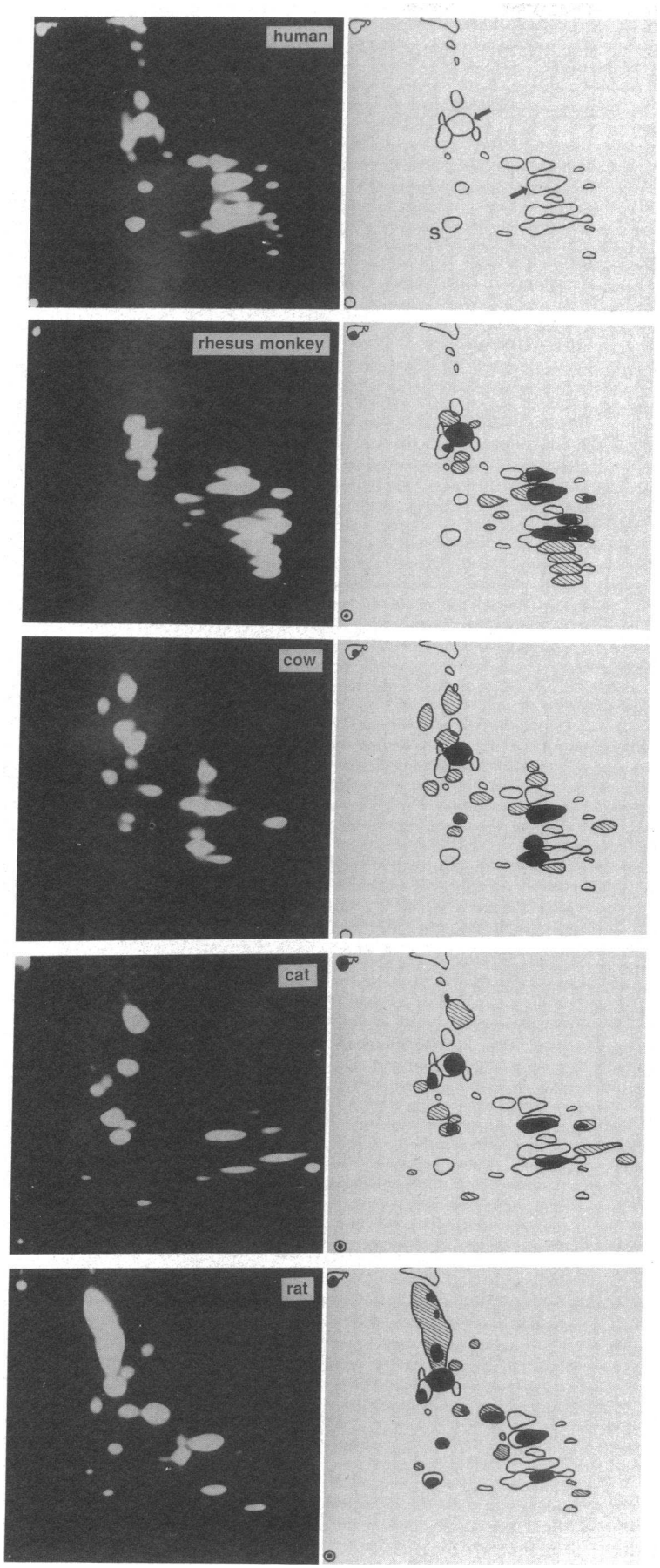

Figure 2. Two-dimensional iodopeptide maps of M_r 32,000 Rh polypeptides isolated from erythrocytes from humans and four nonhuman species. The purified polypeptides were denatured in SDS, labeled with ^{125}I, digested with chymotrypsin, and analyzed in two dimensions (see Methods): electrophoresis (*horizontal*); TLC (*vertical*); the origin was at the lower left. Autoradiographs of the two-dimensional iodopeptide maps are on the left. Drawing of the autoradiograph of the human preparation is at the upper right; the iodopeptide corresponding to the surface-labeled domain described by Blanchard et al. (11) is denoted "S"; two iodopeptides shared by all species are denoted by arrows. Composite drawings comparing autoradiographs of nonhuman proteins and the human Rh polypeptides are on the right: open figures represent iodopeptides specific for human Rh polypeptides; hatched figures represent iodopeptides specific for the indicated species; solid figures represent iodopeptides common to both human and the indicated nonhuman preparation.

existence of such proteins supports the concept that the core M_r 32,000 Rh polypeptides are common to all mammalian erythrocytes and implies that they may play a fundamental role in membrane physiology. Identification of such a specific role has not been made, although contribution to the organization of the phospholipid bilayer is a likely possibility (15). Erythrocytes from a human with the Rh_{mod} syndrome were the only erythrocytes from which the hydroxylapatite method failed to isolate M_r 32,000 polypeptides (data not shown). Presumably the Rh_{mod} mutation leads to decreased synthesis or reduced stability of the M_r 32,000 Rh polypeptides, and this is probably the explanation for the nearly total lack of Rh antigens and the abnormalities of membrane organization characteristic of this syndrome.

Since the cDNA for the Rh polypeptides has not yet been obtained, isolation and two-dimensional iodopeptide mapping is a useful approach for comparing the Rh polypeptides to potentially related proteins. This method is limited, since it will only detect peptide fragments containing ^{125}I-labeled tyrosines. Also, two-dimensional maps tend to accentuate differences between proteins, and migration of each iodopeptide can be drastically altered by a single amino acid substitution. Identical two-dimensional migration of 30–60% of iodopeptides from four other species with human iodopeptides is therefore highly significant. Generally < 10% of iodopeptides derived from unrelated proteins will coincidentally comigrate on two-dimensional maps.

These studies were feasible because of the fortunate, atypical chromatographic behavior of this family of proteins which permitted their isolation by hydroxylapatite chromatography. A nonimmunological approach was essential since Rh serological activity is not present on the nonhuman erythrocytes. The nonhuman M_r 32,000 proteins may not be associated with surface antigens and may not even contain extracellular domains. Unlike the human D, c, and E polypeptides which can be ^{125}I-labeled on the extracellular surface of intact erythrocytes (4, 5), attempts to similarly surface-label the nonhuman erythrocytes failed to consistently label M_r 32,000 membrane components (not shown). Indeed, of the iodopeptides from all nonhuman preparations, only the rat preparation contained an iodopeptide that comigrated with the iodopeptide previously demonstrated to correspond to the surface ^{125}I-labeled domain of the human Rh polypeptides (Fig. 2, *right*). Identification of two iodopeptides common to the preparations from all five species indicates that these portions of the protein may be highly conserved. A common functional domain may exist in the M_r 32,000 protein which requires a highly specific amino acid sequence for biological activity.

It remains to be established if proteins related to the Rh polypeptides exist in nonerythroid tissues. Kidney membranes contain several proteins related to erythrocyte membrane proteins, and membrane proteins of M_r 24,000, 30,000, and 36,000 were isolated by hydroxylapatite chromatography with elution behavior similar to the Rh polypeptides. However, when these kidney membrane proteins were analyzed by two-dimensional iodopeptide mapping there was no significant similarity to the Rh polypeptides (data not shown). The complexity of such nucleated cells may preclude this approach which has proven successful for erythrocytes. It is likely that molecular genetic techniques will provide better understanding of the relationships between Rh-related proteins, identification of their distribution in various tissues, and ultimate elucidation of the normal physiologic function.

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