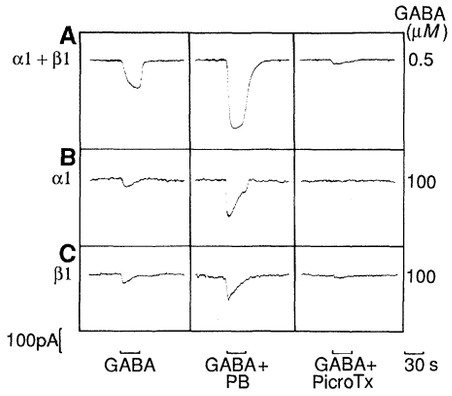

Fig. 3. Pharmacology of the GABA-induced current in cells transfected with either $\alpha_1 + \beta_1$ subunits or the α_1 subunit or the β_1 subunit alone. (**A**) Application of GABA ($5 \times 10^{-7} M$) to a cell transfected with the $\alpha_1 + \beta_1$ subunits and voltage-clamped at −60 mV gave rise to an inward current (left). This current was amplified and increased in duration in the presence of pentobarbital (PB) (50 μM) (middle). After a 5-min washout the current returned to the control level. Picrotoxin (PicroTx) at a concentration of $5 \times 10^{-4} M$ (right) almost completely blocked the GABA-induced current. The same experimental protocol was applied to a cell transfected with the α_1 subunit alone (**B**) or with the β_1 subunit alone (**C**). Note that the concentration used to elicit a detectable inward current in the cells expressing only α_1 or β_1 subunits was $10^{-4} M$ GABA (left traces). Pentobarbital (50 μM) amplified (center traces) and picrotoxin ($5 \times 10^{-4} M$) blocked the inward current (right traces) in cells expressing either the α_1 or the β_1 subunit.

receptor indicates that allosteric sites for barbiturate potentiation are also present on receptors formed from either subunit.

Our results demonstrate the usefulness of expressing neurotransmitter-gated ion channels transiently in mammalian cells. Transient expression, rather than stably expressing cell lines (*19*), should provide for the rapid and simultaneous electrophysiological and pharmacological characterization of many ligand- and voltage-gated ion channels.

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10. Control transfections with the *Escherichia coli lacZ* gene under simian virus 40 (SV40) early promoter control were assayed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), and color formation was determined on paraformaldehyde-fixed cells [J. R. Sanes, J. L. R. Rubenstein, J.-F. Nicolas, *EMBO J.* **5**, 3133 (1987)]. About 40% of the cells were transfected (*9*). In the 293 cell line, positively staining cells were more prevalent in small cell groups, consistent with the notion that only actively dividing cells (that is, groups of cells) are capable of transient DNA uptake and subsequent expression of this DNA.
11. Forty-eight hours after transfection, cells from ten plates (10 cm) were washed twice with phosphate-buffered saline (PBS) and scraped into PBS (10 ml). After low-speed centrifugation (500*g*) the cell pellet was homogenized in a Polytron tissue homogenizer (Brinkmann) in 10 ml of 50 m*M* potassium phosphate, *p*H 7.4, and centrifuged at 50,000*g* for 20 min. Membrane pellets were frozen at −20°C overnight and the wash procedure was repeated three times. The final pellet was resuspended in potassium phosphate buffer, *p*H 7.4, containing 100 m*M* KCl. Homogenate equivalent to 10^6 cells (100 μg of protein) was incubated in a 1-ml reaction volume with [^{3}H]muscimol (Du Pont, 23 Ci/mmol) for 60 min at 4°C or [^{35}S]TBPS (Du Pont, 70 Ci/mmol) for 90 min at 27°C. Samples were filtered on GF/B filters with a Bio-Rad vacuum filter apparatus and washed twice with 5 ml of potassium phosphate–KCl buffer. After drying, filter-retained radioactivity was determined by liquid scintillation.
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15. Electrophysiological responses showed a wide variation around the mean. The 293 cells grow in groups and, by Lucifer yellow injection, are electrically coupled. Thus, it is likely that GABA-induced membrane currents generated in transfected cells can also be recorded from an electrically coupled untransfected cell. This would increase the number of apparently transfected cells and may, in part, explain the wide variation of response amplitudes.
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17. These channels were also seen in HeLa cells expressing single $GABA_A$ receptor subunits, suggesting that no cellular component generates these channels or leads to the formation of heteromeric $GABA_A$ receptors. Similar homomeric channels have recently been observed in oocytes with the use of high-sensitivity electrophysiological techniques [L. A. C. Blair, E. S. Levitan, J. Marshall, V. E. Dionne, E. A. Barnard, *Science* **242**, 577 (1988)]. In addition, glycine-gated, GABA-insensitive homomeric receptor channels were formed when either cells (D. B. Pritchett *et al.*, in preparation) or oocytes (H. Betz, personal communication) expressing the 48-kD rat glycine-receptor subunit were analyzed.
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A 115-kD Polypeptide Immunologically Related to Erythrocyte Band 3 Is Present in Golgi Membranes

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Band 3 multigene family consists of several distinct but structurally related polypeptides which are probably involved in the transport of anions across the plasma membrane of both erythrocytes and nonerythroid cells. A novel member of this family of polypeptides that resides in the Golgi complex was identified with antibodies to Band 3. The Golgi antigen had a larger molecular size and was antigenically distinct from Band 3 in the amino-terminal domain. It was expressed most prominently in cells that secrete large amounts of sulfated proteins and proteoglycans. This polypeptide may participate in sulfate transport across Golgi membranes.

ANION TRANSPORT IS A PROPERTY of many cell types and membranes. In red blood cells, the anion exchanger (Band 3) primarily increases the capacity of the blood to transport CO_2 from the respiring tissues to the lungs by exchanging bicarbonate for chloride (*1*). In epithelial cells such as the kidney tubule intercalated

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cell (*2*) and the gastric oxyntic cell (*3*), bicarbonate-chloride exchange is coupled to proton transport and plays an essential role in the regulation of intracellular *p*H (*4*). Anion translocation in parallel with proton transport has also been described in the Golgi (*5*).

The erythrocyte Band 3 is the only anion transporter characterized at both the protein (*1*) and gene levels (*6*). Antibodies to erythrocyte Band 3 identify immunologically related polypeptides in the basolateral membranes of kidney proximal tubule cells (*7*), intercalated cells (*8*), and gastric oxyntic cells (*9*). Cross-reactive antigens are also present in the plasma membrane of other cell types and, occasionally, in unidentified intracellular structures (*10*).

With a rabbit antiserum to a synthetic peptide, CGLDEYDEVPMPV (*11*), which corresponds to the COOH-terminus of murine Band 3 (anti-Ct) (*6*), we found a Band 3–related polypeptide in the membranes of the Golgi complex where it could regulate Golgi permeability to anions. We verified that the antibody recognized Band 3 in red blood cells by electron-microscopic immunocytochemistry of tissue sections. Strong staining was observed along the cytoplasmic side of the plasma membrane in intact red blood cells and ghosts (Fig. 1, A and B).

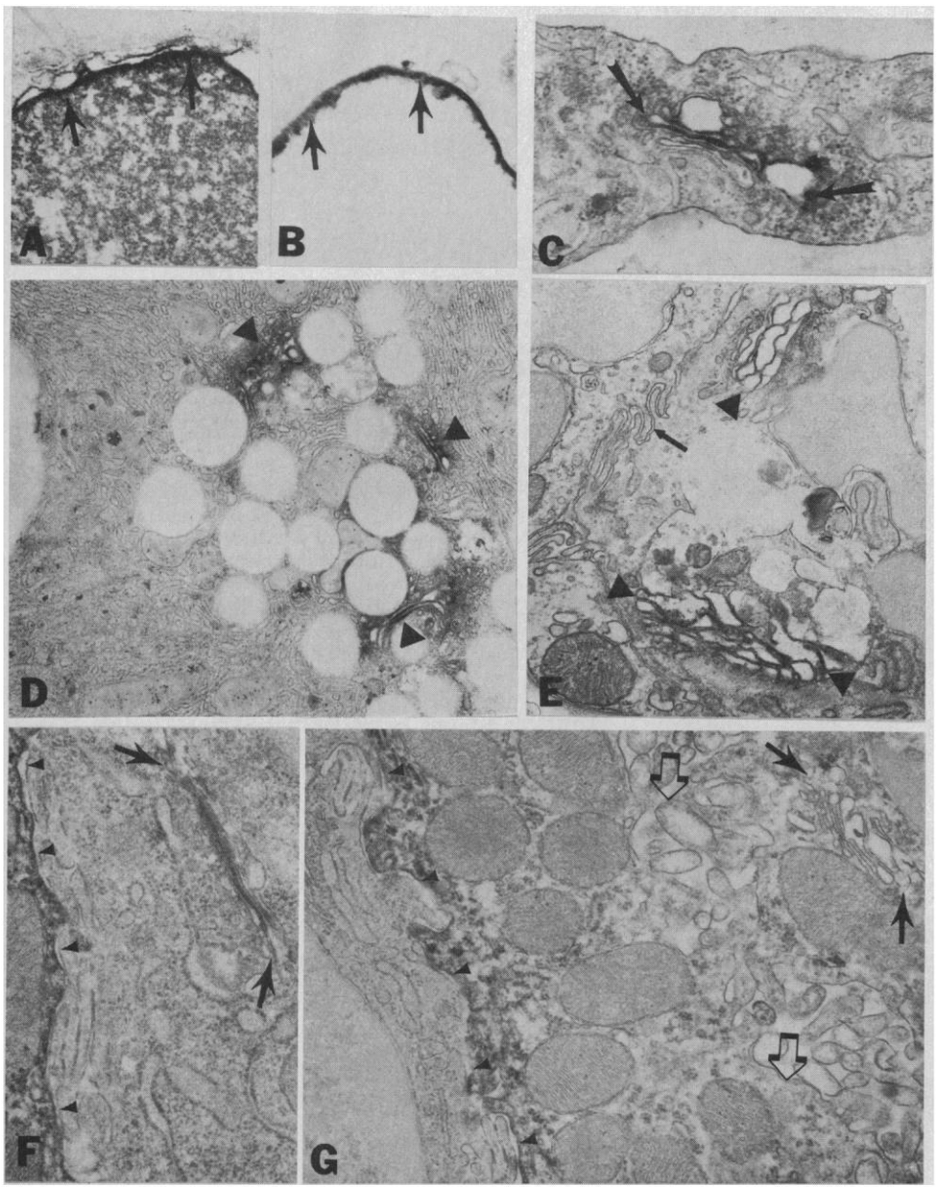

Fig. 1. Immunolocalization of Band 3–related antigens with anti-Ct in various cell types. Adult or 5-day-old Wistar rats were processed for electron microscopic (EM) immunocytochemistry (*25*) with the primary antiserum diluted 1:100. (**A** and **B**) Peroxidase staining of the cytoplasmic side of the plasma membrane (arrows) in red blood cells (A) and ghosts (B). (**C** to **F**) Immunostaining of Golgi membranes in fibroblasts [arrows in (C)], pancreatic acinar cells [arrowheads in (D)], intestinal mucus-secreting cells [arrowheads in (E)], and a gastric chief cell [arrows in (F)] with no staining of the plasma membrane [(C), arrow in (E), and arrowheads in (F)], despite strong staining of the basolateral membrane in gastric oxyntic cells [left, in (F)]. (**G**) Staining of the basolateral membrane (left arrowheads) with no staining of both the apical canalicular membranes (open arrows) and the Golgi (closed arrows) in a gastric oxyntic cell. Magnifications: (A, B, and C) ×16,000; (D) ×10,000; (E) ×12,000; (F) ×35,000; and (G) ×25,000.

We screened a number of tissues for the presence of cross-reactive intracellular antigens. Within the tissues tested, various cell types demonstrated strong intracellular staining (Figs. 1 and 2). These were, in order of decreasing staining intensity, osteoblasts, chondroblasts, fibroblasts, pancreatic and parotid acinar cells, osteoclasts, endothelial cells, mucus-secreting cells in the intestine, stomach, and trachea, and gastric chief cells. The antigen was confined to the Golgi complex and could not be detected in the plasma membrane of these cells (Figs. 1 and 2). As in erythrocytes, the reaction product was found along the cytoplasmic side of the membranes. The Golgi antigen was not localized to specific cisternae, although a gradient of staining intensity was sometimes observed (see Fig. 1C).

We concluded that an antigen (or antigens) sharing one or more common determinants with erythrocyte Band 3 was present in the Golgi of numerous cell types, all of which could be classified as secretory. The Golgi antigen could not be detected, however, in endocrine cells of the pancreas or the intestine, in hepatocytes, in any cell of the kidney tubule, or even in the kidney intercalated cell and in the gastric oxyntic cell, which expressed a Band 3–related antigen in their plasma membrane (Fig. 1G) (*8, 9*). Thus, the antigen is a Golgi-resident protein and not merely in transit towards the plasma membrane.

We stained some cell lines with anti-Ct and found that a transformed rat osteosarcoma osteoblastic cell line [ROS 17/2.8, (*12*)] had both the most prominent Golgi complex and the strongest staining reaction (Fig. 2, C and D). A polyclonal antiserum to the murine whole Band 3 [anti–whole Band 3 (*13*)] also stained the Golgi complex in these cells (Fig. 2E). Both antibodies, however, recognized the same epitope because the staining with either could be inhibited by excess COOH-terminal peptide (20 μg of peptide per microliter of antiserum) (Fig. 2F). In contrast, an excess of peptide could not completely abolish staining of erythrocyte Band 3 on immunoblots when anti–whole Band 3 antiserum was used (Fig. 3A, lanes 4 and 5). Thus, epitopes present at the NH_2-terminal domain of erythrocyte Band 3, which are recognized by this antibody, are not conserved in the Golgi antigen.

Immunoprecipitation of ^{35}S-labeled total proteins with anti-Ct identified only one

specific band of 115 kD (Fig. 3B, lanes 1 and 2). The 115-kD polypeptide was also the most prominent band detected by immunoblots after sedimentation of total cellular membranes (Fig. 3B, lane 4). In contrast to Band 3 (Fig. 3A), it migrated as a sharp band on SDS–polyacrylamide gel electrophoresis, so it may differ in glycosylation.

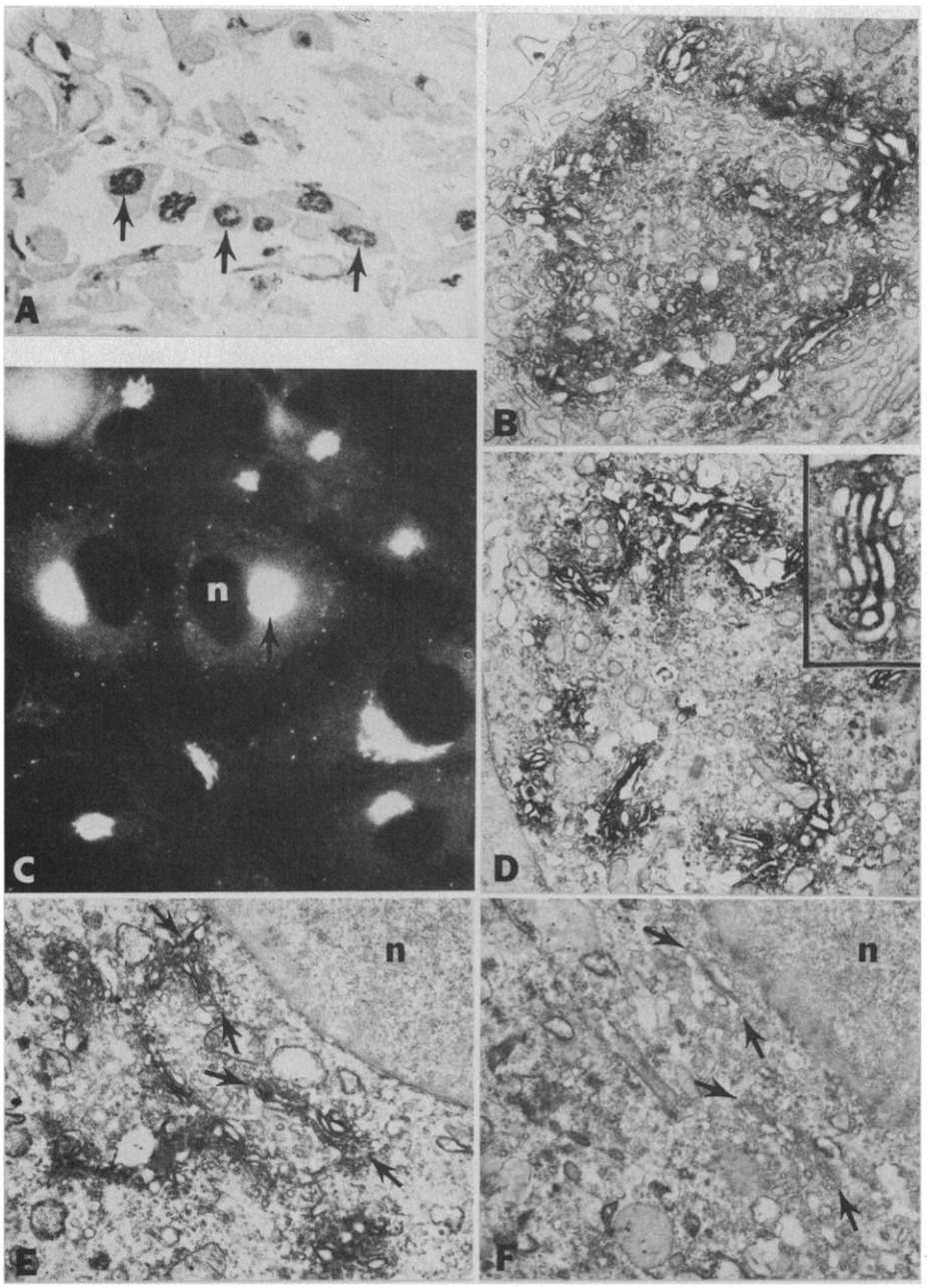

Fig. 2. Immunolocalization of Band 3–related antigens in normal and transformed osteoblasts. (**A** and **B**) Immunolocalization in normal osteoblasts performed in 5-day-old Wistar rat growth plates as described in Fig. 1. (A) Light micrograph of strong peroxidase staining in the Golgi area of osteoblasts (arrows). (B) Low-power electron micrograph of the reaction product, which is restricted to the membranes of the perinuclear Golgi complex. Immunolocalization in rat osteosarcoma cells with anti-Ct (**C** and **D**), anti–whole Band 3 (**E**), or anti–Band 3 and excess peptide (**F**). Transformed rat osteoblasts (ROS 17/2.8) grown on glass cover slips were processed in the presence of 0.05% saponin for immunofluorescence (*26*) or EM immunoperoxidase. Cells in (C) were incubated with primary antibodies (1:100) and then with rhodamine-conjugated goat antibodies to rabbit immunoglobulins (1:100). Immunofluorescence of permeabilized ROS cells shows the strong staining of the Golgi complex (arrow) near the nuclei (n). EM immunoperoxidase staining (D) shows a similar distribution in the ROS cell Golgi area as in normal cells [see (B)]; a higher magnification of one Golgi stack [(D) inset] demonstrates the strict membrane localization of the antigen in the Golgi. Immunolocalization with anti–whole Band 3 in ROS cells (E) and inhibition (F) of the Golgi staining (arrows) in the presence of excess peptide (20 μg/μl) (n, nucleus). Magnifications: (A) ×1,000; (B and D) ×15,000; (C) ×2,000; inset in (D) ×45,000; and (E and F) ×18,000.

This polypeptide was also recognized by anti–whole Band 3, but only in the absence of COOH-terminal peptide (*14*), in agreement with our immunolocalization experiments.

In addition, we have isolated a 2.5-kb partial cDNA clone with a Band 3 cDNA probe from a ROS cell cDNA library.

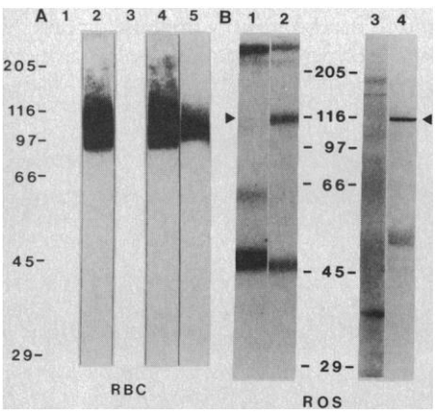

Fig. 3. (**A**) Immunoblot of erythrocyte membrane Band 3. Rat red blood cell (RBC) membranes (*27*) were collected by centrifugation and solubilized by boiling in Laemmli sample buffer (*28*). Solubilized proteins (50 μg per well) were subjected to SDS–9% PAGE (*28*). Separated proteins were transferred to nitrocellulose paper (*29*), the paper was blocked, and individual strips were incubated with 1:1000 dilutions of nonimmune serum (lane 1), anti-Ct (lanes 2 and 3), and anti–whole Band 3 (lanes 4 and 5), either in the absence (lanes 1, 2, and 4) or the presence (lanes 3 and 5) of the COOH-terminal peptide. Bound antibodies were visualized with ^{125}I-labeled protein A (0.5×10^6 to 1×10^6 cpm/ml; Amersham). Molecular size markers on the left (in kilodaltons) correspond to standard proteins (Sigma). (**B**) Immunoprecipitation and immunoblot of the Golgi antigen from ROS 17/2.8 cells. Immunoprecipitations (lanes 1 and 2). Rat osteosarcoma cells (ROS 17/2.8) were grown as in (*12*) and labeled with TRAN[^{35}S]-label (ICN). After unincorporated label was removed, cells were lysed with 1 ml of 50 m*M* tris, *p*H 7.4, 150 m*M* NaCl, leupeptin (10 μg/ml), 1 m*M* phenylmethylsulfonyl fluoride (PMSF), 2 m*M* benzamidine, 2 m*M* EDTA, 1 m*M* dithiothreitol, aprotinin (0.1 U/ml), 1% Triton X-100, 0.2% SDS, and 6*M* urea (lysis buffer). Samples were diluted 1:10 with the lysis buffer without SDS and urea. Immunoprecipitates were formed with the antibodies (1:100 dilutions) and protein A–agarose beads (Sigma) as in (*30*). Eluted proteins were analyzed by SDS-PAGE and fluorography. (Lane 1) Nonimmune serum, (lane 2) anti-Ct. Immunoblotting of total membrane proteins from ROS cells (lanes 3 and 4). ROS cells collected by trypsinization were homogenized on ice in seven times their volume of buffer B (10 m*M* tris, *p*H 7.4, and 10 m*M* KCl) with the above protease inhibitors using a ball-bearing homogenizer (*31*). After removing nuclei by centrifugation at 4°C, membranes were collected by ultracentrifugation. Solubilized proteins were separated by SDS-PAGE and immunoblotted as in (A). Nonimmune serum (lane 3), anti-Ct (lane 4). The arrowheads point to the 115-kD Golgi Band 3–related antigen.

Northern blot analysis showed that this clone was derived from a 4.5-kb mRNA. The deduced amino acid sequence codes for a protein of over 85 kD that is approximately 65% similar to the COOH-terminal membrane domain of mouse erythrocyte Band 3 (*14*).

Thus, the 115-kD polypeptide most likely represents the Golgi antigen. To further verify that this antigen was not present in the plasma membrane, we fractionated ROS 17/2.8 cell homogenates by sucrose density gradient centrifugation. Quantitative immunoblots confirmed that the antigen sedimented mainly in the Golgi fractions and not with the plasma membrane (Fig. 4).

Our finding that a Band 3–related antigen is present in the Golgi of a number of cell types raises two major questions: (i) How does the 115-kD polypeptide differ from erythrocyte Band 3 or previously reported Band 3–related proteins, and (ii) what is its function in the Golgi.

Erythrocyte Band 3 belongs to a multigene family which consists of at least four distinct but homologous members (*15*). The molecular sizes of these gene products vary according to the tissues in which they are expressed. Based on sequence data (*6*), the size of murine erythrocyte Band 3 is 103 kD. Polypeptides of 100 to 115 kD were independently identified in chicken and human kidney (*7, 8*). Polypeptides of around 60 kD were found in other nonerythroid cells (*10*). In the gastric mucosa, there is a Band 3–like protein of 185 kD (*9*). The only sequence data available for a nonerythroid Band 3–related polypeptide, obtained from a human hematopoietic cell line (*16*), also predicts (i) a slightly higher molecular size than that of Band 3, (ii) different potential glycosylation sites, and (iii) an additional 29–amino acid extracellular loop in the transmembrane domain. Although the two sequences diverge toward their NH_2-terminal domains (*16*), the anion transport site of Band 3 (in the COOH-terminal membrane domain) is conserved. The comparable size and similar antigenic characteristics reported here for the Golgi antigen, as well as our preliminary cloning data (*14*), suggest that it belongs to the same gene family as erythrocyte Band 3 and related proteins and could, therefore, be involved in the transport of anions through the Golgi membranes.

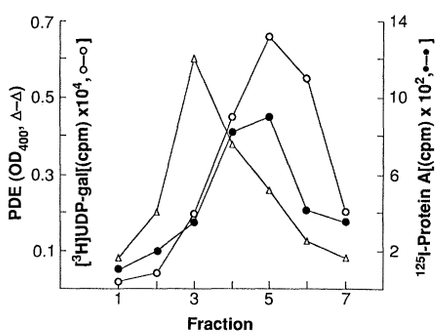

Fig. 4. Sucrose gradient sedimentation of Golgi, plasma membrane, and Band 3–related antigen from homogenized ROS 17/2.8 cells. ROS cells (1×10^8 cells) were trypsinized, resuspended in five times their volume of buffer B with 0.5 m*M* PMSF, leupeptin (10 μg/ml), 2 m*M* benzamidine, and 1.5 m*M* $MgCl_2$, and homogenized on ice with six strokes of the ball-bearing homogenizer. One-tenth volume of 2.5*M* sucrose was added and the postnuclear supernatant was layered on top of the following sucrose solutions (from top): 20, 25, 30, 35, 40, and 55% in buffer B. Ultracentrifuged fractions were collected at each sucrose interface; the last fraction sedimented a few millimeters below the 40 to 55% interface as a sharp band and was also collected. Samples were diluted fourfold with buffer B before determination (in duplicate) of protein concentration (*32*) and enzyme assays. Phosphodiesterase (PDE) (△) (*33*) and galactosyl transferase activities (○) [(*34*) with minor modifications] were analyzed. The amount of the Golgi antigen (●) in each fraction was quantitated by dot-blot on nitrocellulose paper, processed as in Fig. 3 for immunoblots, and counted for ^{125}I radioactivity. Specific binding of protein A was calculated by subtracting nonspecific binding (dots with no samples) from the total binding of each sample. The figure represents typical results (expressed per 10 μg of protein) from three independent experiments.

Numerous intracellular organelles (*17*), including the trans Golgi cisternae (*18*), are acidified by an electrogenic proton pump (*5, 17*). Protons are known to affect the anion transport of Band 3, which exchanges bicarbonate for chloride at neutral *p*H and cotransports sulfate and protons at slightly acidic *p*H (*19*). Bicarbonate-chloride exchange usually requires the presence of carbonic anhydrase in the cytosol (*20*); all cells known to express Band 3 or related proteins in their plasma membrane are enriched in this enzyme (*2, 3*). In contrast, the cells in which we found intense Golgi staining are not enriched. These cells, however, synthesize and secrete high amounts of sulfated proteins and proteoglycans (*21*) and incorporate radioactive sulfate in their Golgi complex (*22*). Although sulfation of newly synthesized proteins is known to occur within the lumen of the Golgi cisternae (*23*) by the action of sulfotransferases and with 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as the cosubstrate (*23, 24*), the exact nature of the sulfate or PAPS transporter is not known.

We suggest that the Golgi Band 3–related polypeptide may be involved in sulfate or PAPS transport, participates in the maintenance of *p*H in this organelle, or both.

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