Catabolism of the Anion Transport Protein in Human Erythrocytes†

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ABSTRACT: We identified the catabolic products of protein 3 in human erythrocytes. Protein 3, the major protein of the erythrocyte membrane, functions in anion transport and reacts covalently with tritiated 4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid ([^{3}H]DIDS), a very selective inhibitor of anion transport. In this study, [^{3}H]DIDS was used to label protein 3 in the membranes of normal cells and those from a donor heterozygous for a variant of protein 3, defined by its elongated amino-terminal end. Both types of cells contained [^{3}H]DIDS-labeled peptides other than protein 3. A protein fragment of 60K molecular weight was found in normal cells, whereas both 60K and 63K fragments were identified in cells from the heterozygote. These peptides are identical with those generated by treatment of intact erythrocytes with Pronase or chymotrypsin. A polyclonal rabbit antibody specific for the purified 60K fragment of protein 3 was used to detect this protein and its products in the erythrocyte membrane. Autoradiographs of membrane peptides that were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose, and allowed to react with the monospecific antibody showed, in addition to protein 3, a 60K fragment and fragments in the 40K region and in the 20–30K region. Cells containing the protein 3 variant yielded two fragments showing a 3K difference in molecular weight in all three regions, demonstrating that degradation of protein 3 is identical in normal erythrocytes and those heterozygous for the variant. This observation also confirms the common derivation of the fragments from protein 3. Since the amino-terminal end of protein 3 is elongated in the variant molecule, we conclude that all of the fragments contain this region. The 60K and 40K fragments, therefore, are clearly derived from cleavage at the bilayer, the former at the outside of the cell and the latter at the cytoplasm–membrane interface. The lower concentration of the 60K fragment in highly enriched reticulocyte fractions compared with more mature erythrocytes suggests an accumulation of catabolic products with maturation.

Protein catabolism represents a distinct mechanism of posttranslational cellular control by which the concentration of any protein is regulated by its rate of degradation. The half-life, or turnover, of proteins has been widely studied in mammalian organisms and has been found to vary greatly. The mature mammalian erythrocyte affords a useful model for the study of integral membrane protein catabolism. Its membrane is easy to isolate in pure form and has been characterized more fully than any other biological membrane. Even though the mature erythrocyte has lost all of its subcellular organelles, and hence its biosynthetic capacity, proteins continue to be degraded and can be studied without interference from newly formed proteins. There is a progressive loss of most of the glycolytic enzyme activities with age (Seaman et al., 1980). The enzyme acetylcholinesterase, a membrane protein that is exposed on the outside of the cell, loses about half of its activity as it ages (Herz et al., 1972).

Despite clear evidence for loss of biological activities in aging erythrocytes, little is known about the fate of membrane proteins once their activity is lost. To address this question, we focused our studies on the major membrane protein of the human erythrocyte, protein 3. Protein 3 performs two key functions in the membrane: it facilitates anion transport and is the binding site on the cytoplasmic surface of the membrane for the cytoskeletal complex and cytoplasmic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase, aldolase, and hemoglobin [for recent reviews, see Maeara & Cantley (1983) and Jennings (1984)]. It not only binds proteins on the cytoplasmic side of the membrane but also may be involved in binding on the outside of the cell, where a portion of the molecule, a large oligosaccharide chain, is exposed.

In order to identify catabolic products of protein 3, we have used site-specific chemical modification and immunochemical techniques. Two previously described properties of protein 3 have allowed us to define the regions of the molecule from which the catabolic peptides are derived. First, the protein 3 molecule is very selectively modified by the anion transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)[1] (Rothstein, 1984). The site of covalent DIDS attachment is located close to where band 3 emerges from the membrane bilayer and is contained in the 60K amino-terminal portion of the molecule which can be generated by treatment of intact cells by protease digestion. Second, we took advantage of heterozygotes for a variant form of protein 3 which is elongated by approximately 3 kDa (Mueller & Morrison, 1977; Morrison et al., 1981). We have localized this elongation to the amino-terminal 23-kDa portion of the molecule. Thus, we are able to use these two markers to determine the part of the protein 3 molecule from which the catabolic fragments were derived. A preliminary report of this work has appeared (Grant et al., 1982).

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[1] Abbreviations: H_2DIDS, dihydro-4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid; PBS, phosphate-buffered saline; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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Experimental Procedures

Materials. Reagent-grade chemicals were used wherever possible. Tritiated dihydro-4,4′-diisothiocyano-1,2-diphenylethane-2,2′-disulfonic acid (H_2DIDS; specific activity, 0.5 Ci/mmol) was a generous gift of Drs. Saul Ship and Aser Rothstein. The dried reagent was dissolved to a concentration of 1 mCi/mL with distilled water and stored in small aliquots at −70 °C.

Human erythrocytes were collected in an acid–citrate–dextrose solution by personnel in the hospital's blood bank. The whole blood was centrifuged at 3000*g* for 10 min and the plasma supernatant removed. The cells were resuspended in 10 volumes of isotonic phosphate-buffered saline (PBS) and then recentrifuged. This procedure was repeated 3 times, followed by a final wash in PBS. In some instances, the erythrocytes were passed through a cellulose column to ensure separation of all leukocytes (Beutler et al., 1976).

[^{3}H]H_2DIDS Labeling and Proteolytic Digestion of Intact Red Cells. The red cells were washed 3 times as described above and twice with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) and 0.145 M NaCl, pH 7.4 (Hepes–saline). The packed cells were diluted with 3 volumes of Hepes–saline and incubated for 30 min at 37 °C with 4 μCi of [^{3}H]H_2DIDS per milliliter of cell suspension. The cells were then washed 3 times with 10–15 volumes of Hepes–saline containing 0.5% bovine serum albumin (BSA) and twice with PBS, pH 7.4. The labeled cells were diluted 1:10 with PBS and digested with 100 μg/mL Pronase for 1 h at 37 °C. The suspension was incubated for an additional 15 min at 37 °C with 0.8 mM phenylmethanesulfonyl fluoride (PMSF), followed by dilution with 1 volume of cold PBS containing 0.4 mM PMSF and then centrifugation. The packed cells were washed 3 times with 20 volumes of cold PBS with 0.4 mM PMSF. Stroma were prepared by lysing and washing the cells until they were white with 80 volumes of cold (4 °C) 7 mM phosphate buffer, pH 7.4, containing 0.4 mM PMSF.

Gel Electrophoresis. Membranes were boiled for 3 min with gel sample buffer, and the solubilized proteins were separated on 7.5–15% gradient slab gels by using the discontinuous buffer system of Laemmli (1970), as previously described (Mueller & Morrison, 1977). Gels stained with Coomassie blue R-250 and destained (Mueller & Morrison, 1977) were prepared for fluorography as described by Bonner & Laskey (1974). Radioactive bands were detected by exposure to Kodak XR-5 medical X-ray film at −70 °C.

Density Fractions. The cells were separated from the plasma by low-speed centrifugation. Fifty percent of the plasma was removed. This plasma, spun free of platelets and leukocytes at 40000*g* for 1 h, was later used to resuspend the erythrocytes, which had been passed through a cellulose column to reduce possible contamination from leukocytes and platelets (Beutler et al., 1976). Cells obtained in this way were centrifuged again to remove the diluted plasma that remained with the cells. The red cells were then resuspended in platelet-free plasma to a hematocrit value of 50% and separated into density fractions by centrifugation at 30 °C for 1 h in an angle centrifuge at 30000*g*. The top and bottom 2% of the cells obtained by this modification of the Murphy (1961) procedure were then washed free of plasma by centrifugation in PBS as described earlier. The dense and light fractions were further separated into the least dense and most dense fractions on a self-forming Percoll gradient (Vettore et al., 1980). Each fraction comprised less than 1% of the initial volume of red cells. Cells obtained by this procedure were then washed free of Percoll in PBS, pH 7.4, at 4 °C, and their membranes were prepared as described previously.

Immunochemical Procedures. Fresh red cells were collected and washed as described in the previous section. Washed cells were digested with Pronase, their protease activity was inhibited, and their membranes were prepared as noted earlier. Washed membranes were stripped of peripheral exogenous proteins by extraction with 0.1 N NaOH and then washed, usually 3 times, with hypotonic phosphate containing 0.4 mM PMSF and 1 mM EDTA to neutralize the base. After the final wash, the stripped membranes were resuspended in half the original volume of the same buffer. Resuspended membranes were boiled for 3 min with sample buffer, and proteins were separated on sodium dodecyl sulfate (SDS)–polyacrylamide gels. The band corresponding to the 60K transmembrane fragment of protein 3 was visualized by incubating the gels in cold (4 °C) 0.5 M KCl for 10 min and excising the relevant portion of the gel with a sharp knife. The gel slice was homogenized with equal volumes of PBS and complete Freund's adjuvant, and approximately 100–200 μg of the homogenate was injected intramuscularly into the rear flanks of female New Zealand rabbits. Rabbits were boosted bimonthly with incomplete Freund's adjuvant.

Immunoblot Technique. Components that reacted with the antibodies to the 60K fragment were determined by the immunoblot technique. Membrane proteins separated on SDS–polyacrylamide gels, as outlined earlier, were transferred to nitrocellulose paper (Gershone & Palade, 1983; Towbin et al., 1979). After 3 h at 0.5 A or overnight at 0.1 A, the nitrocellulose was removed, rinsed for 1 h with PBS, and treated in 10 mM Tris buffer, pH 7.4, 150 mM NaCl, and 0.02% NaN_3 containing 2% casein to block nonspecific protein binding to the nitrocellulose. The nitrocellulose was then incubated overnight with specific rabbit antibody diluted in the same buffer. Unbound antibody was removed by washing 6 times (30 min each) with casein buffer; bound antibody was detected by incubating the membranes with ^{125}I-labeled protein A (1×10^6 cpm/mL for 1 h). Bound radioactivity was detected by autoradiography of the dried nitrocellulose with Kodak X-Omat AR film.

In order to ensure that our antibody was monospecific, we produced affinity-purified immunoglobulin. The protein 3 region of a nitrocellulose blot was cut out. The antibody reacting with protein 3 was dissociated from the paper by treatment with 4 M NaSCN. The supernatant containing the immunoglobulin was then dialyzed to remove the thiocyanate and concentrated by lyophilization.

Results

Intact erythrocytes, treated with [^{3}H]H_2DIDS, careful to inhibit protease activity, were isolated and their [^{3}H]H_2DIDS-labeled components identified (Figure 1). Protein 3 was clearly labeled in membrane preparations from normal donors, as was another component with an apparent molecular weight of 60K.

Figure 1 also shows the membrane peptides of a heterozygote whose erythrocytes contained both the normal and variant forms of protein 3 (Mueller & Morrison, 1977). The variant form has a molecular weight which exceeds that of the normal protein by 3K as a result of the elongated amino-terminal region. Besides protein 3, [^{3}H]H_2DIDS labeling extended to two other components, one of 60K and the other of 63K molecular weight. These components appeared identical in size with the components generated when [^{3}H]H_2DIDS-labeled intact red cells were treated with Pronase. Thus, it would appear that the 60K and 63K fragments preexist in the membrane and interact irreversibly with [^{3}H]H_2DIDS.

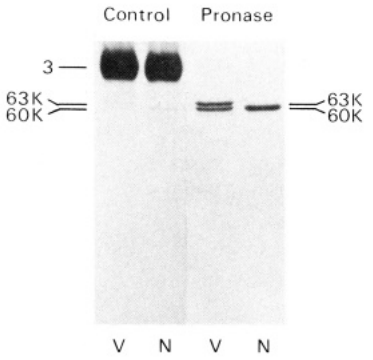

FIGURE 1: Intact red cells from a normal donor (N) and from a donor heterozygous for the band 3 variant (V) were labeled with [^{3}H]-H_2DIDS and digested with Pronase as described under Experimental Procedures. Samples of stroma from control, [^{3}H]H_2DIDS-labeled, and Pronase-digested, [^{3}H]H_2DIDS-labeled cells were solubilized and the proteins separated by SDS gel electrophoresis on a 7.5–15% acrylamide gradient slab gel. The radioactive components were detected by fluorography.

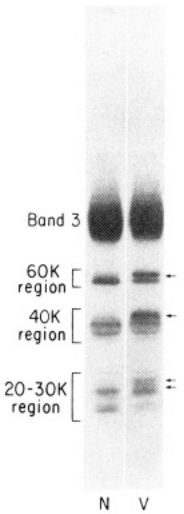

FIGURE 2: Immunoblot of membrane proteins from normal donors (N) and a donor heterozygous for the elongated variant of protein 3 (V). The isolated proteins were separated according to the method of Laemmli (1970) on an SDS–polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose sheet was then stained as outlined under Experimental Procedures with rabbit anti-protein 3 serum. The immunoglobulin was localized by use of ^{125}I-labeled protein A. An autoradiograph was prepared as outlined under Experimental Procedures. The arrows denote the variant forms of protein 3 catabolic products.

Figure 2 shows the membrane from a normal and a heterozygote donor that were carefully prepared in the presence of protease inhibitors. The heterologous antisera interacted not only with protein 3 but also with a number of other peptides that contain antigenic determinants of protein 3. Membranes from cells of the donor with the variant form of protein 3 contained the same immunoreactive peptides as did membranes from normal cells, as well as additional peptides indicated by the arrows. The presence of two fragments always differing in size by 3K indicates that the two forms of protein 3 are being degraded in a similar manner, strengthening the idea that the fragments are derived from protein 3.

To further confirm the specificity of our antibody, we produced affinity-purified antibodies against protein 3. When the SDS-separated peptides of stroma were stained with the purified immunoglobulins, the monospecific antibody produced the same staining pattern as the unfractionated immunoglobulin preparation (Figure 2), establishing that the fragments

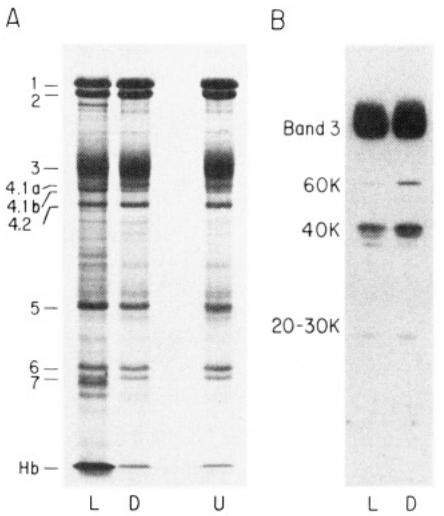

FIGURE 3: Comparison of the polypeptide composition of most dense and least dense erythrocytes. The cells were separated on the basis of their density, and their membranes were isolated, the peptides were separated, and an immunoblot was prepared as described in Figure 2. In panel A, the peptides were visualized by staining with Coomassie blue. In panel B, the peptides were transferred to nitrocellulose and identified by the immunoblot procedure outlined in the text. L designates the membrane peptides derived from the least dense fraction of red cells, D those derived from the most dense fraction, and U those from unfractionated red cells.

identified in this study were in fact generated from protein 3.

Erythrocyte membranes separated by density centrifugation were analyzed for their polypeptide composition on SDS–polyacrylamide gels. The polypeptides in the least dense and most dense fractions are shown in Figure 3A. Staining with Coomassie blue distinguished all membrane peptides, most notably those in the 4.1a and 4.1b fractions. By comparison with membrane from mature red cells, reticulocyte membranes have a higher content of the 4.1b band and thus a higher 4.1b:4.1a ratio (Jackson et al., 1981; Sauberman et al., 1979), as can be seen in the figure. Figure 3B shows the immunoblot obtained with these cells. The same polypeptide fragments of protein 3 are present in both fractions, but the amount of the 60K fragment appears to be very low in the least dense fraction. Although the gel contained equivalent amounts of these membrane fractions, it was not possible to compare the different fragments in a quantitative manner. That is, the polyclonal antibody could not be used because the location and number of epitopes with which it reacts are unknown.

Discussion

These results, obtained with immunochemical techniques and [^{3}H]H_2DIDS labeling, demonstrate that protein 3 in human erythrocytes is degraded in vivo. The 60K amino-terminal end of this protein extends through the lipid bilayer of plasma membranes; hence, Pronase digestion of intact cells generates a 60K peptide derived from protein 3. This suggests that the 60K peptide is the product of cleavage at a site just outside or close to the outside of the membrane bilayer (Figure 4), similar to the site of cleavage by Pronase or chymotrypsin (Mueller & Morrison, 1977). The fact that the 60K fragment retains its [^{3}H]H_2DIDS binding site and the variant region which is on the amino-terminal portion of band 3 clearly indicates that it is generated by cleavage on or near the outside of the bilayer.

Earlier investigations of erythrocyte membrane components that interact with [^{3}H]H_2DIDS have also shown that the anion transport inhibitor not only labels protein 3 but also labels a second component which, until now, has not been identified

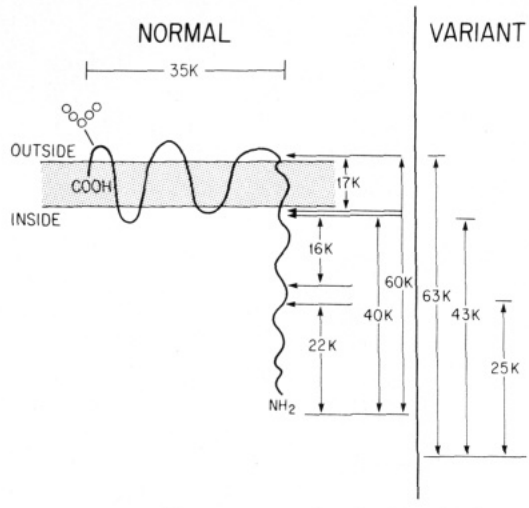

FIGURE 4: Schematic diagram comparing the degradation products of protein 3 and its variant in relation to the membrane bilayer.

as a fragment of protein 3. It was assumed to be a sialoglycoprotein (Cabantchik & Rothstein, 1974; Lepke et al., 1976; Ship et al., 1977).

The lower molecular weight fragments remaining with the membrane are derivatives from the cytoplasmic part of band 3. Since these catabolic fragments could have been generated during isolation of the membrane, we were careful to use erythrocytes freed of leukocytes and platelets. In addition, the membranes of fresh isolated cells were prepared as quickly as possible, in the presence of EDTA and PMSF, to inhibit protease activity. That well-defined protein 3 fragments could be identified indicates that cleavage during catabolism occurs at selective sites. Even though hydrolysis takes place at these sites, the newly generated products remain with the membrane, probably held there either by the secondary and tertiary structure of protein 3 or by the disulfide bonds. It is important to remember that protein 3 in the membrane is for the most part a dimer and that the peptide chain is not spread out as depicted in the two-dimensional scheme shown in Figure 4 with the amino terminus sticking out. the chain is more likely coiled in a fashion so as to hold the proteolytic fragments together. If both of the chains of the undenatured protein 3 are cleaved at the membrane interface, there may be a loss of the cytoplasmic portion but not if it interacts with other membrane proteins. We have presented evidence for such interactions (Hsu & Morrison, 1983). Finally, if the cytoplasmic domain of protein 3 interacts with the lipid bilayer, that may be sufficient to bind the fragments to the membrane.

There appears to be less than complete specificity at the cleavage sites. For example, although the 60K fragment clearly is the major catabolic product of this size in normal membrane, it is always accompanied by lesser amounts of a slightly larger fragment. This is also true for the other fragments of the variant. In the 40K region of the gels, there are always at least two major fragments; the same is true in the 30–20K region, perhaps reflecting the orientation of the protein with respect to the bilayer. Thus, all copies of protein 3 may not be identical. This is clearly the case for the carbohydrate of band 3. Binding of other proteins on the cytoplasmic surface could also account for conformational changes, leading to preferential cleavage at a particular site. Similar observations have been made for the 60K fragment in other immunochemical studies of protein 3 (Kay et al., 1983; Lorand et al., 1983; Drenckhahn et al., 1984; Lutz et al., 1984). Alternatively, these components may reflect heterogeneity of protein 3.

A number of investigators [e.g., see Ross & McConnell (1978) and Ideguchi et al. (1982)] have reported variable forms of protein 3 which they attribute to glycosylation or phosphorylation of the molecule. Protein 3 is very heterogeneous with respect to size. From the highest molecular weight to lowest molecular weight species, there may be as much as a 20K difference. In part, these differences may not all be due to heterogeneity of carbohydrate or phosphorylation but could be due to degradation of protein 3 by proteolysis. Our findings suggest that some of this diversity may come from degradation of the protein.

The data also establish that two of the sites of cleavage are at the membrane interface, since both the 60K and 40K fragments contain the amino-terminal end of protein 3. The 40K fragment is similar in size to the fragment generated by mild trypsin hydrolysis of open erythrocyte membranes (Appell & Low, 1981; Steck et al., 1976).

The only well-established neutral endoprotease present in the mature red cell is a calcium-activated protease (Anderson et al., 1977; Galovtchenko-Matsumoto et al., 1982; King & Morrison, 1977; Murachi, 1983; Tarone et al., 1979). Although found primarily in the cytoplasm, this protein is probably in the membrane as well. The concentration of calcium required for its activation is outside the physiological range; however, in the microenvironment of the membrane, it is possible that the enzyme is activated. Thus, the cleavage sites of protein 3 may depend on localization of the molecule within the cell. If the protease involved is membrane bound, it may cleave only those parts of the protein 3 peptide chain that are in contact with the membrane bilayer. Cleavages at the 60K and 40K sites are clearly at such sites. Although the cleavage may not result from the action of the same enzyme on the outside and inside of the cell membrane, the membrane interface may offer a preferred site for protease activity. Accordingly, the conformation of protein 3 may be such that the region of the amino-terminal end between 20K and 30K may also be in contact with the membrane.

Does protein 3 catabolism depend upon the age of the erythrocyte? The distribution of catabolic products in the most dense and least dense fractions showed no appreciable qualitative differences. The same peptide fragments appear to be present in all fractions. However, the amounts of the protein 3 fragments differed when the preparations were highly enriched for reticulocytes. Thus, it would appear that there is an accumulation of catabolic products of protein 3 as the cell ages from a reticulocyte to a mature erythrocyte. The data, however, suggest that the 60K fragment is not the presumptive senescent signal (Kay et al., 1983; Kay, 1984). Kay (1984) has indicated that the epitope for that antigen involves a portion of the transmembrane segment as well as the glycoprotein-containing carboxyl-terminal part of protein 3. The 60K fragment we have identified clearly does not contain the latter part of protein 3. This is consistent with the data of Lutz et al. (1984).

Although there is an apparent change in the relative amounts of the fragments of protein 3, the absolute differences cannot be evaluated with our polyclonal antibody, because the epitopes bound by the antibody are unknown. A more quantitative investigation is now under way using monoclonal antibodies.

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