Biosynthesis of Reticulocyte Membrane Proteins by Membrane-Free Polyribosomes
(rabbit/hemin/globin/gel electrophoresis)

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ABSTRACT Intact rabbit reticulocyte cells synthesize two predominant species of membrane polypeptides; at least 10 reticulocyte membrane polypeptide species are not produced by the cells. Cell-free extracts of reticulocytes, free of any membranes or membrane-bound polyribosomes, synthesize large amounts of these two membrane polypeptides; one of these polypeptides appears to be modified, probably by loss of 20-40 amino acids, after it is incorporated into the membrane. Deprivation of hemin results in inhibition of synthesis by lysates of membrane proteins, globin, and all other cytoplasmic proteins.

The protein composition of mammalian erythrocyte membrane is now fairly well established. There exist 7-9 major components (1-8); the major species include two polypeptides of molecular weight greater than 200,000 (4), a protein of molecular weight 100,000, and a sialoerythrocyte protein which contains the MN, A, and B blood-group activities (8). The latter two species are the only membrane proteins found on the cell surface (3, 9-12); they may also penetrate to the interior surface of the membrane (13-15). Little is known, however, about the biosynthesis of erythrocyte membrane proteins: the types of erythrocytic cells that synthesize each protein, the type of polysome (free or membrane-attached) that synthesizes the proteins, and the factors that regulate their synthesis.

The reticulocyte is the penultimate cell in the erythropoietic series; it has no nucleus, and makes no RNA or DNA. About 90% of protein produced is α and β-globin chains (16, 17). The protein composition of reticulocyte membranes is very similar to that of erythrocyte membranes (my unpublished observations). In this paper, I show that reticulocytes synthesize only 2 major and 2-3 minor membrane proteins; most of the membrane proteins are no longer being synthesized in significant amounts at this stage.

In most mammalian cells a fraction of the polyribosomes are firmly attached to membranes; these are the site of synthesis of most, if not all, proteins that are eventually transported out of the cell, such as trypsinogen, chymotrypsinogen, albumin, and other serum proteins (18-22). It is not at all clear that this is the sole function of these polyribosomes.

Burka has claimed that rabbit reticulocytes contain membrane-bound polyribosomes (23) that synthesize most of the nonglobin proteins made by the cell (24). By contrast, we showed recently that membrane-free reticulocyte polyribosomes will produce precisely the same globin and nonglobin proteins as does the intact cell (17). In this paper I show, using dodecyl sulfate–gel electrophoresis and peptide mapping procedures, that a membrane-free lysate makes large amounts of the two membrane polypeptides produced by the intact cell; one of these proteins appears to be modified, probably by removal of 20-40 amino acids when it is incorporated into the membrane.

MATERIALS AND METHODS

Materials. Acetylphenylhydrazine was purchased from Sigma, and [35S]methionine (30,000 Ci/mol) from Amerham/Searle. Sources of all other chemicals were detailed (17, 25-27).

Reticulocytes. Rabbits were made anemic as described (25), except acetylphenylhydrazine was used in place of phenylhydrazine. Blood was taken from the ear, and the cells were washed by centrifugation four times; after each of the first two washes the top quarter of the packed cells was removed, in order to insure that no white cells contaminated the preparation.

Labeling of Whole Cells. 0.5 ml of packed reticulocytes were resuspended in 4.5 ml of medium (27), which also contained 90 μCi/ml of [35S]methionine. Incubation was at 30° for 40 min. Incorporation of radioactivity into protein was linear with time during this period; per ml, there was 4 × 10⁷ dpm incorporated into protein. The cell suspension was poured into 15 ml of ice-cold medium; the cells were recovered by centrifugation (6000 × g, 10 min), then lysed in 1.5 ml of cold 5 mM potassium phosphate (pH 8.0) (4). After centrifugation at 20,000 × g for 15 min, the supernatant was removed and saved. The pellet, consisting predominantly of membranes, was washed by centrifugation four times with a large excess of phosphate buffer (4), at which point it was colorless. The membranes were resuspended in 1.5 ml of cold phosphate buffer.

Preparation of reticulocyte lysates and conditions for cell-free protein synthesis were detailed (25-27). Reaction mixtures containing [35S]methionine (80 μCi/ml) and hemin (20 μg/ml) were incubated at 30° for 40 min; incorporation into protein was linear with time for 30 min, yielding 6 × 10⁷ dpm of protein per ml.

Acrylamide Gel Electrophoresis. 50 μl of membrane suspension or lysate was added to 0.2 ml of sample buffer (17, 28), which contained 6 M urea and 1% sodium dodecyl sulfate. The polyacrylamide gels (7.5%) contained 6 M urea and 0.1% Na dodecyl SO₄; detailed conditions for gel electrophoresis were described in the Experimental Procedures.
phoresis, staining with Coomassie blue, and radioautography were detailed (17).

Elution of Radioactive proteins from Gels and Digestion with Trypsin. 10 Gels each of ^35S-labeled membranes and labeled cell-free reaction product were subjected to electrophoresis for twice the usual period, so that bands B and E were well separated from neighboring polypeptides. Regions of gel corresponding to these bands were excised, and protein was eluted by shaking for 36 hr at 37° with 20 ml of 0.5% Na dodecyl SO4. The eluates were diluted with four volumes of water, and then made 0.01 M with respect to sodium phosphate (pH 6.4). The solution was then passed through a 2-ml column of hydroxypatite, previously equilibrated in the same dodecyl SO4 buffer (29). More than 95% of the radioactivity adsorbed to the column and could be eluted with buffer [0.6 M sodium phosphate (pH 6.4)–0.1% dodecyl SO4] in a volume of about 4 ml. This procedure was necessary to concentrate the proteins, to lower the detergent concentration, and to remove polyacrylamide that otherwise would interfere with paper ionophoresis. To this solution was added 0.2 ml of Escherichia coli S-30 extract (30) as carrier, and trichloroacetic acid to 5%. Protein was recovered by centrifugation, then washed by centrifugation five times with 5% CH3COOH to remove traces of detergent. Digestion of the protein with trypsin and paper ionophoresis of the product at pH 3.5 was detailed (31). Strips, 1 cm wide, were cut and counted in a liquid scintillation counter.

RESULTS

Proteins synthesized by intact reticulocytes

Blood, containing about 70% reticulocytes, was withdrawn from a rabbit made anemic by subcutaneous injection of acetylphenylhydrazine; the cells were washed four times by centrifugation, and the top quarter of the cell pellet, including the “buffy coat” of white cells, was purposely discarded. After they were labeled in vitro with ^35S-methionine for 30 min, the cells were lysed with an equal volume of water and membranes were prepared by repeated centrifugation from dilute phosphate buffer (4). An amount of membrane and cytoplasm preparation derived from the same amount of cells was dissolved in buffer containing 6 M urea and 1% Na dodecyl SO4, then analyzed by electrophoresis in polyacrylamide gels; after they were stained with Coomassie blue, the gels were sliced longitudinally and subjected to radioautography (see Methods and Fig. 1).

Reticulocytes synthesize two major membrane proteins (bands B and E, Fig. 1, column δ). Both of these bands correspond to a membrane protein visualized by the Coomassie Blue stain (column δ). By comparison of the mobility of these proteins with proteins of known molecular weight, we determined the molecular weight of B to be 53,000 ± 2000 and of E to be 33,000 ± 2000 (17). That our membrane preparations contain little globin (major band at bottom of gel) is evidence that they are largely free of cytoplasmic proteins. The protein composition of our reticulocyte membrane preparations is the same as that determined for erythrocyte ghosts from the same rabbit (data not shown); they appear very similar to those of others’ preparations of erythrocyte ghosts analyzed by different gel systems (3–7). In particular, they contain two major components of molecular weight greater than 200,000 and one component of molecular weight about 100,000. Neither these nor the majority of other membrane proteins are made by reticulocytes (Fig. 1, column δ). Similar results were obtained with ^14C-lysine (data not shown).

As expected, the vast majority of the labeled cytoplasmic protein is globin (column δ); the cells also make, as noted previously, a discrete number of other cytoplasmic proteins (17). They contain little, if any, labeled proteins corresponding to membrane bands B or E.

Proteins synthesized by reticulocyte lysates

Unlabeled reticulocytes from the same rabbit used above were lysed in an equal volume of water, and clarified twice by centrifugation at 20,000 × g for 15 min to remove cell membranes. (In unpublished experiments, we showed that the same results were obtained using only one centrifugation; a slight membrane contamination of the lysate thus does not influence our results.) Such lysates, when supplemented with hemin, will synthesize globin and other reticulocyte proteins for at least 1 hr at a rate comparable to that of the intact cell (17, 32–34). Fig. 1, column δ, shows that such a lysate will synthesize globin and all other cytoplasmic proteins made by intact cells (17); further, such a lysate also produces two proteins B and E, which correspond specifically to the two proteins that are labeled by whole cells and are found exclusively in the membrane fraction (column δ). Whereas the protein E produced by the lysate (column δ) comigrates on these gels with the authentic membrane E (column δ), band B produced by the lysate migrates as if it has a molecular weight 2000–4000 g/mol greater than that of the authentic membrane band B (columns δ and δ). The difference in mobility between labeled bands B (lysate) and B (membrane) is reproducible, and has been obtained with preparations from three different rabbits.

To further demonstrate that the membrane-free lysate indeed makes authentic membrane proteins, we eluted from the gels bands B, B, and E (labeled with ^35S-methionine), digested them with trypsin, and analyzed the resultant peptides by paper ionophoresis at pH 3.5 (Fig. 2). Band E, whether synthesized by the lysate or isolated from cell mem-
branes, yields very similar peptide profiles. It is also apparent that band B2, synthesized by the lyase and labeled with [35S]methionine has a profile very similar to that of authentic membrane band B2 synthesized by intact reticulocytes. That the membrane protein B2 migrates with a mobility greater than that of B1 is explained most simply by the loss, probably due to digestion by an endopeptidase, of 20-40 amino acids from B1 before the protein is incorporated into the membrane. Possibly, the peptide at 52 cm found in the digest of membrane, but not in lysate protein (Fig. 2a and b), is a result of such a modification. Additional reasons for the qualitative differences between the digests of the two B proteins include (a) contamination of one (or both) by small amounts of other proteins of the same molecular weight and (b) modification (e.g., glycosylation) of some amino-acid residues in the membrane B2 species.

This result confirms our earlier finding (17) that membrane-free reticulocyte lysates synthesize precisely the same proteins, in essentially the same relative amount, as do whole cells. Further, two of the major nonglobin proteins produced by such lysates are authentic membrane proteins.

**Further evidence for cleavage of protein B1**

In the experiment depicted in Fig. 3, membranes were analyzed after labeling of whole cells with [35S]methionine for different periods. After 5 min of labeling, there is more of species B1 than of B2 in the membrane; thereafter, the amount of B1 remains virtually constant, while that of B2 (and E) increase considerably. Hence, species B1 appears kinetically to be a precursor of B2. Furthermore, when cells labeled for 10 or 15 min are treated with emetine (to block further protein synthesis) and membranes are isolated after an additional incubation, species B1 disappears and there is a corresponding increase in the amount of species B2 (Fig. 3). As a control, it can be seen that the amount of species E is unchanged during the "chase" period. This is direct evidence that species B1 is converted into B2 after B1 is incorporated into the membrane. There is no appreciable pool of B (or E) not attached to membranes, for there is no net increase in the amounts of these proteins during the chase period.

**DISCUSSION**

The major problem with these experiments, and in fact with much of the current work on the protein composition of membranes, is the inability to differentiate between "authentic" membrane proteins and cytoplasmic proteins that are "sticky" and bind nonspecifically to membranes during the extraction procedure. It is obvious that the sialoylglycoprotein in red cell membrane preparations is an authentic membrane protein, since it contains the A, B, and N blood-group antigens found on the cell surface. Labeling studies showed that one other protein (molecular weight 100,000) is found at the cell surface (3, 8-12). Bretscher showed further that different parts of these proteins are labeled by chemical reagents that act from the outside or the inside of the membrane (13, 14); hence, these proteins appear to span the membrane and must be considered authentic membrane proteins. The case for the other components visualized in a membrane preparation such as that of Fig. 1, column 6, rests largely on an operational definition of a membrane protein: they are not removed from the membrane by extensive washing in solutions such as 5 mM phosphate buffer (pH 8.0) (3-8). Further, none of the membrane proteins depicted in Fig. 1, including labeled species B1 and E, is removed from the membrane during 10 min of incubation at 37° in either 0.5 M NaCl or in 0.1 mM EDTA (pH 8.0) (unpublished studies). Also, membrane...
proteins are not found in any significant amount in the cytoplasm, and authentic cytoplasmic proteins, such as globin, do not contaminate the membrane preparation (Fig. 1).

An additional unpublished experiment suggests that species B or E are not merely "sticky" cytoplasmic proteins: a sample of the cell-free reaction products labeled with [35S]methionine was mixed with an equal volume of nonradioactive ghosts, and incubated for 10 min at 0°C. Less than 10% of the radioactivity in species B1 or E (or any other labeled proteins) was recovered in the reisolated membrane fraction.

Given the necessarily operational definition of a membrane protein, the present results show clearly that rabbit reticulocytes synthesize only 2 major and 2–3 minor membrane proteins; the majority of reticulocyte membrane proteins are no longer being made in significant amounts by these terminal cells. A decision as to whether this synthesis represents turnover of existing membrane proteins or synthesis of components that are specific to reticulocytes and erythrocytes must await studies of membrane composition and synthesis in the nucleated precursor hematopoetic cells. Likewise, we do not know whether the other membrane proteins are also turning over, but at a rate too slow to be seen in our experiments. It should be noted that there is extensive turnover of membranes in cultured mammalian cells, even when they are in a stationary, nongrowing state (36).

One of the surprising results of this study is that, at least in rabbit reticulocytes, membrane proteins are synthesized on polyribosomes not attached to cell membranes. I emphasize that the procedure for lysis of these cells is extremely gentle (osmotic lysis with distilled water or 1 mM MgCl₂), and it is very unlikely that membrane-bound polysomes would be dislodged by this procedure. Since most of the reticulocyte and erythrocyte membranes are converted to large vesicles (ghosts) by osmotic lysis, and since studies using phase-contrast microscopy indicate that over 99% of the membrane vesicles are removed by the two centrifugations used, it is extremely unlikely that any membrane-bound ribosomes remain in the cell supernatant. However, I cannot eliminate the possibility that there exist polysomes attached to small pieces of membrane.

It appears that newly synthesized membrane proteins must, for some period, be free in the cytoplasm before incorporation into membrane. Further, one of the membrane proteins (B₁) undergoes an alteration—probably proteolytic cleavage—apparently soon after it is incorporated into the membrane (Fig. 3).

It should be noted that this model is different from the case of proteins that are excreted from cells and are produced by membrane-bound polysomes. In such polysomes the large ribosomal subunit, which contains the nascent polypeptide chain, is specifically bound to the endoplasmic reticulum membrane, and the nascent chain passes through the membrane (18, 37–39).

Whether rabbit reticulocytes do contain membrane-bound polysomes is an open question. Burka (23) isolated ribosomes from a membrane fraction and showed that they synthesize, in vitro, predominantly nonglobin proteins (24). By contrast, we showed that a reticulocyte lysate, free of membranes, will synthesize precisely the same globin and nonglobin proteins—including the two membrane proteins (Fig. 1)—as are made by the intact cell (17). Because of possible contamination of the membrane fraction by a small amount of whole cells, we have not tested membranes directly in "cell-free" protein synthesis.

It is by now well known that continued synthesis of globin by reticulocyte extracts requires the presence of hemin or other metalloporphyrins; in its absence there is produced a potent inhibitor of polypeptide chain initiation (40–42). We recently showed that synthesis of all nonglobin proteins by reticulocyte lysates also requires hemin; further, translation of an exogenous viral mRNA is also inhibited by hemin deprivation (17). Since two of the reticulocyte nonglobin proteins produced are in fact membrane proteins, we conclude that in at least one important aspect synthesis of reticulocyte membrane and cytoplasmic proteins are regulated by a common mechanism.

NOTE ADDED IN PROOF

Labeling of intact reticulocytes in the presence of tosylphenylalanyl-chloromethyl-ketone (TPCK) results in extensive synthesis of membrane protein B₁, but no formation of B₂. Hence, conversion of protein B₁ to B₂ probably involves a proteolytic cleavage.

Woodward et al. (J. Biol. Chem. 248, 1556–1561) showed recently that reticulocyte membrane-bound ribosomes synthesize globin almost exclusively.

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