Erythrocyte Membrane Protein Band 3:

Its Biosynthesis and Incorporation into Membranes

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ABSTRACT   Band 3, a transmembrane protein that provides the anion channel of the erythrocyte plasma membrane, crosses the membrane more than once and has a large amino terminal segment exposed on the cytoplasmic side of the membrane. The biosynthesis of band 3 and the process of its incorporation into membranes were studied in vivo in erythroid spleen cells of anemic mice and in vitro in protein synthesizing cell-free systems programmed with polysomes and messenger RNA (mRNA). In intact cells newly synthesized band 3 is rapidly incorporated into intracellular membranes where it is glycosylated and it is subsequently transferred to the plasma membrane where it becomes sensitive to digestion by exogenous chymotrypsin. The appearance of band 3 in the cell surface is not contingent upon its glycosylation because it proceeds efficiently in cells treated with tunicamycin. The site of synthesis of band 3 in bound polysomes was established directly by in vitro translation experiments with purified polysomes or with mRNA extracted from them. The band-3 poly-peptide synthesized in an mRNA-dependent system had the same electrophoretic mobility as that synthesized in cells treated with tunicamycin. When microsomal membranes were present during translation, the in vitro synthesized band-3 polypeptide was cotranslationally glycosylated and inserted into the membranes. This was inferred from the facts that when synthesis was carried out in the presence of membranes the product had a lower electrophoretic mobility and showed partial resistance to protease digestion.

Our observations indicate that the primary translation product of band-3 mRNA is not proteolytically processed either co- or posttranslationally. It is, therefore, proposed that the incorporation of band 3 into the endoplasmic reticulum (ER) membrane is initiated by a permanent insertion signal. To account for the cytoplasmic exposure of the amino terminus of the polypeptide we suggest that this signal is located within the interior of the polypeptide. A mechanism that explains the final transmembrane disposition of band 3 in the plasma membrane as resulting from the mode of its incorporation into the ER is presented.

The erythrocyte membrane is a well established model for studies on the biochemical composition and structure of plasma membranes (c.f. 41, 58). Band 3, a glycoprotein of molecular weight 95–100,000 which constitutes the anion channel in the plasma membrane, is the most abundant transmembrane protein of the human erythrocyte (6, 7, 19, 57). This protein has been purified and its transmembrane disposition extensively characterized. It spans the membrane more than once (15, 16, 32) and a large amino terminal segment of the polypeptide of at least 40,000 daltons is exposed on the cytoplasmic face of the membrane (21, 59). The location of the carboxy terminal segment is still uncertain, although recent results suggest that it too is exposed on the cytoplasmic surface (42).

Very little is known of the mechanism of the incorporation of band 3 into the plasma membrane. The transmembrane orientation of this protein cannot simply be accounted for by the interrupted vectorial discharge of a nascent polypeptide synthesized in bound polysomes. Such a mechanism is thought to explain the transmembrane disposition of other simple plasma membrane proteins, such as the VSV G protein (39, 53), which cross the membrane once and have their amino termini exposed on the extracellular side of the membrane and

their carboxy termini on the cytoplasmic surface.

Several systems have been described in which the synthesis of mammalian erythrocyte membrane proteins has been detected, such as human (22) and mouse erythroleukemic cells (18, 54) but these do not provide sufficiently high levels of band 3 synthesis to allow detailed studies of the mechanism of insertion of this protein into the membrane.

The spleen of mice made anemic by phenylhydrazine administration becomes a major site of extramedullary erythropoiesis in which there is a relatively synchronous development of a cell population with erythroid characteristics (e.g., reference 14). The mouse spleen system has, therefore, been used by many investigators as model to study erythroid development (34). We have used it to follow the synthesis, glycosylation, and transfer of band 3 to the surface of intact erythroid spleen cells maintained in culture. In addition, we have obtained polysomes and mRNA preparations from the spleens of anemic mice and have been able to demonstrate that synthesis of band 3 occurs in membrane-bound ribosomes and that the nascent polypeptide is cotranslationally inserted into the endoplasmic reticulum (ER) membrane.

MATERIALS AND METHODS

Concanavalin A (Con A) sepharose and protein A sepharose were obtained from Pharmacia Fine Chemicals, Piscataway, N.J., $[^{35}S]$methionine (>800 Ci/mmol) from New England Nuclear, Boston, Mass., phenylhydrazine, trypsin, and chymotrypsin were from Sigma Chemical Company, St. Louis, Mo., polyethylenelmide-coated polyacrylamide beads (Affi-gel 731) from Bio-Rad Laboratories, Richmond, Calif., and trasylol from Mobay Chemical Co., Pittsburgh, Pa.

Purification of Murine Band 3 and Preparation of Antibodies

Methods for the purification of band 3 from Balb/c murine erythrocytes and for the preparation and characterization of specific antibodies have been previously described (54). Most of the experiments required an IgG fraction prepared by ammonium sulfate precipitation of immune serum, but in several cases affinity purified antibodies were used.

Induction of Anemia and Labeling of Spleen Cells

Three intraperitoneal injections of a 0.8% neutralized phenylhydrazine solution were given to female Balb/c mice (0.1 ml per 20 g weight) at 0, 16, and 24 h (14). The spleens were removed after various times and placed in an ice cold Hank's balanced salt solution without Ca^{++} and Mg^{++}. Cell suspensions were obtained by disrupting the tissue with tweezers and pipetting the fragments through a pasteur pipette. After filtering through sterile gauze, cells were collected by centrifugation at 1000 g for 5 min, washed once by recentrifugation and resuspended in a small volume of the same solution. Nucleated cells were counted in a hemocytometer after staining with crystal violet.

For metabolic labeling, cell suspensions (5 × 10⁶ nucleated cells/ml) were incubated with leucine- or methionine-free Ham's F12 medium (28) or RPMI (Gibco Laboratories, Grand Island Biological Co., Grand Island, N.Y.), supplemented with 10% dialyzed fetal calf serum, 100 μg/ml streptomycin sulfate, 100 U/ml penicillin and $[^{35}S]$methionine (10–50 μCi/ml, sp act 800 Ci/mmol) or $[^3H]$leucine (25 μCi/ml, 55 Ci/mmol). After incubation for the desired time at 37°C in a 5% CO_2 incubator, cells were collected by centrifugation for 5 min at 1000 g at room temperature and washed three times with a Ca^{++}- and Mg^{++}-free Hank's balanced salt solution that contained phenylmethylsulfonylfluoride (PMSF) (100 μg/ml). This protease inhibitor was omitted when protease digestion of whole cells was to be carried out. Resuspended cells were lysed by sonication in 0.25 M NaCl, 15 mM Tris-HCl, pH 7.4, 0.02% NaN₃, 1% SDS.

Con A Sepharose Chromatography of Labeled Spleen Proteins

Labeled spleen cell lysates (from 2 × 10⁷ nucleated cells) were diluted with column buffer (0.15 M NaCl, 0.01 M Tris HCl, pH 7.2) containing trasylol (50

U/ml) and PMSF (100 μg/ml) and were applied to a 0.5 × 3 cm Con A sepharose column previously washed with 0.2 M methyl-α-D-mannopyranoside in column buffer and then equilibrated with column buffer. After sample application and extensive washing until no further radioactivity came off the column, the bound material was eluted with 0.2 M methyl-α-D-mannopyranoside in column buffer containing 0.05% SDS. After the addition of 0.3 A₂₆₀ U/ml of crude yeast RNA as a carrier, protein was precipitated with 10% cold TCA, recovered by centrifugation and resuspended by sonication in 200 μl of 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4, 2% SDS. When necessary, the pH was adjusted to neutrality by the addition of Tris base. Aliquots were taken to measure radioactivity, for electrophoretic analysis and for immuneprecipitation.

Isolation of a Crude Membrane Fraction

After incubation for labeling, cell suspensions were immediately cooled by dilution with 3 vol of an ice cold Ca^{++}- and Mg^{++}-free Hank's balanced salt solution containing PMSF (100 μg/ml). All subsequent steps were performed at 4°C. Cells were collected by centrifugation, washed twice with the same solution, resuspended (2.5 × 10⁷ nucleated cells/ml) and swollen by incubation for 5 min in 10 mM NaCl, 10 mM Tris, pH 7.4, 1.5 mM $MgCl_2$, before disruption with 40 strokes of a tight fitting type B Dounce homogenizer, which resulted in >90% cell lysis. A postnuclear supernatant (PNS) was prepared (5 min at 1200 g in an IEC PR2 centrifuge International Equipment Co., Needham Heights, Mass.) and a membrane fraction was recovered from the PNS by sedimentation (100,000 g for 30 min) and dissolved in 2% SDS.

Treatment of Spleen Cells with Proteases

Spleen cell suspensions were rapidly chilled by tenfold dilution with ice cold Ca^{++}- and Mg^{++}-free Hank's balanced salt solution. Cells were collected (1000 g for 5 min at 4°C), washed once and incubated for 30 min at 4°C in 0.3 ml of the same solution containing chymotrypsin or trypsin at the indicated concentrations. To inactivate the proteases incubation was continued with 100 U trasylol (10 min at 4°), followed by 100 μg/ml PMSF (5 min at 4°). Cells were collected (1000 g for 10 min.) and sonicated in 2% SDS, 0.25 M NaCl, 50 mM Tris, pH 7.4 to dissolve total cell protein.

Preparation of Free and Membrane-bound Polysomes

Free and membrane-bound polysomes were prepared from spleens of anemic mice by an adaptation of the procedure developed by Ramsey and Steele (47, 48, 49) for rat liver polysomes. After passage through a tissue press the pulp was homogenized in polysome buffer (250 mM KCl, 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, and 3 mM DTT) containing 250 mM sucrose and the homogenate was centrifuged in a SW27.1 rotor (Beckman Instruments, Fullerton, Calif.) that was first run for 2 min at 740 g and then accelerated to 31,000 g for the remaining 12 min. The supernatant (S1) was saved as a source of free polysomes. The large sediment (P1), containing all particulate fractions including nuclei, was either processed directly for guanidine hydrochloride extraction of RNA or was used to obtain bound polysomes. In the latter case, the pellet was resuspended by homogenization in 30 ml of a 50% rat liver high speed supernatant (RLHSS) (47) adjusted to 250 mM KCl and 20 mM $MgCl_2$. Membranes were solubilized by addition of 0.11 vol of 10% Triton and a brief homogenization, and nuclei were removed by centrifugation at 15,000 g in a Sorvall HB/4 rotor (DuPont Instruments). 0.11 vol of 13% deoxycholate (DOC) was added to the supernatant and insoluble material was removed by centrifugation for 10 min at 24,000 g. Bound polysomes were recovered from this supernatant and free polysomes from the S1 fraction by centrifugation through discontinuous sucrose gradients containing RLHSS, prepared in Ti60 tubes. The gradients consisted of layers of 2 M sucrose (7.5 ml) and 1.38 M sucrose (8.5 ml) both in 10 mM HEPES, 4 mM $MgCl_2$, heparin (0.5 mg/ml), 250 mM KCl, 3 mM DTT, containing 25% RLHSS in the 1.38 M sucrose, and 9% RLHSS in the 2.0 M sucrose. After centrifugation for 22 h at 175,000 g the polysome pellets obtained were rinsed with 50 mM Tris, 50 mM KCl, 5 mM $MgCl_2$ and suspended in the same solution at a concentration of 16A₂₆₀ U/ml.

Purification of mRNA

Spleens of anemic mice were removed 112 h after the beginning of induction of anemia by phenylhydrazine and mRNA was prepared by two different methods. In the first, a cell suspension was prepared as described above, cells were washed twice, and total cell RNA was extracted with phenol according to Merkel et al. (43). The aqueous phase was removed and, after the NaCl concentration was adjusted to 0.3 M, 2 vol of ethanol (−20°) were added. DNA

was removed by winding on a glass rod and, after overnight incubation at −20°C, the RNA was recovered by centrifugation from the remaining alcohol suspension, reprecipitated with ethanol, and dissolved in water.

In the second method total RNA was extracted from spleens or from polysome pellets or supernatants with guanidine hydrochloride by a modification of the procedure of Cox (13). Particulate samples were homogenized in 8 M guanidine-HCl containing 50 mM Tris, pH 7.5, 10 mM EDTA, and the RNA precipitated with one-half volume of ethanol at −20°C for 1.5 h, was redissolved in 6 M guanidine, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and reprecipitated. This procedure was repeated three times, and the RNA was then dissolved in H₂O and reprecipitated several times with ethanol. In all cases poly A(+) mRNA was purified by oligo dT cellulose chromatography (1) and used for translation experiments.

Fractionation of the mRNA

The poly (A) mRNA (5–15 A₂₆₀ U) was heated in 1 mM EDTA, 0.5% SDS at 70°C for 10 min and immediately cooled in an ice bath. Tris-HCl, pH 7.4 was added to 10 mM and the sample was fractionated on a 10–30% sucrose gradient in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5% SDS, which was centrifuged in a SW41 rotor (Beckman Instruments) at 38,000 rpm for 16 h. Samples were collected dropwise from the bottom after piercing the tube, diluted twofold with water, brought to 0.25 M NaCl, and the mRNA was precipitated with 2 vol of cold ethanol without a carrier. The mRNA precipitate was dissolved in water and used for translation.

In Vitro Protein Synthesis

In vitro translation was carried out either in the nuclease-treated rabbit reticulocyte (45) at 37°C or in a wheat germ lysate extract at 25°C (50). Rabbit reticulocyte lysates, were prepared according to Palmiter et al. (44), passed through Sephadex G-25, and supplemented with stripped rat liver tRNA (60 µg/ml) and 0.2 mM spermidine. When membranes were present, translation in the reticulocyte lysate was also carried out at 28°C.

Dog pancreas microsomal membranes were prepared and treated with EDTA as described by Shields and Blobel (55). Before use, the membranes (86 A₂₆₀/ml) were suspended in 20 mM Hepes, pH 7, 30% glycerol, 1 mM DTT and treated with 25 U/ml of miccococal nuclease for 10 min at 25°C, followed by inactivation of the nuclease with EGTA.

Proteolytic Digestion to Test for Insertion of Band 3 into Membranes

Aliquots of translation mixtures (240 µl) cooled on ice, received 20 µl of 10 mM CaCl₂ and 30 µl of trypsin, chymotrypsin (0.5 mg/ml each) before incubation at 4°C for 3 h. Trasylol (50 µl of 10⁴ U/ml) was then added and incubation was continued for 10 min.

Indirect Immuneprecipitation using Protein A Sepharose

The procedure was a modification of that of Goldman and Blobel (26). Samples in 2% SDS were placed for 2 min in a boiling water bath and, after cooling to room temperature, 4 vol of solution A (0.19 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.4, 2.5% Triton, 100 U/ml trasylol) and 5 µl of anti-band-3 IgG (40 mg/ml) or normal rabbit IgG were added. After incubation at room temperature for 1 h, 10 µl of protein A sepharose beads was added and incubation continued for 2 h at room temperature with shaking. The protein A sepharose beads were then collected (12,800 g for 3 min) and washed five times with solution A containing 0.1% SDS. Bound proteins were dissolved in 100 µl of 1 M DTT in 10% sucrose, 10% SDS, 20 mM EDTA, 50 mM Tris-phosphate, pH 6.7 by boiling for 2 min. Beads were removed by centrifugation at 12,800 g for 3 min and the supernatant was applied to a 5–11% gradient SDS polyacrylamide gel.

Gel Electrophoresis

Gel electrophoresis was carried out in 5–11% SDS polyacrylamide gradient slabs gels (38) according to a modification of the procedure of Maizel (40) and the distribution of radioactive protein was determined by fluorography (5).

RESULTS

Band 3 Synthesis in Spleen Cells of Anemic Mice

The spleens of mice treated with phenylhydrazine undergo a large increase in weight and become predominantly eryth-

ropoietic organs in which up to 80% of the nucleated cells belong to the erythroid series (14, 20, 56). The erythroid differentiation that accompanies the anemia resulting from phenylhydrazine injections is a relatively synchronous process (11), and it is therefore possible to follow the development of the capacity of erythroid cells to synthesize band 3 in this system.

Spleen cell suspensions, obtained from mice sacrificed at various times after the beginning of phenylhydrazine treatment (injections at 0, 16, and 24 h) were labeled with [³⁵S]methionine in tissue culture media under conditions in which the kinetics of amino acid incorporation were linear for ~8 h. The time of appearance of band 3 during the course of anemia was determined by specific immuneprecipitation followed by SDS polyacrylamide gel electrophoresis and fluorography (Fig. 1). Band 3 synthesis could not be detected in cells from control animals or after 64 h of treatment but was readily apparent 89 h after the initial injection of phenylhydrazine. Synthesis of band-3 protein appeared to be maximal 89–118 h after the induction of anemia when radioactivity in the immuneprecipitates represented 0.35% of the total incorporation (over the background precipitated with nonimmune serum). Previous studies (11) have shown that, at this time, the concentration of proerythroblasts in the spleen reaches a maximum while the population of basophilic erythroblasts is still increasing. The time of maximal band 3 synthesis coincides with that reported for maximum sp act of globin mRNA (9), although the highest rate of globin synthesis occurs at ~160 h (34).
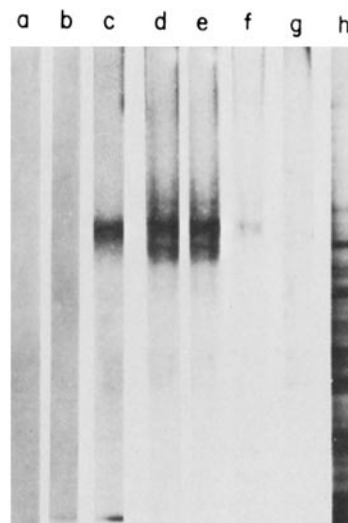

FIGURE 1   Time-course of appearance of band 3. Spleen cell suspensions from anemic mice sacrificed at various times after the beginning of phenylhydrazine treatments were labeled at a cell density of 5 × 10⁶ nucleated cells/ml for 8 h with [³⁵S]methionine in 10 ml of methionine-free F12 medium supplemented with 10% dialyzed fetal calf serum. Aliquots of lysates (100,000 cpm corresponding to ~10⁶ cells) and immuneprecipitates (10⁶ cpm from lysates of ~10⁷ cells) obtained with anti band 3 antibodies were analyzed by SDS gel electrophoresis and fluorography as described in Materials and Methods. The immuneprecipitate from spleen cells of control mice is shown in lane a. Immuneprecipitates from spleen cells of anemic mice sacrificed at 64, 89, 94, 118, and 160 h after the first phenylhydrazine injection are shown in lanes b–f. A control using normal serum is shown for the 94-h sample in lane g. Lane h shows the profile of total [³⁵S]methionine labeled proteins in the 89 h sample. The lower bands in lanes d and e most likely represent degradation products of band 3 generated during immuneprecipitation. Such bands were observed infrequently.

We initially obtained suggestive evidence (data not shown) that the protein identified as band 3 by immuneprecipitation was also a plasma membrane component in the spleen from the observation that the protein was measurably enriched (four- to sixfold) in a crude plasma membrane fraction obtained by lysis of cells that were attached to polyethyleneimide coated polyacrylamide beads (31). For a more rigorous demonstration that this protein is localized in the plasma membrane we took advantage of the fact that in the intact erythrocyte membrane, murine band 3, as is the case with its human counterpart, is resistant to digestion by exogenous trypsin but is sensitive to chymotrypsin (8, 54), which generates two fragments of 32,000 and 64,000 daltons. We found that when intact spleen cells, labeled for sufficiently long periods, were incubated with chymotrypsin under appropriate conditions (200 μg/ml at 4°C for 30 min) band 3 was quantitatively cleaved by the protease without causing cell lysis or any significant changes in the electrophoretic profile of total [^{35}S]methionine-labeled proteins. Immuneprecipitation with anti-band-3 antibodies showed that at least five immunereactive polypeptide fragments of band 3 that remained associated with the cells were generated by the chymotrypsin treatment (Fig. 2). Apparent molecular weights of these fragments were: 27,000, 29,000, 35,000, 50,000, and 68,000. On the other hand, incubation with trypsin at the highest concentrations that did not lead to cell lysis (100 μg/ml) did not result in cleavage of band 3.

These results show conclusively that band 3 in spleen cells is transferred to the cell surface although the susceptibility to chymotrypsin is somewhat different than in mature erythro-cytes in which mainly two cleavage products (32,000 and 64,000 mol wt) are obtained (8, 54). It is, however, quite possible that two of the fragments obtained in our experiments correspond to these two major chymotryptic fragments of erythrocyte band 3.
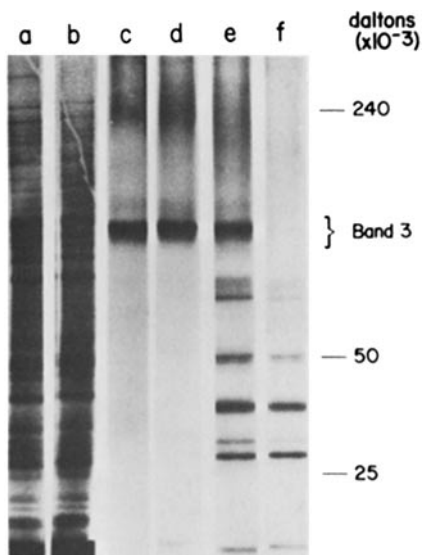

FIGURE 2 Accessibility of band 3 in intact spleen cells to exogenous chymotrypsin. Spleen cells (89 h after the beginning of phenylhy-drazine treatment) were labeled with [^{35}S]methionine for 4 h, washed twice and incubated with proteases as described in Mate-rials and Methods. Labeled proteins were analyzed by SDS gel electrophoresis and fluorography. Lane a—total protein profile of untreated cells. Lane b—protein from spleen cells digested with 200 μg/ml of chymotrypsin. Lanes c-f immuneprecipitates obtained with anti band 3 antibodies from spleen cells treated with 0 (c); 60 (d); 100 (e); and 200 (f) μg/ml chymotrypsin for 30 min at 4°C.

As is the case with its human counterpart, murine band 3 is the major Con A-binding protein in the mouse erythrocyte membrane (54). Gel electrophoretic analysis of total cellular proteins and of glycoproteins purified by Con A sepharose showed that the pattern of labeled proteins changed during the course of anemia (0, 88, 136 h). Certain labeled bands that were prominent in the patterns of control spleen (marked * in Fig. 3 A) were almost undetectable in the patterns from spleen cells of anemic mice, whereas several other labeled proteins and glycoproteins (marked by arrowheads in Fig. 3 A) appeared to be specific to erythroid spleen cells. Immuneprecipitation from the Con A-bound glycoprotein fraction and the unbound fraction confirmed that in spleen cells, as is the case with cultured Friend cells (54), band 3 is quantitatively removed by Con A sepharose chromatography from cell lysates (Fig. 3 B). Immuneprecipitation indicated that band 3 represented 5.3% of the radioactivity in the Con A-bound fraction which corre-sponded to a 15-fold enrichment for band 3 by affinity chro-matography.

The glycoprotein nature of band 3 in erythroid spleen cells was also demonstrated by the effect of tunicamycin on the electrophoretic mobility of the immuneprecipitable polypep-tide synthesized in cells treated with this drug, which blocks glycosylation by interfering with the formation of dolichol bound N-acetyl glucosamine derivatives (61, 63). The im-muneprecipitable polypeptide recovered from cells treated with tunicamycin at concentrations (0.5 μg/ml) that did not signif-icantly inhibit protein synthesis had a slightly greater electro-phoretic mobility (corresponding to a difference of 2,000-3,000 daltons) than labeled band-3 immuneprecipitates from control cells, (Fig. 4). Because tunicamycin prevents the addition of core oligosaccharides to nascent polypeptides that occurs in the endoplasmic reticulum (c.f. reference 62) these results suggest that band 3 is synthesized in bound polysomes and inserted cotranslationally into the ER membrane.

It was of interest to determine whether the carbohydrate moieties play an important role in determining the final loca-tion of the protein into the plasma membrane. Tunicamycin-treated cells were labeled for 15 min with [^{35}S]methionine, a time at which a large fraction of the [^{35}S]methionine-labeled band 3 was found protected from exogenous chymotrypsin (Fig. 5 d). When a chase was affected by dilution with 12 μg/ml methionine (2,000-fold increase over label) and further incubation for 4 h, subsequent digestion of these cells with 200 μg/ml of chymotrypsin led to almost complete cleavage of labeled band 3 (Fig. 5 e). The patterns of immuneprecipitable peptides obtained from control and tunicamycin-treated cells differed only in the mobility of one band (labeled * in Fig. 5 c), which presumably represents a segment of the polypeptide exposed on the extracellular surface, which normally bears the oligosaccharide chains. This result indicates that unglycosy-lated band 3 is efficiently tansferred to the cell surface and therefore that the carbohydrate moieties do not play an impor-tant role in directing the polypeptide to the cell membrane.

Synthesis of Band 3 on Membrane Bound Ribosomes

Band 3, synthesized during very short (10 min) in vivo pulses, was resistant to chymotrypsin digestion of intact cells and was found to be exclusively localized in intracellular membranes, which were removed from cell lysates by sedimen-tation (results not shown). This finding was consistent with the
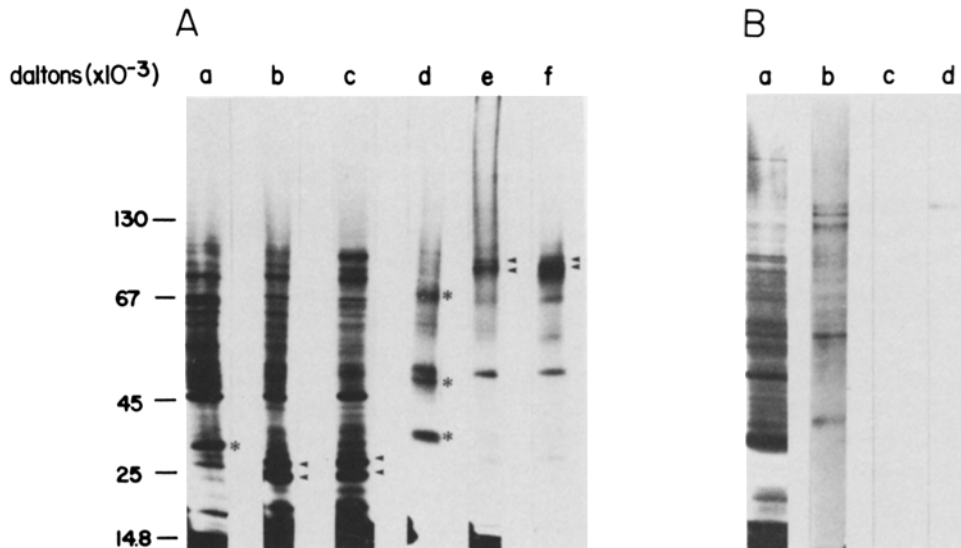
FIGURE 3   Con A sepharose fractionation of spleen cell proteins. *A*, Spleen cell suspensions prepared after various times of anemia 0 (*a* and *d*), 88 h (*b* and *e*), and 136 h (*c* and *f*) were labeled for 8 h with [³H]leucine in tissue culture media. Cell lysates were prepared and aliquots (120–200 μl) containing 700,000 cpm were analyzed directly by SDS gel electrophoresis and fluorography (*a*, *b*, and *c*). The remainder of each lysate was fractionated on a Con A sepharose column as described in Materials and Methods. The Con A-bound fractions, corresponded to 4.2, 3.2, and 2.0% of total [³H]leucine at 0, 88, and 136 h of anemia respectively. Aliquots of the Con A-bound fractions (containing 160,000 cpm) were also analyzed (*d*, *e*, and *f*). Polypeptide bands specific to spleen cells from untreated mice are marked (*) and those that are more prominent in spleen cells from anemic mice are marked by an arrow. *B*. Localization of band 3 in the Con A-bound fraction. Spleen cells from anemic mice sacrificed 89 h after the initial phenylhydrazine injection were labeled with [³⁵S]methionine for 6 h. After Con A sepharose fractionation of the cell lysate the unbound (*a*) and bound fractions (*b*) and their corresponding immuneprecipitates (*c* and *d*) were analyzed by SDS gel electrophoresis followed by fluorography.
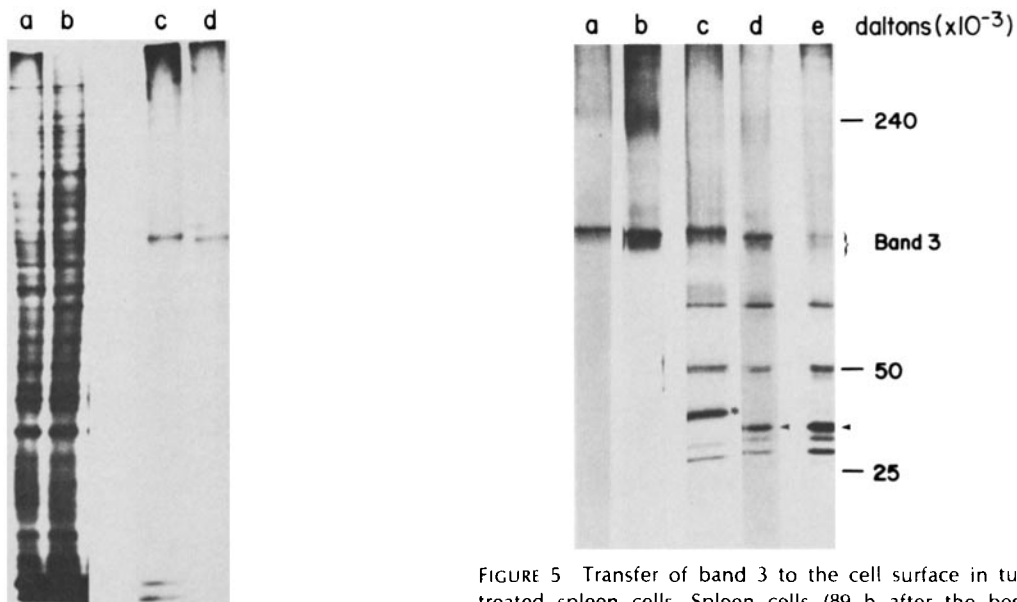

FIGURE 4   Tunicamycin treatment of spleen cells. Spleen cells obtained 89 h after the first phenylhydrazine injection were incubated at 37°C with tunicamycin (0.5 μg/ml) for 3 h before labeling with [³⁵S]methionine for 4 h. Fluorographs of total protein electrophoretic profiles of control (*a*) and treated cells (*b*) and the corresponding immuneprecipitates (*c* and *d*) are shown.

FIGURE 5   Transfer of band 3 to the cell surface in tunicamycin treated spleen cells. Spleen cells (89 h after the beginning of phenylhydrazine treatment) were labeled with [³⁵S]methionine as controls (*a* and *c*) or in the presence of 0.5 μg/ml tunicamycin (*b*, *d*, and *e*). Labeling was for 4 h (*a*, *b*, and *c*), for 15 min (*d*), or for 15 min followed by a 4 h chase (*e*). Lysates of intact cells (*a* and *b*) or cells treated with chymotrypsin (200 μg/ml at 4°C for 30 min) (*c*, *d*, and *e*) were analyzed by immunoprecipitation with anti band 3 antibodies followed by gel electrophoresis and fluorography.

notion that the protein is synthesized on bound polysomes and cotranslationally inserted in the ER membranes.

To directly ascertain the subcellular site of synthesis of band 3, free and membrane bound polysomes were isolated from spleens of anemic mice and their capacity to synthesize band 3 was assayed by the use of in vitro translation systems. A previous report (10) indicated that membrane-bound polysomes, prepared from spleen by standard fractionation procedures, have low protein synthesis activity. We adopted a cell
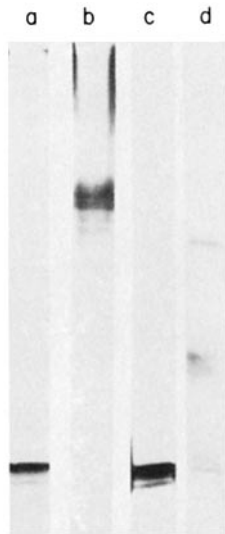
FIGURE 6 In vitro synthesis of band 3 by membrane bound polysomes. Free and membrane bound polysomes from the spleens of anemic mice were prepared as described in Materials and Methods, but a small piece (0.1 g) of rat liver was included in the initial homogenization. The polysomes (5.5 A_{260} U suspended in 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$) were used to program translation in reticulocyte lysates (200 μl total volume), incubated at 27° for 60 min. Immunoprecipitates obtained from systems programmed with free (a and c) or bound polysomes (b and d) using anti band 3 IgG (a and b) or anti albumin IgG (c and d) are shown after analysis by SDS-gel electrophoresis followed by fluorography.

fractionation based on that developed by Ramsey and Steele (47, 48, 49) for rat liver in which homogenization is carried out in a medium containing 250 mM KCl, a concentration of KCl that has been reported to release loosely bound polyribosomes (48) from microsomal membranes. This method also uses RLHSS to inhibit endogenous nucleases. When incubated in the reticulocyte lysate cell-free translation system, free- and membrane-bound polysomes showed comparable, high in vitro amino acid incorporation activities (approximately 30 times above background), but only membrane bound polysomes yielded a product immuneprecipitable with anti-band-3 antibodies (Fig. 6). The quantitative removal of membrane bound polysomes from the free polysome fraction was demonstrated in control experiments in which a small piece of rat liver was added to mouse spleens before cell fractionation and the distribution of albumin synthesizing polysomes assessed. Fig. 6 shows that no band-3 or albumin mRNA activity was detected in free polysomes. It should be noted that the band representing the polypeptide immunoprecipitated with anti-band-3 antibodies is unusually diffuse. This electrophoretic behavior of the in vitro synthesized band 3 may result from partial degradation or premature termination of polypeptide growth in the reticulocyte lysate, or from completion of synthesis of nascent chains partially glycosylated in vivo.

The site of synthesis of band 3 on membrane-bound polysomes was confirmed using mRNA-dependent translation systems. Because of mRNA extracted from purified membrane-bound polysomes was a poor template for in vitro translation preparations, we first demonstrated that while band-3 mRNA activity was easily detectable in preparations of total cellular mRNA extracts, no band-3 mRNA template activity was present in a highly active fraction of mRNA from free polysomes (Fig. 7A). Subsequently, active mRNA contained in mem-

brane-bound polysomes was obtained by the method using guanidine hydrochloride, directly from the crude particulate fraction (P1) sedimented in the first step of the fractionation procedure which serves as the source of membrane-bound polysomes. Translation products of mRNA extracted from total spleen, from this particulate crude membrane fraction, from free polysomes, and from a supernatant devoid of polysomes, as well as the respective immuneprecipitates are compared in Fig. 7B. It can be seen that only total mRNA and mRNA from P1, a source of bound polysomes, directed the cell-free synthesis of an immuneprecipitable polypeptide corresponding to band 3. The site of synthesis of band 3 in bound polysomes suggests that the polypeptide is cotranslationally inserted into microsomal membranes.

It should be noted that, whereas at optimal conditions of anemia, immuneprecipitated band 3 from spleen cells represents up to 0.35% of the total [^{35}S]methionine labeled protein, band 3 represented at least an order of magnitude lower fraction of the cell-free translation products. This is probably due either to difficulties in obtaining undergraded high mol wt mRNA, or to a preferential translation of lower molecular weight messengers, including that for globin, in the reticulocyte lysate.

For subsequent characterization of the product of in vitro translation, mRNA preparations enriched for band-3 mRNA were obtained by fractionation of poly A (+) mRNA from spleen cells of anemic mice through sedimentation in sucrose gradients of low ionic strength. Band-3 mRNA sedimenting at ~25S, was recovered in fractions that contained ~3% of the total translation activity and at least a 30-fold enrichment was effected by this fractionation procedure (data not shown). When the gradient-purified mRNA was used as template, band 3 represented 1% of the total incorporated radioactivity, a fraction that was adequate for further studies on the characterization of the cell-free product and its incorporation into microsomal membranes.

To assess the possibility that nascent band-3 polypeptides are proteolytically processed during their insertion into the ER membrane, the electrophoretic mobility of band 3 synthesized in vitro was compared with the mobilities of the immuneprecipitable polypeptides synthesized in vivo in control cells and in cells treated with tunicamycin (Fig. 8). It was found that the products of cell-free translation (Fig. 8c and d) were of slightly higher mobility than the mature protein synthesized in spleen cells (Fig. 8a). The mobility of the in vitro product, however, was indistinguishable from that of the unglycosylated polypeptide synthesized in tunicamycin-treated cells (Fig. 8b). The resolution of the SDS gradient gels used is such that a 2,500 mol wt difference could have been detectable as separate bands (1 mm apart) even in the high molecular weight range of band 3. These results, therefore, suggest that band 3 is not synthesized as a large polypeptide precursor that is processed proteolytically either co- or posttranslationally. This conclusion must be substantiated however, by direct comparison of amino terminal sequences of the in vitro synthesized and mature polypeptides. This is not attainable at the moment because the amino terminus of mature band 3 is blocked (16) and no specific sequence information on this protein is yet available.

A direct demonstration of the cotranslational glycosylation of band 3 during its insertion into the ER membrane was achieved in a cell-free translation system supplemented with dog pancreas microsomal membranes. When membranes were present during translation, but not when they were added
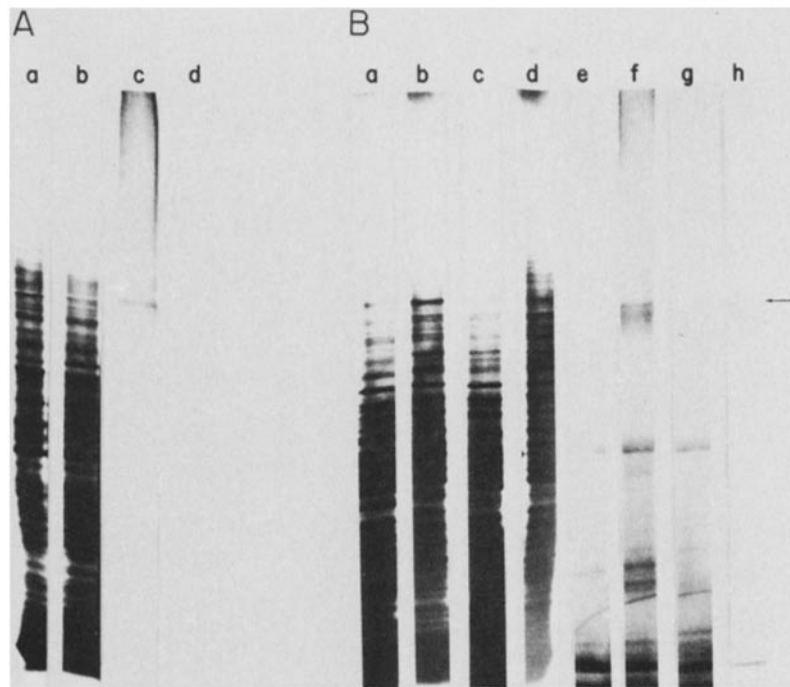
FIGURE 7 Distribution of band 3 mRNA in cell fractions from spleen cells. Translation was carried out in the reticulocyte lysate as described. A. Translation products of total spleen mRNA (a and c) and free polysomal mRNA (b and d) are shown. Total mRNA was prepared by phenol extraction of spleen cells according to Merkel et al., (43). Lanes a and b show total translation products present in 5 μl aliquots of each translation mixture whereas c and d show the products immunoprecipitated with anti band 3 antibodies from 143 μl of translation mixture. B. Translation products of free (a and e), bound (b and f), nonpolysomal (c and g), and total mRNA (d and h). The guanidine hydrochloride procedure described in Materials and Methods was used to prepare total mRNA from the spleen of animals sacrificed 118 h after initiation of anemia or to prepare RNA from the crude particulate fractions obtained in the first step of cell fractionation, which contains microsomes bearing membrane bound ribosomes. After separating free polysomes, as described in Materials and Methods, RNA was extracted by the guanidine HCl method from both the supernatant fluid (diluted 3 times with 8 M guanidine HCl) and pellet fractions to yield nonpolysomal RNA and RNA of free polysomes. 10 μg of oligo d(T) cellulose purified mRNA were used for translation in 200 μl of translation mixtures containing reticulocyte lysate. Total [^{35}S]methionine labeled products in a 5 μl aliquots are shown in lanes a to d and the corresponding immunoprecipitates from the remainder of the translation mixtures are shown in lanes e–h. The arrow marks the position of the translation product specifically immunoprecipitated with anti band 3 antibodies.

posttranslationally, a fraction of the immunoprecipitated product was converted into a form that migrated with a slightly lower mobility (Fig. 10) as expected for cotranslational addition of oligosaccharide chains. The extent of glycosylation (~30%) of the chains that were affected corresponded to the capacity of the same membranes (not shown) to process and to sequester within the microsomal lumen, human placental lactogen, a secretory protein containing a transient amino terminal signal (4).

Cotranslational incorporation of band 3 into the microsomal membranes was also demonstrated by the partial resistance of the polypeptide to proteolysis when a mixture of trypsin and chymotrypsin was added after translation in the presence of membranes had been completed. A 36,000 dalton immunoprecipitable polypeptide was recovered with the membranes, which, it can be presumed, represents a portion of band 3 that is contained within the membrane and/or is exposed on the luminal face of the vesicles. This polypeptide was not protected from proteolysis when membranes were omitted or were added posttranslationally immediately before protease treatment. The fact that a portion of the cotranslationally inserted band 3 was still accessible to proteases, as expected for a transmembrane protein, should be contrasted with the complete inaccessibility to proteases of secretory proteins, which in similar in vitro systems are transferred to the microsomal lumen (3).

DISCUSSION

The results presented in this paper elucidate important aspects of the mechanism of biosynthesis of band 3 concerning both the initial incorporation of the polypeptide into intracellular membranes and its ultimate transfer to the plasma membrane. We demonstrated that band 3 is synthesized on membrane-bound ribosomes and that the polypeptide is cotranslationally inserted into the ER membranes. Concurrently with this process, band 3 acquires its core oligosaccharides and a transmembrane disposition with respect to the phospholipid bilayer which, most likely, is maintained during its transfer to the cell surface. It should be emphasized, however, that band 3 does not have the simple transmembrane disposition of most other well studied viral (33, 51) and cellular (12, 35, 46, 64, 66) plasma membrane glycoproteins, which have their amino terminal ends exposed on the extracellular surface of the membrane and the COOH terminal portions on the cytoplasmic side. Not only does band 3 cross the membrane more than once (15, 32) but a large (40,000-daltons) amino terminal segment of the protein is exposed on the cytoplasmic face of the plasma membrane (21). This transmembrane disposition cannot be explained as resulting from the interruption of a simple vectorial discharge across the ER membrane, such as that which appears to occur during the insertion of the G protein of VSV

into the ER membrane, which is initiated by an amino terminal transient insertion signal but is thought to be halted by a distal halt or stop transfer signal (39, 51).

Because our observations make it unlikely that newly synthesized band 3 undergoes co- or posttranslational proteolytic cleavages that could expose the amino terminus on the cytoplasm, we propose that the insertion of band 3 into the membrane is initiated by a cotranslational insertion signal that is
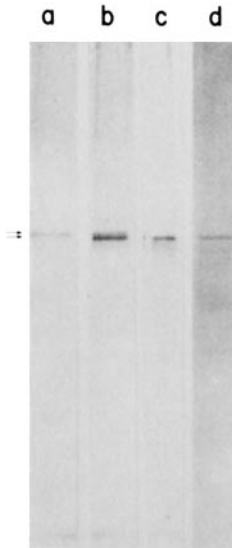

FIGURE 8 Electrophoretic mobilities of mature, unglycosylated and in vitro synthesized band 3. Immunoprecipitates obtained with anti band 3 antibodies from [^{35}S]methionine labeled control spleen cells (a) or cells treated with tunicamycin (0.5 μg/ml) cells (b), are compared with immunoprecipitates obtained from wheat germ (c) and rabbit reticulocyte (d) translation mixtures programmed with sucrose gradient enriched band-3 mRNA. The position of glycosylated and unglycosylated forms are indicated by arrows. The upper, weaker band in lane b represent the glycosylated protein arising from incomplete inhibition by tunicamycin.

located in the interior of the polypeptide, but is functionally equivalent to the transient or permanent signals found in other membrane polypeptides synthesized in bound polysomes. Such an interior signal would trigger association of the synthesizing ribosome with the membrane relatively late in translation, leaving the 45,000-dalton amino terminal segment preceding the signal permanently exposed on the cytoplasmic side of the membrane (Fig. 9). The functioning of such an internal signal for cotranslational insertion would be completely analogous to that of amino terminal signal segments in other membrane and secretory proteins that, during protein synthesis, are thought to adopt a loop disposition within the membrane, with their amino termini remaining on the cytoplasmic surface of the ER (30, 37, 60). The interior insertion signal of band 3 would be expected to remain as a permanent transmembrane segment of the mature protein, which, however, crosses the membrane at least one more time. Once inserted in the membrane the final complex disposition of band 3 could result from a conformational rearrangement that would leave specific domains of the polypeptide exposed on each side of the membrane. Alternatively, by analogy with the situation with other membrane polypeptides that have a simple transmembrane disposition, band 3 could have a halt-transfer signal that interrupts the vectorial discharge initiated by the preceding interior cotranslational insertion signal and leaves subsequent regions of the polypeptide exposed on the cytoplasmic side of the membrane. Of course, in this way, a sequence of multiple halt transfer and interior insertion signals could determine multiple crossings of the membrane, as illustrated in Fig. 9.

Having demonstrated that newly synthesized band 3 is incorporated into the ER membrane, we studied the subsequent transfer of the polypeptide to the cell surface using cultured spleen cells that were labeled in vitro. All band-3 molecules synthesized in the erythroid cells were incorporated into the plasma membrane, as indicated by their susceptibility to digestion by extracellular chymotrypsin. However, the peptide pattern generated by this digestion was somewhat different from that of band 3 in mature erythrocytes, in which chymotrypsin
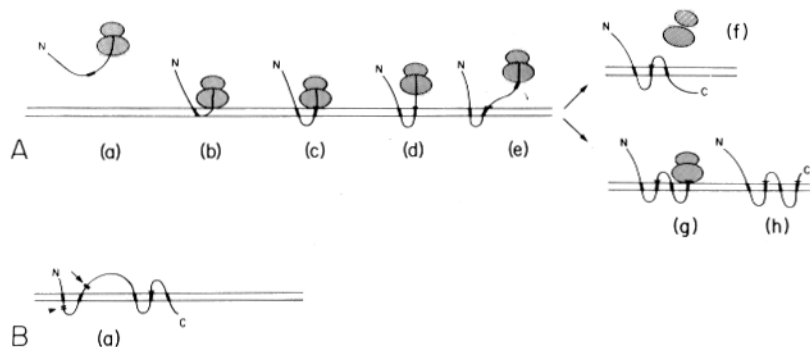

FIGURE 9 Models to account for the cytoplasmic exposure of a large amino terminal segment of a transmembrane protein which crosses the membrane more than once. A. An interior insertion signal (■) (a), (b) initiates the incorporation of the polypeptide into the membrane leaving a large amino terminal segment exposed on the cytoplasmic face. A halt transfer signal (T) (c), (d) prevents passage of the polypeptide through the membrane leaving distal regions on the cytoplasmic side until a subsequent insertion signal reinitiates passage through the membrane (e). This would lead to exposure of the COOH terminal end of the polypeptide on the luminal aspect of the membrane (f), unless a second halt transfer signal (g, h) is present in the polypeptide. B. Cytoplasmic exposure of the amino terminus could result from cleavage on the cytoplasmic side of the membrane (arrow) of a polypeptide that is initially inserted in the membrane via an amino terminal signal, which is soon followed by a stop transfer signal. The first insertion signal itself would be a transient feature removed by cotranslational cleavage (arrowhead) from the nascent chain. Our results do not favor this model because the size of the primary translation product of band-3 messenger RNA was indistinguishable from that of the mature but unglycosylated polypeptide synthesized in cells treated with tunicamycin. This makes it unlikely that a sizable amino terminal segment (40-50 amino acids) is removed proteolytically.
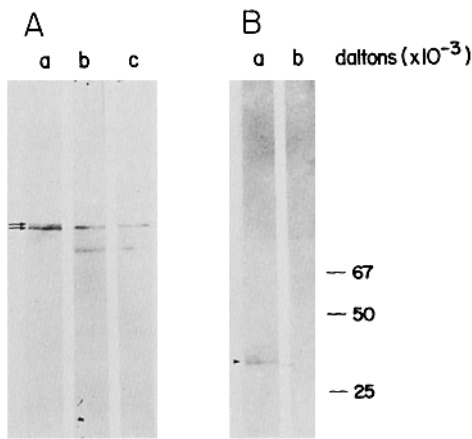
FIGURE 10 Cotranslational insertion of band 3 into dog pancreas microsomal membranes. A. Wheat germ translation mixtures (75 μl) programmed with sucrose gradient purified band 3 mRNA received: (a) 2 A₂₆₀/ml of nuclease treated dog pancreas microsomal membranes added before translation for 120 min. at 25°C; (b) no addition; In (c) dog pancreas membranes (2 A₂₆₀/ml) were added only after translation for 120 min and incubation with 3 μl of RNase A (0.5 mg/ml) for 10 min at 25°. The mixture was then incubated for 30 min more. Immunoprecipitates obtained with anti-band 3 antibodies were analyzed by gel electrophoresis and fluorography. The bands in the lower part of lanes (b) and (c) appear to represent incomplete translation products, specifically immunoprecipitated by anti-band 3 antibodies when translation is carried out in the wheat germ system in the absence of membranes. B. Effect of proteolysis on band 3 incorporated in dog pancreas microsomal membranes. Nuclease treated unstripped dog pancreas membranes (5 A₂₆₀/ml) were added to mRNA translation mixtures programmed with enriched band 3 mRNA, before (a) or after (b) translation, as described above. Translation mixtures were cooled on ice, CaCl₂ was added to a final concentration of 0.77 mM and 30 μl of a mixture of trypsin and chymotrypsin (each 0.5 mg/ml) was added before incubation at 4° for 3 h. Before immunoprecipitation with anti-band 3 antibodies, proteases were inactivated with 50 μl of trasylol (10⁴ U/ml, Mobay Chemicals) and the mixture brought to 2% SDS. Immunoprecipitates were analyzed by SDS gel electrophoresis followed by fluorography.

digestion generates mainly two fragments of 32,000 and 64,000 daltons from the mature protein (54), whereas five different immuneprecipitable polypeptides were produced by chymotrypsin digestion of spleen cells labeled in culture before cell lysis occurred. This may result from differences in conformation or in accessibility to the protease. It is thought that in mature erythrocytes, band 3 is contained within dimeric or tetrameric complexes (36, 57, 67) that function in anion transport (7), and it is possible that this quaternary structure is yet to be formed in the immature spleen cells, which probably have a lower concentration of monomeric subunits. This could be responsible for conformational differences reflected in the sensitivity to the protease. It is also possible that although functional band-3 complexes may be assembled in the immature cells, differences in other plasma membrane components affect the sites exposed to protease cleavage.

Band 3 is a glycoprotein that contains asparagine-linked oligosaccharide chains and its synthesis was affected in a predictable fashion by treatment of cells with tunicamycin. The nonglycosylated band-3 polypeptide, with a slightly faster electrophoretic mobility than the mature protein was, however, effectively transferred to the cell surface where, after a 30 min chase, it became completely sensitive to digestion by chymo-

trypsin. This observation indicates that, as is the case with several other viral (27, 52) and cellular (23) membrane proteins, the carbohydrate moieties attached to the polypeptide are not necessary for its incorporation into the plasma membrane, and therefore do not serve as sorting out signals directing the transfer of band 3 from the ER to the cell surface. A similar conclusion has been reached for several secretory proteins (17, 62). In some instances, however, inhibition of glycosylation has been shown to impair secretion (29) or incorporation of a protein into the plasma membrane (24), although this may result from altered solubility properties of the unglycosylated product, which may not be compatible with operation of intracellular distribution mechanisms (25). It should be noted, however, that unglycosylated band 3 was somewhat more susceptible to digestion by the extracellular protease than the normal protein. The patterns of immuneprecipitable digestion products generated from the mature and unglycosylated proteins appeared identical, except that the apparent molecular weight of a single fragment was reduced from 38,000 to 34,000 by tunicamycin treatment. It is therefore likely that this segment contains the major asparagine-linked carbohydrate chain of the mature protein.

An important question concerning the biosynthesis of plasma membrane glycoproteins is whether the orientation of the mature proteins is attained during their insertion into the ER membranes. Proteolysis of the microsomes containing the inserted in vitro synthesized band 3 yielded only one immuneprecipitable polypeptide of ~36,000 daltons. From the disposition of band 3 in the plasma membrane of mature erythrocytes one would have expected a more complex pattern of proteolytic fragments. The presence of only one immuneprecipitable fragment is probably due to the fact that the antiserum used, which was preadsorbed with intact erythrocytes, is directed primarily against the cytoplasmically exposed segments of band 3, which appear to be the more immunogenic portions of the molecule (Marchesi, unpublished data). It would be of considerable interest to directly compare the orientation of band 3 in the ER membrane with that of the mature protein in the cell surface of spleen cells to rigorously evaluate the basic assumption of the model presented here. It is perhaps worth reiterating that this model does not preclude that changes in the conformation of membrane polypeptides that could alter the exposure of specific peptide segments may occur after the initial insertion in the ER, as a result of subsequent interactions with other specific integral and peripheral membrane proteins.

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