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Polypeptides immunologically related to band 3 are present in nucleated somatic cells

(band 3-like protein/cell-surface capping/cytoskeleton/peptide mapping)

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ABSTRACT **Band 3, the major transmembrane polypeptide of erythrocytes, mediates the exchange of anions (chloride and bicarbonate) across the membrane. We suspected that band 3 was present on nucleated somatic cells as well as erythrocytes because the senescent cell antigen that is immunologically related to band 3 is present on lymphocytes, platelets, adult liver cells, and embryonic kidney cells; and antibodies prepared against the senescent cell antigen isolated from leukocytes react with erythrocyte band 3. For this reason, we examined human fibroblasts, lung cells, neutrophils, mononuclear leukocytes, squamous epithelial (mouth) cells, lung squamous epithelial carcinoma, mouse neuroblastoma cells, and rat hepatocytes for immunoreactive forms of band 3 by using monospecific antibodies to erythrocyte band 3. The results demonstrated that polypeptides sharing common antigenic determinants with erythrocyte band 3 are present in nucleated somatic cells as determined by immunofluorescence, immunoelectron microscopy, and immunoautoradiography. Peptide mapping revealed substantial sequence homology between erythrocyte band 3 and the band 3-like protein of leukocytes. Immunofluorescence studies indicate that the band 3-like proteins in nucleated cells participate in antibody-induced cell surface capping.**

Band 3, the major transmembrane polypeptide of erythrocytes, mediates the exchange of anions (chloride and bicarbonate) across the membrane (1, 2) and appears to be the binding site for hemoglobin (3) and the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (4), aldolase (5), and phosphofructokinase (6). Band 3 binds to band 2.1, which simultaneously binds to spectrin, thereby linking the cytoplasmic surface of the plasma membrane to the internal cytoskeleton (7). In addition, it may contain the glucose transport protein (8, 9) and is thought to be involved in the transfer of water across the membrane (10).

We suspected that band 3 was present in nucleated somatic cells as well as erythrocytes because the senescent cell antigen that is immunologically related to band 3 (11–13) is present on lymphocytes, platelets, adult liver cells, and embryonic kidney cells (14). Furthermore, antibodies prepared against the senescent cell antigen isolated from leukocytes (WBC) react with erythrocyte band 3 (13); and other cells are known to transport anions (15).

As a test of this hypothesis, primary cultures of human fibroblasts, lung cells, neutrophils, mononuclear WBC, squamous epithelial (mouth) cells, lung squamous epithelial carcinoma, mouse neuroblastoma cells, and rat hepatocytes were examined for the presence of immunoreactive forms of band 3 by immunofluorescence, immunoelectron microscopy, and immunoautoradiography. Band 3-related polypeptides were demonstrated in all of these cells. Peptide mapping indicates that these polypeptides share substantial sequence homology with erythrocyte band 3.

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MATERIALS AND METHODS

Cell Preparation. Cultures of normal human fibroblasts (foreskin) were obtained from the Cytogenetic Laboratory, Scott and White Hospital. Human diploid lung cells (ATCC 153), mouth squamous epithelial carcinoma cells (SW 2224), lung squamous epithelial carcinoma (SW 1271), hypernephroma (SW 156), rhabdomyosarcoma (SW 80), and mouse neuroblastoma cells (HTZ) were maintained both in culture and in liquid nitrogen in this laboratory. Human mononuclear WBC were isolated on Percoll density gradients as described (16). Hepatocytes, isolated from rat livers by elutriation, were a gift from Matthew Heil and Justine Garvey.

Antibody Isolation and Characterization. Rabbit antibodies to purified band 3 were prepared as described (13). Antibodies were characterized by immunoautoradiography by utilizing a gel overlay method and immunoblotting technique (13). Both IgG antibodies to band 3 and anti-band 3 antiserum were used for these studies (13).

Indirect Immunofluorescence. Indirect immunofluorescence was performed both on fixed cells and on living cells in suspension. For the former method, cells were grown on coverslips in Leighton tubes. They were washed with Dulbecco's phosphate-buffered saline (P_i/NaCl) and fixed with 5% acetic acid/95% ethanol at −20°C for 15 min. This fixation procedure renders the cell membrane permeable to antibodies while preserving cell structure. Therefore, cytoplasmic components are accessible to antibodies. Fixed cells were washed twice over 30 min with P_i/NaCl, incubated with antibodies to band 3 or preimmune serum for 30 min at 24°C in a moist chamber, washed, and incubated with fluorescein isothiocyanate (FITC)-conjugated protein A (Pharmacia). After P_i/NaCl washes, coverslips containing cells were mounted on glass slides with buffered glycerol and sealed with clear nail polish (17). Living cells viewed in suspension were grown in tissue culture flasks in L-15 medium and trypsinized with 100 μg of diphenylcarbamyl chloride-treated trypsin per ml for 3 hr at 37°C. Cells were collected, washed three times with P_i/NaCl, and incubated with serum obtained prior to immunization ("preimmune serum") or antibodies to band 3 for 30 min at 4°C or room temperature (17). After extensive washing with P_i/NaCl, cells were incubated with FITC-protein A, washed, and examined with a Zeiss phase–contrast epifluorescence microscope. For capping studies, cells were incubated at 37°C for 0, 5, 10, 15, and 30 min and then examined.

Abbreviations: FITC, fluorescein isothiocyanate; WBC, leukocytes.

Immunoelectron Microscopy. A buffy coat preparation of human peripheral blood was washed four times with P_i/NaCl containing 5 mM D-glucose and incubated with trypsin at 100 μg/ml in P_i/NaCl containing 500 μg of ATP per ml overnight at 24°C. After four washes with P_i/NaCl/glucose, 50-μl aliquots of these cells were incubated with rabbit antibodies to band 3 or with preimmune serum for 1 hr at 24°C. They were washed with P_i/NaCl/glucose containing 1% bovine serum albumin and incubated with gold colloid-conjugated to the goat Fab fragment of anti-rabbit IgG prepared by published procedures (18–20) for 1 hr at 24°C. Additional controls consisted of cells incubated only with Fab anti-rabbit IgG–gold colloid. Cells were washed with P_i/NaCl containing 1% bovine serum albumin and 0.02% polyethylene glycol (M_r 20,000), fixed with 1% glutaraldehyde, and postfixed with 1% osmium tetroxide (21–23). They were transferred to conical capsules, centrifuged, resuspended in liquid agar, and centrifuged into a pellet. After the agar hardened, the tip containing the cells was dehydrated through a graded series of ethanol solutions and equilibrated with propylene oxide (23). The cell–agar pellet was embedded in Epon-Araldite plastic. Sections were cut on an LKB Ultratome III, stained with uranyl acetate and lead citrate, and photographed with a RCA EMU 3F electron microscope.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Cells were lysed and membranes were washed with 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM diisopropylfluorophosphate, 1 mM EGTA, and 1 mM EDTA as protease inhibitors (13). All procedures were performed at 0–4°C. A low-speed centrifugation (1,000 × *g*) was used to remove nuclei, and a high speed centrifugation (27,000 × *g*) was used to collect membranes (14). Membrane proteins were analyzed on 6–25% $NaDodSO_4$/polyacrylamide gradient gels or 7% $NaDodSO_4$/polyacrylamide gels utilizing the discontinuous buffer system of Laemmli (24).

Immunostaining of Membrane Proteins. Immunoautoradiography was performed by the immunoblotting technique of Towbin *et al.* (25) with the modifications as described (13) or by the gel overlay method (13). For immunoautoradiographic studies, cells removed from tissue culture flasks with rubber policeman were compared to those obtained by trypsinization. No difference in the molecular weight of polypeptides stained with anti-band 3 was observed between these methods of cell isolation.

One-Dimensional Partial Proteolytic Peptide Mapping. Peptide maps were obtained by limited proteolysis in $NaDodSO_4$/polyacrylamide gels by the method of Cleveland *et al.* (26). Membrane proteins were separated by $NaDodSO_4$/polyacrylamide gradient gel electrophoresis and lightly stained with Coomassie blue. Band 3 and band 3-related polypeptides from nucleated cells were cut from gels. Gel slices were equilibrated with electrophoresis buffer and placed in individual wells of a 6–20% polyacrylamide gradient gel. Glycerol (20%), trypsin (5 μg), and Pyronin Y were added to each well. Gels were subjected to electrophoresis until the Pyronin Y was at the interface between the stacking and separating gels. The current was turned off for 30 min to allow digestion of proteins and then continued until the Pyronin Y was 1 cm from the lower edge of the gel. Gels were stained with Coomassie blue and then with silver stain.

RESULTS

Indirect Immunofluorescence and Immunoelectron Microscopy of Cells with Antibodies to Band 3. In order to test the hypothesis that band 3 was present on nucleated somatic cells, immunofluorescence and immunoelectron-microscopy studies were performed. Phase-contrast and surface immunofluorescence studies of live mouth squamous epithelial cells incubated with antibodies to band 3 revealed diffuse labeling evenly distributed over the entire plasma membrane (Fig. 1 *a* and *b*). Warming cells to 37°C resulted in capping of the antibodies (Fig. 1 *c* and *d*). Fluorescence staining was not observed when preimmune serum was substituted for antibodies to band 3 or when cells were incubated with FITC-protein A without an incubation with antibodies or serum (Fig. 1 *e* and

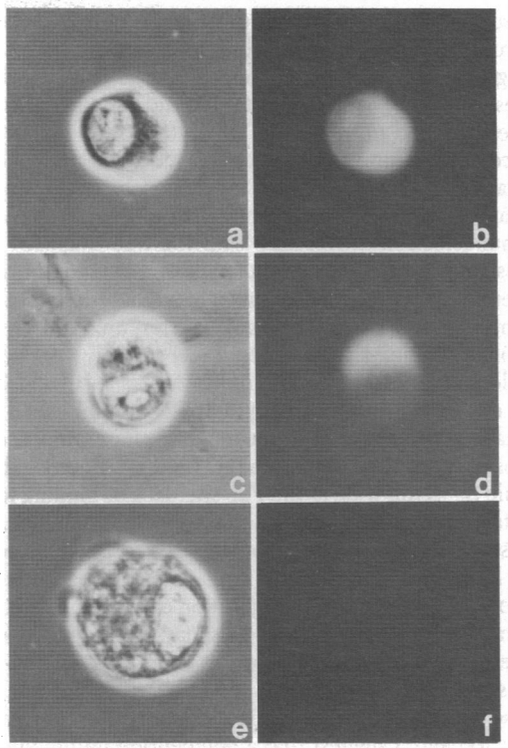

FIG. 1. Phase-contrast (*Left*) and immunofluorescence (*Right*) microscopy of mouth squamous epithelial cells (SW 2224) stained with rabbit antibodies to band 3 (*a–d*) or preimmune serum (*e* and *f*). Cells incubated with antibodies to band 3 were incubated with FITC-labeled protein A at 4°C (*a* and *b*) and at 4°C followed by a 10-min incubation at 37°C (*c* and *d*).

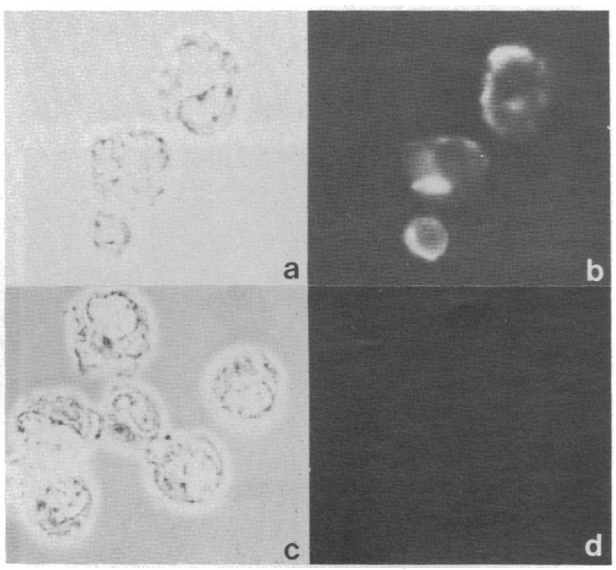

FIG. 2. Phase-contrast (*Left*) and immunofluorescence micrographs (*Right*) of lung carcinoma cells (SW 1271) incubated with monospecific antibodies to band 3 (*a* and *b*) or preimmune serum (*c* and *d*) followed by incubation with FITC-labeled protein A at 4°C. They were then incubated at 37°C for 15 min.

f). Absorption of antisera with purified band 3 conjugated to Sepharose or erythrocyte membranes abolished fluorescence staining of cells. Lung carcinoma cells, also labeled with antibodies to band 3, exhibited patching and capping of antibodies when warmed to 37°C (Fig. 2). Cytoplasmic immunofluorescence of fixed lung epithelial cells (Fig. 3) and normal human fibroblasts (not shown) revealed labeling of both the cell membrane and a fine reticular network within cells.

Immunoelectron microscopy with the Fab fragment of antibodies conjugated to gold colloid showed labeling of neutrophil membranes (Fig. 4) and monocyte membranes (not shown) with antibodies to band 3 but not with preimmune serum or gold colloid alone. Erythrocytes were sparsely labeled. Immunoelectron-microscopy data indicate that anti-band 3 antibodies do not bind to intact erythrocytes (unpublished data). Enzymatic treatment of erythrocytes with, for example, α-chymotrypsin, is required to initiate IgG binding. Because the IgG molecule is ≈9 nm wide at the Fab site, lack of binding to band 3 in intact erythrocytes may be due to steric hindrance. Neutrophils, which were heavily labeled, are very short-lived cells with a lifespan of ≈24–48 hr after entering the circulation. Therefore, changes in the configuration of the band 3-like molecule in the membrane of these cells may have occurred during the overnight incubation, rendering band 3 accessible to antibodies.

These surface and cytoplasmic binding studies utilizing antibodies to band 3 suggest that a polypeptide immunologically related to erythrocyte band 3 is present in both the membrane and cytoplasm of human mouth and lung carcinoma cells and in normal human lung cells, fibroblasts, neutrophils, and monocytes.

Immunoautoradiographic Identification of Erythrocyte Band 3-Related Polypeptides in Other Somatic Cells. Antibodies to erythrocyte band 3 were used to detect band 3-related polypeptides in membranes of human lung cells and WBC by an immunoautoradiographic gel-staining technique (13). Membrane proteins were separated on a polyacrylamide gradient gel. The gel was fixed, equilibrated with a physiological ionic strength buffer, incubated with antibodies to band 3, and then stained with ^{125}I-labeled protein A. Comparison of the Coomassie blue-stained gel with the corresponding immunoautoradiogram (Fig. 5*a*) showed that anti-band 3 antibody stains band 3 in erythrocyte ghosts and band 3 and its M_r ≈60,000 and ≈35,000 fragments in ghosts prepared from α-chymotrypsin-treated erythrocytes. Antibodies to band 3 stained polypeptides of M_rs ≈ 60,000, 48,000, and 38,000 in membranes of cultured human lung cells (Fig. 5*a*). Examination of membranes from freshly drawn human WBC revealed staining of a band of M_r ≈95,000 with antibodies to band 3 (Fig. 5*b*). Band 3-related polypeptides of M_rs 69,000 and 60,000 were demonstrated in membranes of neoplastic cells maintained in culture (Fig. 5*c*). Rat hepatocytes that were not maintained in culture had an additional band with a molecular weight greater than that of erythrocyte band 3 (Fig. 5*c*).

The relatively low level of radioactivity detected in immunoblots of nucleated somatic cells probably reflects a difference

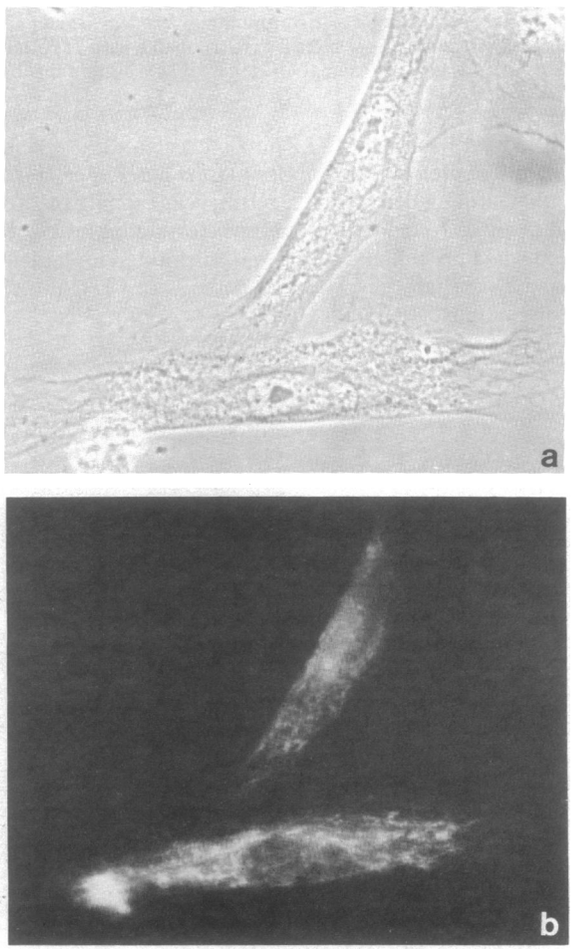

FIG. 3. Phase-contrast (*a*) and immunofluorescence micrographs (*b*) of normal human lung cells (ATCC 153) grown on coverslips inside Leighton tubes and fixed with acetic acid/ethanol prior to incubation with antibodies to band 3.

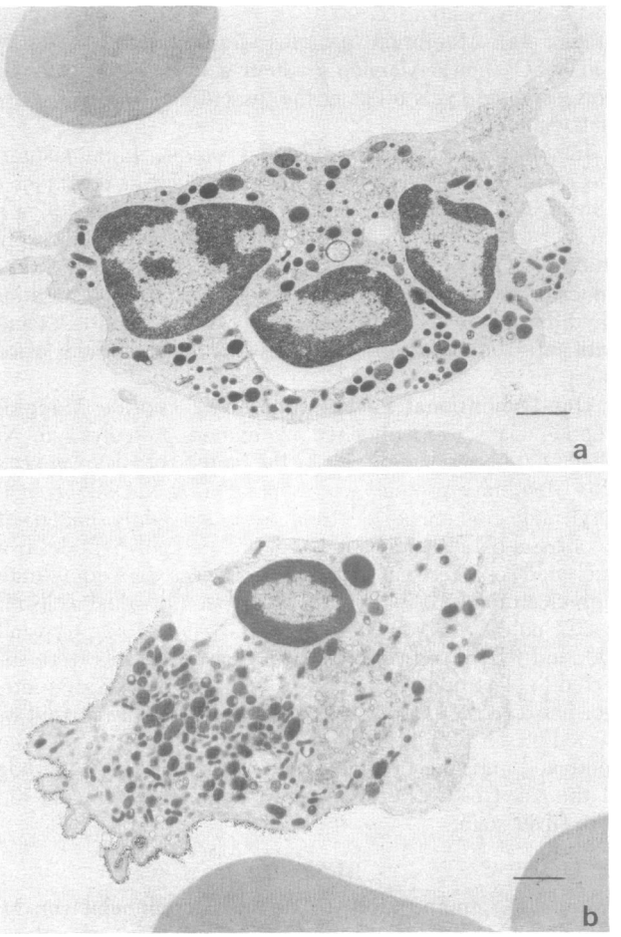

FIG. 4. Immunoelectron microscopy of normal human neutrophils incubated with preimmune serum (*a*) or antibodies to band 3 (*b*) followed by incubation with Fab fragments of the goat IgG fraction of anti-rabbit IgG heavy chain conjugated to gold colloid. (Bar = 1 μm.)

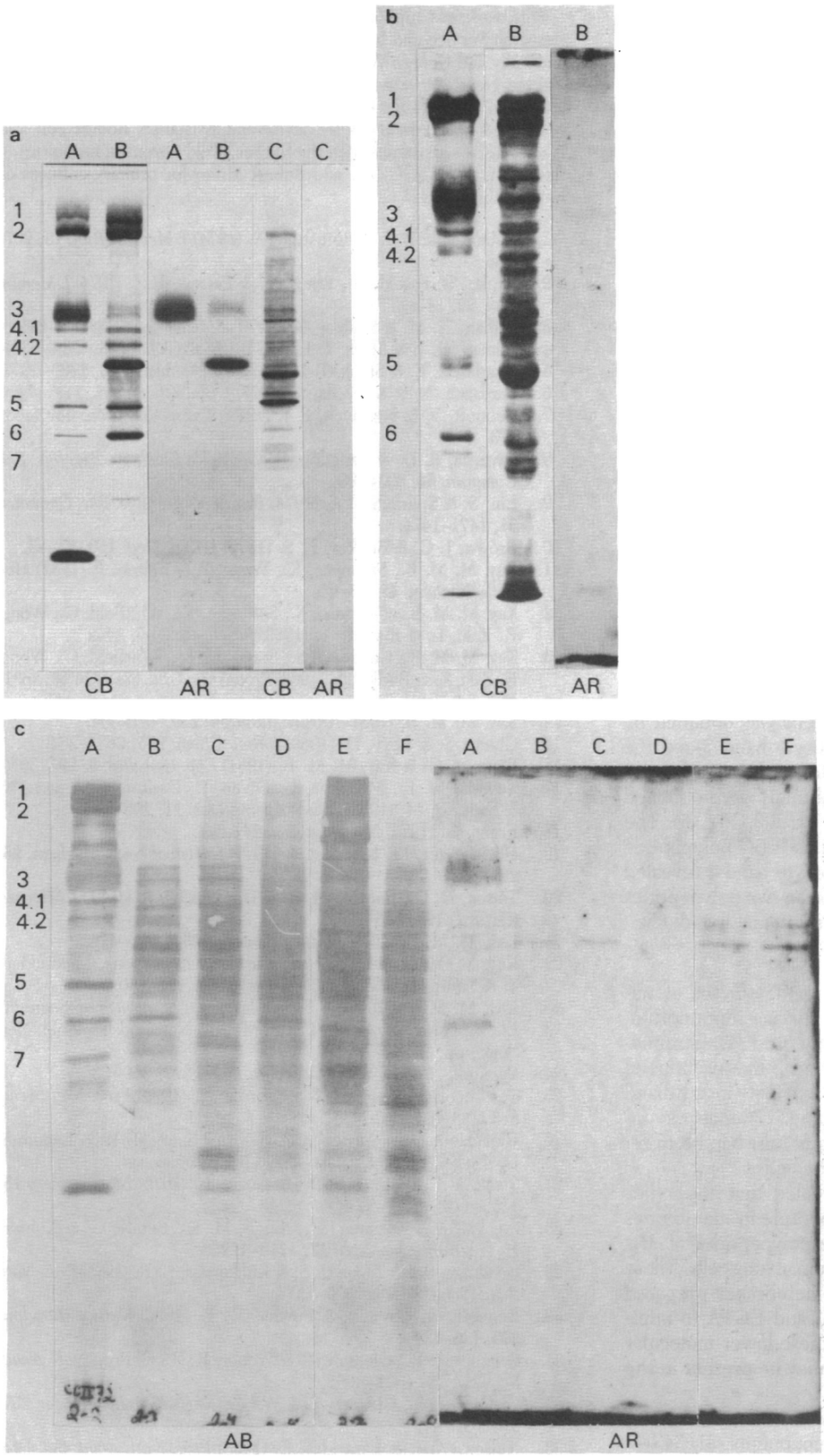

FIG. 5. (*a*) Immunoautoradiographic characterization of band 3 and related peptides in membranes of intact human erythrocytes (lanes A), α-chymotrypsin-treated intact erythrocytes (lanes B), and normal human lung cells (ATCC 153) (lanes C) by using antibodies to human erythrocyte band 3. CB, Coomassie blue; AR, autoradiograph. Proteins were subjected to electrophoresis on $NaDodSO_4$/6–25% polyacrylamide gradient gels. Gels were incubated with anti-band 3, followed by ^{125}I-labeled protein A, dried, and autoradiographed for 5 days at −60°C (13). Treatment of intact erythrocytes with α-chymotrypsin (200 μg/ml) overnight at 24°C produces a band 3 fragment of M_r ≈60,000 (the CH 55 fragment described by Steck in ref. 27) that contains the amino terminus and another of M_r ≈35,000 that contains the carboxyl terminus (27, 28). Rabbit antibodies to band 3 bind to both fragments. Neither preimmune serum nor protein A binds to erythrocyte proteins under the conditions used (13). (*b*) Detection of band 3-related polypeptide in WBC membranes with antibodies to band 3. Lanes: A, erythrocyte membranes; B, WBC membranes; CB, Coomassie blue-stained gel; AR, immunoautoradiography with anti-band 3. Membrane proteins were separated by electrophoresis on $NaDodSO_4$/polyacrylamide (7%) slab gel using the discontinuous system of Laemmli (24). Immunoautoradiography by the gel overlay technique was performed as described (13). Autoradiographs were exposed for 3 days. (*c*) Detection of band 3-related polypeptides in membranes from cultured neoplastic cells (lanes B–D and F) and rat liver hepatocytes (lane E) by using antibodies to band 3. Lanes: A, erythrocyte membranes; B, hypernephroma (SW 156); C, rhabdomyosarcoma (SW 80); D, carcinoma of the mouth (SW 2224); E, rat hepatocytes; F, mouse neuroblastoma. AB, Amido black stain; AR, immunoautoradiography with anti-band 3. Membrane proteins were separated by electrophoresis on $NaDodSO_4$/polyacrylamide (12–25%) gradient gel using the discontinuous system of Laemmli (24). Immunoblotting was performed as described (13, 25). Autoradiographs were exposed for 24 hr at −80°C. Antibodies to erythrocyte band 3 stain polypeptides of M_rs ≈69,000 and 60,000 in membranes of cultured neoplastic cells and M_rs ≈150,000, 69,000, and 60,000 in membranes of hepatocytes isolated by elutriation from rat livers.

in the amount of band 3 present in erythrocytes and that present in nucleated cells. Erythrocyte band 3 represents ≈25% of the total membrane protein and appears as a major band on Coomassie blue-stained gels. In contrast, band 3-related polypeptides in nucleated cells are present in small amounts. Thus, the difference in staining patterns between the cell types probably is indicative of a difference in concentration of the protein.

The presence of proteins with molecular weights less than that of erythrocyte band 3 that stain with anti-band 3 antibodies in membranes of cultured (but not freshly obtained) nucleated cells prepared with diisopropylfluorophosphate, EGTA, and EDTA suggests that these polypeptides may be naturally occurring immunoreactive forms of band 3 in these cells. However, proteolysis cannot be excluded, even though potent protease inhibitors were used in all solutions and procedures were conducted at 0–4°C.

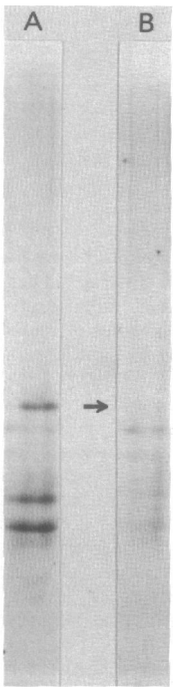

FIG. 6. One-dimensional partial proteolytic maps of erythrocyte band 3 (lane A) and its immunologically related polypeptide from WBC (lane B) obtained by the technique of Cleveland *et al.* (26) with silver staining to visualize peptides.

Comparative Partial Proteolytic Mapping of Erythrocyte Band 3 and the Band 3-Related Polypeptide from Nucleated Cells. Additional evidence for the occurrence of a band 3-related polypeptide in nucleated somatic cells was obtained by comparative peptide mapping of erythrocyte band 3 and the WBC polypeptide to which antibodies to erythrocyte band 3 bind. Partial proteolytic one-dimensional maps were obtained by using the technique of Cleveland *et al.* (26) (Fig. 6). The tryptic maps of erythrocyte band 3 and the WBC polypeptide that is immunologically related to erythrocyte band 3 revealed a number of common peptides. Although the two polypeptides are not identical, they share substantial sequence homology.

DISCUSSION

Antibodies to erythrocyte band 3 bind to the surface of nucleated somatic cells as determined by surface immunofluorescence and immunoelectron-microscopy studies. Immunofluorescence studies indicate that the band 3-like proteins in nucleated cells are mobile because they participate in anti-band 3-induced cell surface patching and capping. Cytoplasmic immunofluorescence studies reveal binding of anti-band 3 to reticular structures within nucleated somatic cells.

Immunoautoradiographic analysis revealed that antibodies to band 3 react with a M_r ≈95,000 polypeptide in membranes of freshly isolated WBC and with three polypeptides of M_rs ≈60,000, 48,000, and 38,000 in cultured human lung cells. These polypeptides were present in lung cell membranes prepared with diisopropylfluorophosphate, EDTA, and EGTA to minimize artifactual proteolysis. Therefore, these lower molecular weight immunoreactive forms of band 3 may be present in the membranes of cells cultured *in vitro*.

Cheng and Levy have found that the anion transport system of rat hepatocytes is composed of two components, M_r 54,000 and M_r 43,000 (15). Thus, band 3-like proteins in nonerythroid cells may be variants of different molecular weights adapted to each cell type and its environment and functions.

Polypeptides related to erythrocyte senescent cell antigen (14), spectrin (29–34), bands 2.1 (35) and 4.1 (36, 37), and actin (38) have been demonstrated in nucleated cells. The band 3-like protein present in nucleated cells may be the attachment site for the cytoskeletal protein network in the plasma membrane as it is in erythrocytes.

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