HORMONALLY REGULATED PHOSPHOPROTEIN OF TURKEY ERYTHROCYTES

Localization to Plasma Membrane

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ABSTRACT

The catecholamine-stimulated cotransport of sodium and potassium ions across the plasma membrane of the turkey erythrocyte was previously found to be associated with increased $^{32}$P incorporation into a high molecular weight protein. To determine the subcellular localization of this phosphorylated protein, which we have termed goblin, a new method has been developed for isolation of pure plasma membranes from turkey erythrocytes. With this method, it has been demonstrated that goblin is located in the plasma membrane. Goblin is not extracted by solutions of low or high ionic strength but is partially extracted by nonionic detergents, indicating that it is not a component of turkey erythrocyte spectrin and suggesting that it may be an intrinsic protein of the plasma membrane. The data are compatible with a possible role for goblin in the hormonal control of ion movements across the plasma membrane.

KEY WORDS catecholamines - cyclic AMP - cation transport - avian erythrocyte - plasma membrane - phosphoprotein

Turkey and duck erythrocytes respond to stimulation by $\beta$-adrenergic agonists with an increase in the transmembrane movement of sodium and potassium ions and, under appropriate conditions, with a net uptake of ions and water (11-16, 20-22, 34, 37-39, 41). These altered ion movements appear to involve a mechanism which cotransports sodium and potassium (21, 41) and which is functionally distinct from the sodium-potassium pump (12, 15, 21, 41). This response to $\beta$-adrenergic agonists takes place on a time scale of minutes, and appears to be mediated by an increase in the intracellular level of cyclic AMP (13, 14, 22, 34).

Cyclic AMP has been shown to mediate the hormonal regulation of several soluble enzyme activities through cyclic AMP-dependent changes in protein phosphorylation (33), and it has been proposed that changes in protein phosphorylation (24) mediate many, if not all, of the physiological effects of cyclic AMP in eukaryotic cells. However, it has not yet been established that the effects of cyclic AMP on membrane function also involve cyclic AMP-dependent protein phosphorylation. Turkey erythrocytes, available in large quantity as a homogeneous cell population, appear to be an excellent system for determining whether cyclic AMP can regulate plasma membrane function by controlling the phosphorylation of specific membrane proteins.

When turkey erythrocytes were preincubated in
42P, to label the intracellular ATP, subsequent exposure to either β-adrenergic agonists or cyclic AMP caused an increased incorporation of 42P into a high molecular weight polypeptide, with an apparent mol wt on SDS-polyacrylamide gels of ~240,000 (38). This increased 42P incorporation correlated with increased ion fluxes under a number of experimental conditions (37, 38). Although the subcellular location of the high molecular weight protein was not established, it was hypothesized that the state of phosphorylation of this protein might regulate sodium-potassium cotransport across the plasma membrane of turkey erythrocytes (38). Mature turkey erythrocytes differ from mature mammalian erythrocytes in that they still contain a prominent nucleus, a few mitochondria, and other organelles. The work reported here was undertaken to localize the high molecular weight phosphoprotein within the turkey erythrocyte.

Various techniques have been reported to fragment hypotonically swollen or lysed avian erythrocytes in such a way as to permit the separation of plasma membranes from nuclei by differential centrifugation. These include Dounce (7, 17) and Polytron (6, 50) homogenizations, and disruption by French press (3) and nitrogen cavitation (46). For the small volumes of highly radioactive erythrocytes needed for our studies, sonic disruption of ghosts proved to be preferable to the above methods. We have found that sonically disrupted erythrocyte ghosts can be used as a starting preparation for isolating a satisfactory plasmalemmal fraction in which more than half of the high molecular weight phosphoprotein is recovered at high concentration. Results obtained by differential extraction suggest that the phosphoprotein is either an intrinsic protein of the plasmalemma or tightly bound to such a protein.

MATERIALS AND METHODS

Buffered solution A consisted of 157.5 mM NaCl, 2.5 mM KCl, 11.1 mM glucose, and 10 mM (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES),1 adjusted to pH 7.4 with NaOH. Buffered solution B consisted of 10 mM MgCl2 and 10 mM Tris-HCl, pH 8. Freshly drawn turkey blood was washed as previously described (38). The erythrocytes were suspended at 10% (vol/vol) in solution A and were equilibrated at 39°C for 60 min in a shaking water bath. Human blood was also used in some experiments. All conditions used for human blood were identical to the conditions used for turkey blood except that (a) solution A was modified in that it contained 2 mM orthophosphate in addition to the other constituents given above, and (b) incubations were carried out at 37°C rather than 39°C.

Protein Phosphorylation

To study protein phosphorylation, 100 µl of carrier-free 42P, (2.0–2.5 mCi) in Tris-HCl was added to an aliquot of the 10% cell suspension (final vol, 2.4 ml), which was maintained for 3–6 h at 39°C. The suspension was then divided into two 1.2-ml aliquots. 12 µl of a 10–4 M solution of dl-isoproterenol was added to one aliquot (final concentration 10–9 M), and both the control and the isoproterenol-treated cells were incubated for an additional 20 min at 39°C. After this incubation, a 40-µl aliquot was removed from both the control and hormone-stimulated cells and prepared for SDS-gel electrophoresis. The remaining erythrocytes were hypotonically lysed and then either fractionated or subjected to various extraction procedures, as described below. The cell fractions—ghosts, nuclei, plasma membranes, etc.—were re-suspended in solution B to the original volume of starting material, and 40-µl aliquots were removed for SDS-gel electrophoresis.

The aliquots of the 42P-labeled samples were solubilized for SDS-gel electrophoresis by adding them to tubes containing 100 µl of "stop solution" (9% SDS, 30 mM Tris-HCl, pH 7.8, 3 mM EDTA, 10% glycerol, a small amount of pyronin Y as a tracking dye) and sufficient water to bring the final vol to 300 µl. Immediately after addition of the sample aliquots, the tubes were transferred to a boiling water bath for 20 min. After removal from the boiling water bath and cooling, 100 µl of dithioerythritol (75 mg/ml) was added to each tube.

A modification of the above procedure was made for the lysis supernate (see Results), which was necessarily more dilute than the other fractions. Thus, a proportionately larger aliquot of supernate was added to a tube containing 100 µl of stop solution. The tube was then boiled until the volume was reduced to that of the other boiled samples (slightly <300 µl). Subsequent treatment of the lysis supernate was identical to that of the other samples.

Polyacrylamide Gel Electrophoresis

After overnight storage of the samples at room temperature, 50- to 100-µl aliquots were subjected to SDS-polyacrylamide gel electrophoresis. The gel system used was that described by Laemmli (25). The gels were prepared as previously described (27) except that the separation portion of the gel contained 6% acrylamide (wt/vol) in 0.25 M sucrose. We shall refer to these gels as "Laemmli gels." After being run, the gels were stained, destained, dried, and subjected to autoradiography (44). The amount of radioactive phosphate incorporated into

1 Abbreviations used in this paper: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; PCA, perchloric acid; PMSF, phenylmethylsulfonyl fluoride.
the individual polypeptide bands was quantitated by scanning the autoradiographs with a microdensitometer (23, 44) and, when indicated, by scintillation counting of the gel bands as well.

The apparent mol wt of polypeptide bands on the SDS gels were estimated by comparing the migration of the bands of interest with the migration of the following standard proteins: phosphorylase a (92,500), bovine serum albumin (68,000), immunoglobulin G-heavy chain (50,000), aldolase (40,000), immunoglobulin G-light chain (25,000), and cytochrome c (13,500). As an additional standard the mol wt of band 1 of human spectrin was taken to be 240,000 (18).

**Processing of Fractions for Microscopy**

Aliquots of intact cells in solution A and the various cell fractions in solution B were centrifuged (30 s at 12,000 g, except for the plasma membrane fraction which was centrifuged for 30 min at 150,000 g) to yield small pellets 1–2 mm in diameter and <1 mm in thickness. After removal of the supernate the pellets were fixed for 2 h at 4°C with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0, and postfixed for 1 h at 4°C with 1% OsO₄ in the same buffer. The samples were stained overnight in block with uranyl acetate, dehydrated, and embedded in Epon (29). Sections cut with a diamond knife on a Sorvall (Porter-Blum) microtome MT-2 (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) were contrasted with uranyl acetate and lead citrate (45). Micrographs were taken at magnifications ranging from ×3,000 to ×40,000 with a Siemens 102 or a JEOL 100CX electron microscope.

**Other Assays**

Cold TCA (10% final concentration) was used to precipitate duplicate aliquots of the various cell fractions. The TCA precipitate of one of these aliquots was dissolved in 1 N NaOH and assayed in triplicate for protein according to the method of Lowry et al. (28), using bovine serum albumin as the standard. The TCA precipitate of the other aliquot was extracted with 0.5 N perchloric acid (PCA) for 20 min at 75°C. This PCA extract was assayed in triplicate for DNA according to the procedure of Burton (5).

The procedure of Alexander et al. (1), as modified (39), was used to measure the binding capacity of [3H]alprenolol present in 200-μl aliquots of the erythrocyte cell fractions suspended in solution B. Specific [3H]alprenolol binding was calculated as the difference in the binding which occurred when identical samples were incubated with 10⁻⁸ M [3H]alprenolol (a saturating concentration) in the absence or presence of 10⁻⁴ M dl-isoproterenol.

For this and all other centrifugations, time was counted from the moment the centrifuge reached top speed.

Succinate-cytochrome c reductase activity was measured as described by Yamashita and Racker (48). The assay medium differed from that of Yamashita and Racker in that it contained KCN, at a final concentration of 1 mM, and in that Sigma type III cytochrome c was used in place of the type II preparation.

**Extraction of the Erythrocyte Ghosts**

Ghosts were prepared by hypotonic lysis and washing as described under the heading “Cell Fractionation” in the Results section. Three extraction conditions were examined: low ionic strength, high ionic strength, and nonionic detergents. For the low ionic strength extraction, the pelleted ghosts obtained from 100 μl of packed cells were suspended in ice-cold “low-ionic strength solution” (3 mM β-mercaptoethanol, 0.03 mM phenylmethylsulfonylfluoride (PMSF), previously adjusted to pH 8.5 with NH₄OH) to a final vol of 1 ml. This ghost suspension was dialyzed overnight at 4°C, with one change, against 1 liter of the same low ionic strength solution. The material remaining in the dialysis bag was subjected to centrifugation (150,000 g, 30 min). The supernate was removed and saved. The pellet was resuspended in 1 ml of ice-cold low ionic strength solution, by repeated passage through a small-bore needle.

For the extractions with high ionic strength medium or nonionic detergent, respectively, 0.1 ml of a 20% (vol/vol) suspension of ghosts in solution B was diluted with 0.6 ml of either 1 M NaCl or 0.3% Ammonyx-LO and incubated on ice for 2 h. Following incubation, both samples were centrifuged at 150,000 g for 30 min. The supernates were removed and saved. The pellets were resuspended to 0.7 ml with 1 M NaCl or 0.3% Ammonyx-LO, respectively.

**Materials**

Ammonyx-LO was obtained from Onyx Chemical Co. (Div. of Millimeter Onyx Corp., Jersey City, N.J.). Nonidet P-40 was obtained from Particle Data Inc. (Elmhurst, Ill.). Triton X-100, cytochrome c, and dl-isoproterenol-HCl were obtained from Sigma Chemical Co. (St. Louis, Mo.). 32P (carrier-free) and [3H]alprenolol (sp act 32.65 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). All other reagents were of the highest purity commercially available.

**RESULTS**

Previous work had shown that brief exposure of turkey erythrocytes to the β-adrenergic agonist isoproterenol resulted in a marked stimulation of 32P incorporation into a high molecular weight phosphoprotein (38). In the gel system described by Fairbanks et al. (10), this high molecular weight phosphoprotein comigrated with the lower band of a high molecular weight protein-staining doublet reminiscent of the spectrin doublet of human...
erythrocytes. To better resolve the high molecular weight proteins of both turkey and human erythrocytes, we employed the gel electrophoresis system described by Laemmli (25) (Fig. 1). This gel system resolves a high molecular weight doublet in human erythrocytes (Fig. 1, arrows, left-hand lane). In agreement with previous work (35, 36, 47) using the gel system of Fairbanks et al. (10), $^{32}$P is incorporated into a protein in human erythrocytes which comigrates with the lower band of this doublet; isoproterenol does not alter the amount of $^{32}$P incorporated into this protein. In turkey erythrocytes, however, Laemmli gels resolve a high molecular weight triplet (arrows, right-hand lane). On the basis of evidence presented below, we have tentatively identified the highest and lowest bands of this triplet as turkey spectrin (right-hand lane, light arrows). The middle band of the triplet (right-hand lane, heavy arrow) comigrates with the isoproterenol-responsive phosphoprotein. We have christened this high molecular weight phosphoprotein "goblin" because, in the company of spectrin, it "haunts" the higher regions of the gel, and because it is found in the erythrocytes of an avian species with a characteristic vocalization.

The autoradiograph in Fig. 1 and the traces obtained from it by scanning microdensitometry (not shown) reveal that $^{32}$P was incorporated into a number of proteins of the turkey erythrocyte, both in the absence and the presence of isoproterenol. In the experiment illustrated in Fig. 1, isoproterenol stimulated $^{32}$P incorporation into goblin by a factor of 2.4. (The increased incorporation of $^{32}$P into the lower band of spectrin was contributed by a proteolytic degradation product of goblin [manuscript in preparation].) In five additional experiments, isoproterenol stimulated the incorporation of $^{32}$P into goblin by a factor of 2.7 ± 1.4. Scintillation counting of gel slices containing $^{32}$P-goblin gave results qualitatively similar to those obtained by densitometry of the autoradiographs (data not shown). This stimulation is comparable to that reported by Rudolph and Greengard (38) using the Fairbanks et al. (10) gel system.

**Cell Fractionation**

To determine whether goblin is a plasma membrane protein, turkey erythrocytes were preincubated in $^{32}$P, to label intracellular ATP and the cells were divided into two equal aliquots, one of which was stimulated for 20 min with isoproterenol. Both the control and isoproterenol-treated cells were fractionated according to the procedure described below. All manipulations were carried out at 4°C.

**Hypotonic Lysis:** Turkey erythrocyte ghosts were prepared by rapidly diluting 1 ml of a 10% (vol/vol) cell suspension into a 15-ml Corex tube (Corning Glass Works, Science Products Div., Corning, N. Y.) containing 10 ml of solution B, as the hypotonic lysis solution. The tube was vortexed vigorously during the addition of the cell suspension. The ghosts produced by this procedure were sedimented by centrifugation at 12,000 g for 15 s in a Sorvall SS-34 rotor. The supernate was
removed and stored on ice. The ghosts were washed by resuspension in 5 ml of solution B followed by a recentrifugation. The supernate from this wash step was pooled with the supernate from the initial hypotonic lysis.

The pellet obtained upon hypotonic lysis of intact avian erythrocytes consists of two layers (cf. reference 6). The upper layer, which comprises the major portion of the pellet, is white to light pink in color and consists of erythrocyte ghosts. The lower layer is a small white button of material which firmly adheres to the bottom of the tube, and is probably composed of leukocytes (6, 10). In some experiments precautions were taken to prevent contamination of the erythrocyte ghosts with material from this lower layer of the pellet. These precautions had no obvious effect, however, on the protein staining pattern or autoradiograph of the resulting plasma membrane fraction.

**SONICATION**: The pellet of the washed ghosts, still in the 15-ml Corex tube, was resuspended to 1 ml with solution B. The sample was then sonicated for 10 s at a setting of 3 (meter reading = 20) on a model W140 sonifier with Special Microtip (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). These sonication conditions maximized fragmentation of plasma membranes while minimizing disruption of nuclei, as judged by phase contrast microscopy.

**LOW AND INTERMEDIATE SPEED CENTRIFUGATIONS**: Unfragmented cells and nuclei were pelleted by centrifugation at 900 g for 10 min to yield a “nuclear fraction.” The supernate, which contained the plasma membrane fragments and had a cloudy white appearance, was carefully transferred to another tube. Neither washing the 900-g pellet by resuspension and centrifugation nor a second 10-s sonication of the resuspended 900-g pellet significantly increased the yield of plasma membranes. The few nuclei left in the 900-g supernate were removed, together with most mitochondria and many plasmalemmal fragments, by centrifugation at 12,000 g for 30 s. The resultant pellet will be referred to as the “mixed membrane fraction.”

**HIGH SPEED CENTRIFUGATION**: The plasma membranes present in the 12,000-g supernate were pelleted by centrifugation for 30 min at 150,000 g (47,000 rpm with a Beckman 50 Ti Rotor [Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.]). Both supernate (150,000-g supernate) and pellet (plasma membrane fraction) were retained for further analysis.

**Morphology of Cells and Cell Fractions**

The intact erythrocytes (Fig. 2) appear as biconvex discs which, with the exception of a prominent nucleus, contain little in the way of internal structures except for a few mitochondria, some remnants of endoplasmic reticulum, and a few polysomes. After hypotonic lysis the cells lose their soluble contents, but in most cases the nuclei and other subcellular components remain within their plasma membranes. The hypotonic lysis removes from the nuclei the interchromatin material (mostly hemoglobin) and causes the heterochromatin masses to swell and occasionally herniate through the nuclear envelopes, which are otherwise retained over large areas (Fig. 3).

In general, the nuclei isolated in the nuclear fraction have the same appearance as in the ghost preparation. Most of them appear intact and are still surrounded by their envelopes; fragmented or disrupted nuclei and chromatin masses are relatively rare (Fig. 4). Thus, the sonication procedure does not appear to cause significant breakage of the nuclei. The electron micrograph shown in Fig. 4 is representative of the lower 3/4 of the nuclear fraction pellet. The bottom of this pellet contains a few leukocyte fragments, and the top of the pellet contains a layer of unfragmented or only partially fragmented ghosts.

The mixed membrane fraction (not illustrated) consists primarily of plasma membrane fragments, generally larger but otherwise identical to those to be described in the plasmalemmal fraction. In addition, it contains, scattered throughout the pellet, a noticeably greater number of mitochondria than does the plasmalemmal fraction and, collected at the bottom of the pellet, a few nuclei.

The plasma membrane fraction is illustrated in Figs. 5 and 6. The fraction consists primarily of plasmalemmal fragments of different sizes. The small fragments are apparently reorganized into closed vesicles, whereas the large fragments—which predominate—appear as membrane sheets with free margins that generally tend to roll inwards. Irrespective of size and geometry, the fragments show an obvious “vertical” (transmembrane) asymmetry (Fig. 6); their outer surface is smooth, whereas their inner surface is “decorated” with small globular or fibrillar masses which range in size from 100 to 400 Å. By analogy with findings made on mammalian (human) erythrocytes, this infrastructure probably consists of peripheral...
membrane proteins, e.g. spectrins and actin (30, 32, 42, 43). The membrane itself has the usual trilayered structure and measures ~90 Å in thickness. The same structural details can be seen in the intact plasmalemma of the ghosts, before sonication (Fig. 3, insets). In that case, it is clear that the asymmetry described applies only to the plasmalemma. Hence, the asymmetry can be used as a morphological marker in assessing the homogeneity of the plasmalemmal fractions. By this criterion, the contamination of our plasmalemmal fraction by other (intracellular) membranes is negligible. Fewer than 5% of the profiles seen in sections of the plasma membrane fraction can be recognized as contaminants which include mitochondria, small smooth vesicles (presumably of endoplasmic reticular derivation), Golgi elements, coated vesicles, and small masses of heterochromatin (Fig. 5). The estimated area of contaminant membranes is ~2%.

**Chemical Analysis of Cells and Cell Fractions**

The protein and DNA contents of the cell fractions of turkey erythrocytes are presented in Table 1. Nearly all of the DNA present in the intact cells is recovered with the ghosts, and the DNA present in the ghosts is in turn largely recovered in the nuclear fraction. The DNA/protein ratio is two orders of magnitude lower in the plasma membrane fraction than in the ghosts. Thus, both morphological and chemical criteria indicate that the plasma membrane fraction is not significantly contaminated by nuclear-derived material.

The capacity of the various fractions to specifi-
Figure 3  Erythrocyte ghosts. Nuclei (n), mitochondria (m) and a few other subcellular components are retained within the collapsed plasma membrane (pm). Except for the extraction of their hemoglobin and some disruption of their heterochromatin (ch1), most nuclei appear intact. Some heterochromatin fragments herniate at the periphery of the nuclear profiles (ch2). The two insets show the plasmalemma of the ghosts and its infrastructure (if) at higher magnification. x 8,500; insets, x 100,000.

cally bind the β-adrenergic antagonist alprenolol is also summarized in Table I. From the alprenolol binding capacity of the ghosts, and the figure $6 \times 10^9$ turkey erythrocytes/ml packed cells (2), one calculates the presence of ~850 β-receptors/cell, within a factor of 2 of previously published results (4, 26, 39). If it is assumed that β-receptors are present only in the plasma membrane of the intact cells, and therefore serve as a marker for the plasma membrane, then nearly half of the cells’
plasma membrane is recovered in the plasma membrane fraction; the remainder is recovered with the nuclear and mixed membrane fractions.

To determine whether goblin is present in the erythrocyte plasma membrane, aliquots of the cell fractions were subjected to SDS-polyacrylamide gel electrophoresis using the Laemmli gel system, protein staining (Fig. 7), autoradiography, and densitometric scanning of the resultant autoradiographs. The densitometric measurements revealed that phosphogoblin is present at a comparable level in the cells and ghosts, and that roughly half of the phosphogoblin is recovered in the plasma membrane fraction (Table 1). The presence of phosphogoblin in the nuclear and mixed membrane fractions is consistent with the morphological data indicating the presence of a significant amount of plasma membrane in these fractions.

97% of the phosphogoblin measured in the erythrocyte ghosts was recovered in the derivative nuclear fraction, mixed membranes, and plasma membranes (Table 1). The high recovery of phosphogoblin in the cell fractions is consistent with the failure to detect dephosphorylation of phosphogoblin in any of the cell fractions stored overnight at 4°C, or in plasma membranes incubated for 40 min at 40°C. Furthermore, inclusion of NaF in solution B was neither necessary for, nor did it enhance the isoproterenol-dependent incorporation of 32P into phosphogoblin (data not shown). This result contrasts with studies of in vivo protein phosphorylation in rabbit skeletal muscle, in which inclusion of NaF in homogenization and extraction media was necessary for the

**Figure 4** Nuclear fraction. Representative field for the lower 1/4 of the pellet. The fraction consists of intact nuclei (n), fragmented nuclei (fn) and a few dispersed chromatin masses (ch). A “contaminating” plasmalemmal fragment appears at (pm). The inset shows parts of two nuclear profiles (at higher magnification) to demonstrate the retention of nuclear envelopes (ne). × 8,000; inset, × 20,000.

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detected isoproterenol-stimulated incorporation of phosphate into phosphorylase kinase and glycogen synthetase (33). The turkey erythrocyte thus appears to possess a much less active protein phosphatase activity than that found in mammalian skeletal muscle.

The quantitative distribution of phosphogoblin among the various fractions roughly parallels the amount of plasma membrane in those fractions as measured by alprenolol binding (Table I). Thus, 54 and 46%, respectively, of phosphogoblin and alprenolol binding present in the ghosts are recovered in the plasma membrane fraction. Densitometric measurements of the protein staining patterns of the various cell fractions indicate a close correspondence between the fractionation of phosphogoblin and that of the protein staining band with which it comigrates (data not shown). In addition to this protein band (see heavy arrows in Figs. 1 and 7), located between the two spectrin bands, other major protein staining bands present in the plasma membrane fraction are the two spectrin bands themselves, as well as a band designated band 3 (Fig. 7, bracket; nomenclature of Fairbanks et al. [10]), because it resembles and comigrates with the diffusely staining protein of the same name present in the human erythrocyte membrane. The subcellular distributions of spectrin and of band 3 are similar to that of the protein staining band which comigrates with goblin (Fig. 7). By contrast, several lower molecular weight proteins are present in the cells, ghosts, nuclear fraction, and mixed membranes, but not in the plasma membrane fraction.

Goblin is clearly the major hormone-responsive phosphoprotein present in the plasma membrane.
fraction. However, at least one additional protein (of mol wt ~48,000) can be resolved in the Laemmli gels (Fig. 7, asterisk) whose $^{32}$P content is elevated when intact cells are briefly exposed to isoproterenol.

**Extraction of Goblin from Ghosts**

Based on electrophoretic mobility in Laemmli gels, phosphogoblin appears to be distinct from turkey spectrin (Fig. 1). To further examine possible differences between goblin and turkey spectrin, we determined the extractability of these proteins under a number of conditions. The extraction experiments were carried out with freshly prepared ghosts rather than with the plasma membrane fraction, to avoid any possible artifacts introduced by sonication.

Spectrin is extracted from human erythrocyte ghosts under conditions of low ionic strength (8, 10, 19, 31). Fig. 8 shows the effect of dialyzing turkey erythrocyte ghosts against a low ionic strength medium. The protein staining pattern reveals that a large proportion of the turkey spectrin (Fig. 8a, light arrows) is extracted under these conditions. This similarity to human spectrin supports the use of the term spectrin to describe these two bands in turkey erythrocytes. The protein staining band which comigrates with phosphogoblin (Fig. 8a, heavy arrow), in contrast to turkey spectrin, is retained in the pellet after incubation at low ionic strength. The autoradiograph (Fig. 8b) directly confirms the localization of phosphogoblin to the pellet. As seen in the protein staining pattern of Fig. 8a, turkey band 3, like phosphogoblin and the protein staining band with which it comigrates, is retained in the low ionic-strength pellet. Such behavior is consistent with the notion that turkey band 3 includes intrinsic membrane proteins (49). Neither turkey band 3 nor phosphogoblin is extracted from freshly prepared ghosts by incubation with 1 M NaCl as described in Materials and Methods (not illustrated).
The data for protein and phosphogoblin recovery represent mean values ± SD from five separate fractionation experiments. Those for DNA and alprenolol binding are the values obtained in two separate fractionation experiments. Phosphogoblin in the fractions is expressed as the increment in \(^{32}\)P content which resulted from exposure of intact cells to isoproterenol; it was measured by scanning microdensitometry of autoradiographs (see references 23 and 44 for details). The values for \(^{32}\)P content were normalized to 100% for ghosts. The amount of phosphogoblin in the ghosts was 110% of that in the intact cells. The values given in the Table for alprenolol binding capacity of the ghosts were obtained after the ghosts had been sonicated; in the same experiments the mean binding capacity of nonsonicated ghosts was 11.2 pmol/ml packed cells. Aliquot sizes (normalized to the original volume of packed cells) used to measure alprenolol binding were: 10 \(\mu l\) for the ghosts, nuclear fraction, and plasma membranes; 20 \(\mu l\) for the lysis supernate; and 50 \(\mu l\) for mixed membranes and the 150,000-g supenate. With these aliquots, 8% or less of the total alprenolol added to the assay tube was bound. The ratio of specific to total alprenolol binding was 30% in the nuclear fraction, 51% in the sonicated ghosts, 58% in the mixed membrane fraction, and 75% in the plasma membrane fraction.

All values are normalized to 1 ml of packed cells as the starting material.

**Fig. 9** illustrates the effect of incubating ghosts with the nonionic detergent Ammonyx-LO. Both phosphogoblin and the protein staining band with which it comigrates were partially solubilized by the detergent. The same was true for band 3. In contrast, spectrin remained almost totally in the pellet. The nonionic detergents Triton X-100 and Nonidet P-40 yielded similar results (not illustrated). Extraction yields of phosphogoblin with the three detergents varied between 40 and 80%. Re-extraction of the residual pellet did not significantly increase the yield of solubilized phosphogoblin.

**DISCUSSION**

Evidence has been presented that goblin, a protein of the turkey erythrocyte whose phosphorylation is regulated by catecholamines and by cyclic AMP, is located in the plasma membrane of the erythrocyte. This evidence consists of the demonstration that goblin is highly enriched in a fraction of plasma membranes isolated from intact cells. The fractionation procedure, based on sonication of nucleated erythrocyte ghosts followed by differential centrifugation, produces a plasma mem-

**TABLE I**

| Protein | DNA | Alprenolol binding | \(^{32}\)P phosphogoblin |
|---------|-----|--------------------|-------------------------|
| Cells   | 261 ± 42 | 14.7, 13.5 | Undetectable |
| Lysis supernate | 242 ± 36 | 0.030, 0.072 | Undetectable |
| Ghosts  | 20.2 ± 6.5 | 14.1, 13.3 | 8.1, 8.9 |
| Nuclear fraction | 14.8 ± 3.6 | 13.9, 13.3 | 2.9, 2.3 |
| Mixed membranes | 0.522 ± 0.136 | 0.044, 0.030 | 1.4, 1.0 |
| Plasma membranes | 2.21 ± 0.87 | 0.019, 0.011 | 3.6, 4.2 |
| 150,000-g supernate | 1.21 ± 0.17 | 0.001, 0.003 | Undetectable |

**Figure 7** Protein staining pattern of cell fractions of turkey erythrocytes, as observed upon SDS-polyacrylamide gel electrophoresis. For details see text. The heavy arrow indicates goblin, the light arrows turkey spectrin. The brackets here and in Fig. 8 indicate the protein staining region of turkey erythrocytes which migrates in SDS gels like band 3 of human erythrocytes. The "-" and "+" signs indicate the absence and presence of isoproterenol, respectively. The asterisk indicates a protein, of mol wt ~48,000, whose \(^{32}\)P content is increased by isoproterenol (see text).

**BEAM, ALPER, PALADE, AND GREENGARD** Phosphoprotein of the Turkey Erythrocyte
brane fraction with good purity and yield. This fractionation procedure has advantages of ease and rapidity. The entire procedure can be completed in 90 min or less, and is easily scaled up for large preparations. Phosphoprotein kinase and adenylate cyclase activities are preserved during this procedure (unpublished results). In contrast, coupling between the β-receptor and adenylate cyclase is lost after the sonication step, in agreement with previous results (40).

By morphological criteria, the plasma membrane fraction is largely free of recognizable contaminants such as mitochondria or fragments of nuclei. While the nuclear envelope displays morphological transmembrane symmetry (Fig. 4), the plasmalemma is an asymmetric structure (Fig. 3, inset; Fig. 6), "decorated" on its cytoplasmic surface. Contaminating, symmetric membrane profiles constitute only ~2% of the plasma membrane fraction on visual inspection. In the nuclear fraction, chromatin is predominantly present within morphologically recognizable nuclei, and these nuclei retain, for the most part, their nuclear envelopes (Fig. 4). Thus, if the nuclear envelope were the predominant source of goblin, then the nuclear fraction should be enriched in goblin compared to the plasma membrane fraction. That the opposite is observed (Table I) argues against the notion that the nuclear envelope is the source of goblin.

Intact mitochondria are present in the plasma membrane fraction at a level at least 10-fold lower than in the mixed membrane fraction. The enrichment of goblin in the plasma membrane fraction therefore argues against a mitochondrial origin of this protein. However, one possibility which is difficult to exclude on morphological criteria alone is that mitochondrial fragments distorted beyond recognition are present in the plasma membrane fraction. We attempted to address this issue by assaying the cell fractions for succinate-cytochrome c reductase as a marker for mitochondrial membranes, but the interpretation of the results...
was complicated by two problems. First, as has been previously noted (50), the nucleated ghosts possessed no detectable activity. Second, though sonication of the ghosts successfully unmasked their enzymatic activity, the recovery of activity in the derivative fractions routinely exceeded by some 50% that present in the sonicated ghosts prior to their fractionation.

Certain observations, however, indirectly argue against a mitochondrial membrane localization for the protein goblin: the specific activity of succinate-cytochrome c reductase in the plasma membrane fraction was 3.5-fold lower than in the mixed membrane fraction. This agrees qualitatively with the 10-fold lower frequency of occurrence of mitochondria in the electron micrographs, but contrasts with the specific activities of alprenolol binding and relative specific activities of phosphogoblin, which are similar in the two fractions (Table I). The ratio of phosphogoblin recovery to alprenolol binding recovery remained relatively constant throughout the fractionation (Table I). The same was true for the ratio of Coomassie-blue-staining intensities on SDS-polyacrylamide gels of turkey spectrin and the band which comigrates with phosphogoblin (data not shown). Because alprenolol binding and turkey spectrin are both presumptive markers for the plasma membrane, these fixed ratios are consistent with a plasmalemmal, but not a nuclear or a mitochondrial, origin for both phosphogoblin and the protein staining band with which it comigrates.

Phosphogoblin appears to be distinct from human and turkey spectrin by several criteria. Phosphogoblin migrates in Laemmli gels at a rate different from that of both human and turkey spectrin (Fig. 1). Turkey spectrin is partially extracted from nucleated ghosts in conditions of low ionic strength, whereas phosphogoblin remains in the membrane residue (Fig. 8). Conversely, nonionic detergents can partially extract phosphogoblin but fail to extract spectrin (Fig. 9). By the standard established for the polypeptides of the human erythrocyte membrane (49), the results of our extraction experiments are ambiguous. The incomplete extraction of phosphogoblin by nonionic detergents parallels, however, the incomplete extraction of the band tentatively identified as turkey band 3. Thus, phosphogoblin appears to behave as an intrinsic protein of the plasma membrane. These data do not, however, exclude the possibility that phosphogoblin is a peripheral protein not liberated from the membrane by the manipulations of ionic strength examined in our experiments. Thus, it is conceivable that the extraction of phosphogoblin by nonionic detergents is a consequence of the solubilization of those intrinsic membrane proteins which are the sites of goblin's attachment to the membrane.

The proportion of phosphogoblin solubilized by the various extraction procedures, as indicated by \(^{32}P\) autoradiography, correlated closely with the proportion of the comigrating protein staining band solubilized by these procedures. Moreover, phosphogoblin and this protein staining band are enriched in parallel by chromatography of detergent extracts on columns of alkylamino-agarose, and depleted in parallel by mild proteolysis or chemical cross linking of purified plasma membranes (unpublished results). Thus, phosphogoblin and the protein staining band with which it co-migrates have several properties in common and may, in fact, be identical proteins. The inability of nonionic detergents to completely solubilize phosphogoblin does raise the possibility of nonhomogeneity of the phosphoprotein band. However, limited proteolysis of solubilized and nonsolubilized phosphogoblin by the method of Cleveland et al. (9) yielded identical phosphopeptide maps, indicating that the solubilized and nonsolubilized phosphoproteins were identical (data not shown).

The phosphopeptide maps also revealed that the incremental incorporation of \(^{32}P\) into goblin which is stimulated by isoproterenol occurs at site(s) distinct from the site(s) incorporating \(^{32}P\) in control cells. The addition to isoproterenol-stimulated cells of excess propranolol, a \(\beta\)-adrenergic antagonist, caused (a) reduction of cation cotransport to control levels and (b) removal of \(^{32}P\) from the hormonally regulated site(s). The latter argues (37) that isoproterenol causes an actual increase in the phosphate content of the hormonally regulated site(s) rather than an increased turnover of phosphate in the site(s).

In conclusion, the present work demonstrates that goblin is a protein of the plasma membrane of turkey erythrocytes, and complements other studies (37, 38, manuscript in preparation) which have demonstrated correlations between catecholamine-stimulated sodium-potassium cotransport and \(^{32}P\) incorporation into goblin in these cells. The results are consistent with the hypothesis that phosphorylation of goblin, by a cyclic AMP-dependent protein kinase, may regulate the ouabain-insensitive, sodium-potassium cotransport system of the turkey erythrocyte.
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