Involvement of Rh Blood Group Polypeptides in the Maintenance of Aminophospholipid Asymmetry†

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ABSTRACT: The human erythrocyte (RBC) Rh blood group system consists of a complex of distinct integral membrane polypeptides with physical properties common to the aminophospholipid transporter responsible for the transbilayer movement of phosphatidylserine (PS) in RBC. To assess the involvement of Rh polypeptides in PS translocation, the aminophospholipid translocase was labeled with a photoactivatable PS analogue, ^{125}I-azido-PS, and with an inhibitor of PS transport, ^{125}I-labeled 2-(2-pyridyldithio)ethylamine. The ability of monoclonal Rh antibodies to immunoprecipitate the labeled transporter was determined. Immunoprecipitated Rh polypeptides were found to be labeled with the aminophospholipid translocase markers, suggesting that Rh proteins are involved in the transbilayer movement of PS.

The highly asymmetric transmembrane organization of phosphatidylserine (PS)[1] in RBC is maintained by an ATP- and temperature-dependent, cysteine-containing, aminophospholipid-specific translocase (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985). Recent studies have suggested that the translocase is a M_r 32 000 (Schroit et al., 1987; Connor & Schroit, 1988) nonglycosylated (Connor & Schroit, 1989) integral membrane polypeptide associated with the cytoskeleton (Connor & Schroit, 1990). Interestingly, several 30–32-kDa integral membrane proteins have been identified in RBC as the polypeptides bearing defined Rh alleles (Moore et al., 1982; Gahmberg, 1982; Saboori et al., 1988; Bloy et al., 1988a; Blanchard et al., 1988; Hughes-Jones et al., 1988). These polypeptides, which contain the major RhD antigen and associated E/e and C/c epitopes, are also nonglycosylated (Gahmberg, 1983) cysteine-containing polypeptides (Green, 1967, 1983) associated with the membrane skeleton (Ridgwell et al., 1984; Gahmberg & Karhi, 1984; Paradis et al., 1986; Bloy et al., 1987). In addition, recent studies have indicated that Rh polypeptides are major fatty acid acylated proteins (de Vetten & Agre, 1988) and that their immunoreactivity and sensitivity to proteases is dependent upon complexed lipid (Green et al., 1984; Suyama & Goldstein, 1990). The similarities between the properties of Rh polypeptides and the aminophospholipid transporter suggest that the Rh blood group system may play a role in maintaining membrane phospholipid asymmetry (de Vetten & Agre, 1988). In this report, we show that monoclonal antibodies against defined Rh epitopes (Bloy et al., 1988b) immunoprecipitate a 32-kDa polypeptide specifically labeled with a transportable photoactivated PS analogue, ^{125}I-azido-PS (Schroit et al., 1987), or with an inhibitor of PS transport, ^{125}I-labeled 2-(2-pyridyldithio)ethylamine (Connor & Schroit, 1988). These results suggest that Rh polypeptides are involved in the maintenance of PS asymmetry in RBC.

Experimental Procedures

Materials and Routine Procedures. Red cells bearing defined Rh alleles [D– – (–D–/–D–), R_1R_1 (CDe/CDe), R_2R_2 (cDE/cDE), rr (cde/cde), or no known Rh epitopes (Rh null cells)] were obtained from Gamma Biologicals Inc. (Houston) and from the National Blood Group Reference Center (Paris). Human monoclonal anti-D, -E, and -c of the Rh blood group system were produced from Epstein–Barr virus transformed B lymphocytes from immunized donors (Bloy et al., 1988b). Carrier-free (sp act. ~2000 Ci/mmol) ^{125}I-PDA and carrier-free ^{125}I-N_3-PS were prepared as previously described (Connor & Schroit, 1988; Schroit et al., 1987). The ability of cells to transport PS was determined as described previously (Connor & Schroit, 1988) with fluorescent, NBD-labeled lipids. The fraction of lipid not transported to the cells' inner leaflet was determined by the ability to "back-exchange" lipid accessible to 2% bovine serum albumin.

Membrane Labeling. RBC were washed with PBS before use. The cells (2×10^8/mL) were then mixed with ~1 μCi of ^{125}I-PDA or ^{125}I-N_3-PS in ethanol (final EtOH concentration <1%) and incubated for 20 min at 0 or 37 °C, respectively. The cells were then washed with ice-cold PBS. All experiments with the photolabeled lipid were carried out under a red safelight. These cells were transferred to 1.0-cm quartz cuvettes and irradiated with constant mixing 12 cm for an Osram HBO 100W/2 super-pressure mercury lamp for 2 min. To identify the proteins labeled with the probes, ghosts were prepared by hypotonic lysis (5 mM phosphate buffer, pH 8.0), solubilized with SDS, and subjected to SDS–polyacrylamide electrophoresis (without reductants) using 11% separating and 6% stacking gels with the discontinuous buffer system of Laemmli (1970). The gels were fixed, dried, and autoradiographed with Kodak XAR-5 film.

Immunoprecipitation. Red cells bearing defined Rh alleles were labeled with ^{125}I-PDA or ^{125}I-N_3-PS as described above. Ghosts (~4×10^8) were prepared by hypotonic lysis, and the pellets were resuspended in 400 μL of PBS containing 50 μg of antibody. The suspension was mixed overnight at 4 °C and washed with PBS. The membranes were solubilized in 300 μL of PBS containing 3% (w/v) Triton X-100 at 4 °C for 1

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[1] Abbreviations: ^{125}I-PDA, *N*-[3-(3-[^{125}I]iodo-4-hydroxyphenyl)-propionyl]-2-(2-pyridyldithio)ethylamine; ^{125}I-N_3-PS, 1-oleoyl-2-[[[3-(3-[^{125}I]iodo-4-azidophenyl)propionyl]amino]caproyl]phosphatidylserine; PBS, phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 10 mM sodium/potassium phosphates, 10 mM glucose, pH 7.4); PDA, 2-(2-pyridyldithio)ethylamine; PS, phosphatidylserine; RBC, human red blood cells.

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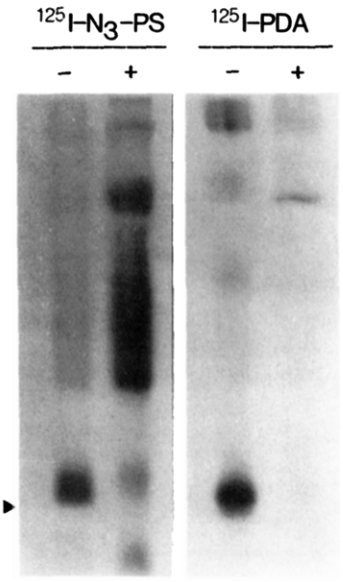

FIGURE 1: Inhibition in the labeling of the 32-kDa polypeptide by PDA. Control RBC (–) or RBC pretreated with 2 mM PDA (+) for 30 min at 0 °C were washed and incubated with ^{125}I-PDA or ^{125}I-N$_3$-PS to label the PS transporter. Ghosts were prepared by hypotonic lysis, and the distribution of ^{125}I-labeled proteins was determined by autoradiography after separation of the polypeptides by SDS–PAGE. The arrow denotes the position of the 31-kDa standard (carbonic anhydrase).

h. Supernatants obtained after centrifugation (20000*g* for 30 min) were incubated with 50 μL of a 50% suspension of protein A–Sepharose (Sigma) for 90 min at 4 °C and washed as described (Bloy et al., 1988b). Sepharose-bound Rh polypeptides were released by boiling with SDS and analyzed by autoradiography after SDS–PAGE.

Results

Association of Photolabeled Substrate and Inhibitor with the 32-kDa Polypeptide. We have previously shown that a labeled substrate (^{125}I-N$_3$-PS) and an inhibitor of PS transport (PDA) bind to 32-kDa polypeptides. To determine whether both probes bind to the same membrane protein, the ability of PDA to inhibit the labeling of the 32-kDa polypeptide by ^{125}I-N$_3$-PS and ^{125}I-PDA was determined. Figure 1 shows that PDA completely abrogated the association of both of these probes to the polypeptide(s). Interestingly, when ^{125}I-N$_3$-PS binding was blocked by PDA, it appeared to diffuse throughout the membrane, resulting in a random distribution similar to that obtained with a phosphatidylcholine analogue (Schroit et al., 1987).

Immunoprecipitation of RBC Labeled with ^{125}I-PDA and ^{125}I-N$_3$-PS. RBC expressing different Rh alleles were labeled with the PS transport inhibitor ^{125}I-PDA. Ghosts were prepared by hypotonic lysis and incubated with monoclonal antibodies against defined Rh antigens. Analysis of immune complexes precipitated with protein A–Sepharose from supernatants obtained from Triton X-100 solubilized ghosts revealed prominent labeling of these proteins with the PS transport inhibitor ^{125}I-PDA (Figure 2). Immunoprecipitation occurred only when monoclonal antibody was incubated with RBC of the appropriate phenotype. Thus, anti-c, anti-D, and anti-E precipitated labeled Rh polypeptides from R_2R_2 (cDE/cDE) cells, but only monoclonal anti-c precipitated polypeptides from rr (cde/cde) cells because these cells do not express the D or E alleles. Similarly, only anti-D precipitated Rh polypeptides from D– – cells since these cells completely lack both the C/c and E/e epitopes.

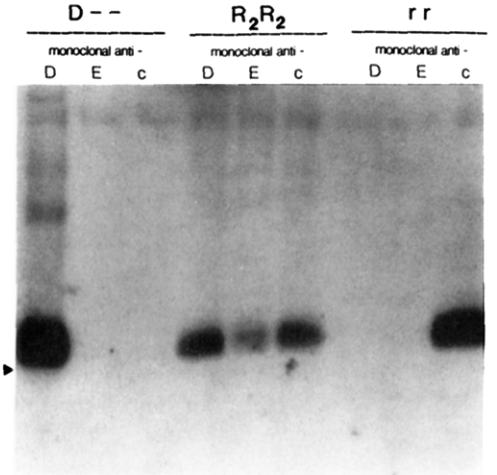

FIGURE 2: Immunoprecipitation of ^{125}I-PDA-labeled aminophospholipid translocase by monoclonal anti-RhD, anti-RhE, and anti-Rhc. Red cells with the phenotypes D– –, R_2R_2 (cDE/cDE), and rr (cde/cde) were labeled with ^{125}I-PDA as described under Experimental Procedures. Ghosts were prepared, and the cells were incubated with monoclonal anti-D, anti-E, or anti-c. Immune complexes precipitated from Triton X-100 lysates with protein A–Sepharose were analyzed for the presence of ^{125}I-labeled Rh polypeptides by autoradiography by SDS-PAGE. The arrow denotes the 31-kDa marker.

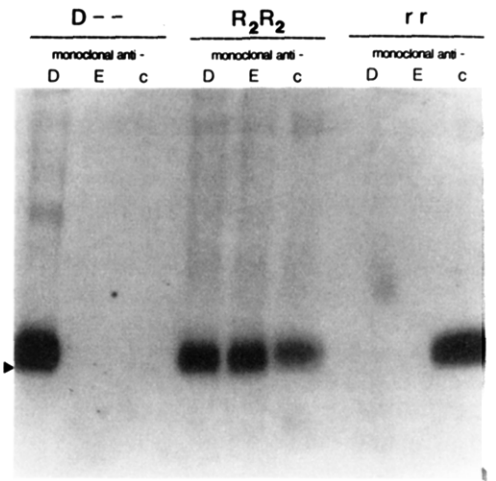

FIGURE 3: Immunoprecipitation of ^{125}I-N$_3$-PS-labeled aminophospholipid translocase by monoclonal anti-RhD, anti-RhE, and anti-Rhc. D– –, R_2R_2, and rr cells were labeled with ^{125}I-N$_3$-PS as described under Experimental Procedures. Immune complexes precipitated from ghosts incubated with anti-D, anti-E, or anti-c were analyzed as described for Figure 2. The arrow denotes the 31-kDa marker.

Similar data were obtained from RBC labeled with the PS analogue ^{125}I-N$_3$-PS. Specific immunoprecipitation of ^{125}I-N$_3$-PS-labeled Rh polypeptides occurred only when the appropriate combination of antibody and RBC phenotype was employed. Figure 3 shows that PS-labeled aminophospholipid translocase was immunoprecipitated from D– – cells only with anti-D. As shown in Figure 2, anti-D, anti-E, and anti-c immunoprecipitated Rh polypeptides from R_2R_2 cells, whereas only anti-c immunoprecipitated polypeptide from rr cells. Other experiments using R_1R_1 (CDe/CDe) cells labeled with ^{125}I-PDA or ^{125}I-N$_3$-PS gave immunoprecipitation profiles identical with those obtained with D– – cells (data not shown).

Association of Photolabeled Substrate and Inhibitor with the 32-kDa Polypeptide in Rh Null Cells. Analysis of the various antibody/RBC combinations (Figures 2 and 3) shows that all of the Rh polypeptides, irrespective of their antigenic phenotype, are labeled by both ^{125}I-PDA and ^{125}I-N$_3$-PS. This

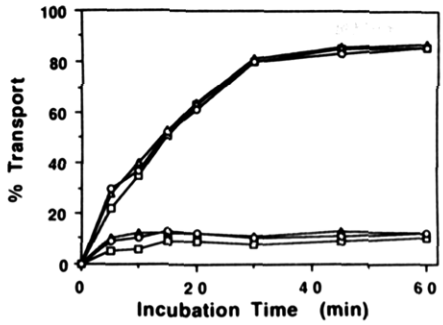

FIGURE 4: Translocation of NBD-PS and NBD-PC from the outer-to-inner membrane leaflet of RBC. R_1R_1 (○), rr (△), or Rh null cells (□) were incubated with NBD-PS (three upper curves) or NBD-PC (three lower curves) (1 μg of lipid/2 × 10^6 cells) at 37 °C. At the indicated intervals, the fraction of NBD-labeled lipid in the inner leaflet was determined by the inability to remove the lipid by back-exchange to BSA.

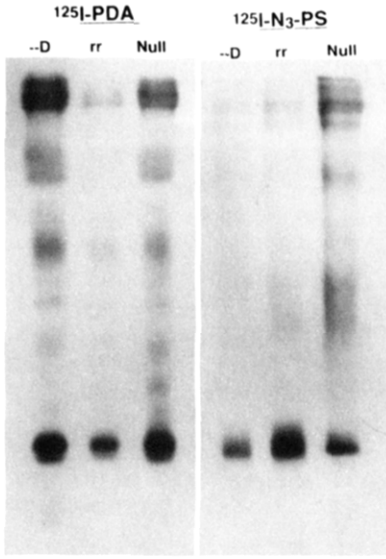

FIGURE 5: Labeling of the 32-kDa polypeptide in D−−, rr, and Rh null cells by ^{125}I-PDA and ^{125}I-N_3-PS. RBC were labeled with the indicated probes, and ghosts were analyzed by SDS–PAGE as described for Figure 1. Increased PDA labeling in the spectrin region of D−− cells is due to the use of cells taken from frozen stock.

finding, combined with the fact that D−− cells do not express C/c or E/e epitopes, suggests that Rh polypeptides are involved in the transbilayer movement of PS in a manner that is independent of antigenic phenotype or presence of specific Rh alleles. To verify this, the ability of Rh null cells, RBC that express no known Rh antigens (Sturgeon, 1970), to transport PS and to be labeled with ^{125}I-PDA and ^{125}I-N_3-PS was assessed. The results shown in Figures 4 and 5 indicate that these cells transport PS and are labeled by both probes in a manner indistinguishable from that of Rh antigen bearing cells.[2]

Discussion

The results presented here implicate the involvement of Rh blood group polypeptides in the transbilayer movement of PS and in the maintenance of PS asymmetry in blood cells. The observation that all RBC, irrespective of Rh phenotype, transport PS and are labeled by both ^{125}I-PDA and ^{125}I-N_3-PS indicates that antigenic epitopes expressed on the cell surface do not play a role in the transport process. Indeed, attempts to inhibit PS transport with appropriate monoclonal (results not shown) or polyclonal antibodies or by digestion of cell surface proteins with a variety of proteases failed to affect PS transport (Connor & Schroit, 1989). This indicates that the portion of the polypeptide critical to its transport activity is buried in the bilayer membrane. These observations suggest that, functionally, the translocase is associated with the Rh system, albeit independent of its serological phenotype. This conclusion is supported by results obtained with Rh null cells, which possess a functional PS translocase but do not express any known Rh antigens (Sturgeon, 1970). Although these cells have defects in ion transport (Lauf & Joiner, 1976; Ballas et al., 1984) and demonstrate increased susceptibility of phosphatidylethanolamine to phospholipase (Kuypers et al., 1984), they still retain normal PS translocase activity (Figure 4) and probe-labeling profiles (Figure 5) and do not express endogenous PS on their surface as determined by their inability to promote prothrombinase complex activity (data not shown).

The observation that Rh null cells are identical with Rh antigen bearing RBC in all aspects of the PS transporters' activity and characteristics raises the possibility that these cells express a functional Rh-like polypeptide/translocase that is immunologically unrelated to known Rh phenotypes (Suyama & Goldstein, 1988). Alternatively, the transporter could be a distinct protein or subunit that is complexed to the antigen-bearing Rh polypeptides. At the present time, however, it is not possible to rigorously exclude either of these possibilities. If Rh polypeptides and the PS transporter are distinct proteins or subunits, the complex would have to be stable in the presence of Triton X-100. This possibility could conceivably be addressed by determining whether the number of labeled molecules corresponds to the number of Rh polypeptides per cell. However, since only isotopic amounts of the probes can be used, quantifying the number of labeled molecules in the immunoprecipitates cannot yield meaningful results. The presence of distinct proteins might be supported should biochemical studies, as opposed to immunological reactivity, indicate that Rh null cells lack any Rh-like polypeptides, although such an observation would not preclude the existence of subunits.

Since the maintenance of PS asymmetry probably represents a fundamental physiological process and Rh-like polypeptides (Saboori et al., 1989)/PS transporter (Connor & Schroit, 1989) are found in all mammalian red blood cells, it is possible that the functional (membrane-spanning) portion of the protein is highly conserved unlike the antigenic diversity observed at the cell surface. This, in fact, is supported by the results of Saboori et al. (1989), who have shown up to 60% cross-species homology between isolated "Rh-like" polypeptides by iodopeptide mapping.

In conclusion, the data presented here demonstrate that the 32-kDa polypeptide responsible for the intrabilayer transport of PS is associated with the Rh blood group complex. Whether the PS transporter and Rh polypeptides are distinct or analogous proteins remains to be determined. The latter possibility, however, is supported by recent cloning data that indicate that the Rh protein is a multi spanning membrane polypeptide, a property common to other membrane proteins associated with transport and channel functions (Cherif-Zahar et al., 1990). The observation that many cells have an asymmetric distribution of PS across the bilayer membrane and specifically transport PS from the outer-to-inner leaflet (Martin & Pagano, 1987; Zachowski et al., 1987; Sune et al., 1987) suggests that antigenically distinct Rh-like proteins might also be present

[2] The data presented in Figures 4 and 5 are representative from one individual. Identical results have been obtained from four Rh null.

in nonerythroid cells as they are in all mammalian RBC (Saboori et al., 1989).

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