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Localization of senescent cell antigen on band 3

(fragments of band 3/two-dimensional peptide mapping/aging antigenic determinants/anion transport region/immunoblotting)

MARGUERITE M. B. KAY

Research and Medical Services (151), and Division of Geriatric Medicine, Department of Medicine, and Departments of Biochemistry and of Microbiology and Immunology, Texas A & M University, 1901 South First Street, Olin E. Teague Veterans Center, Temple, TX 76501

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ABSTRACT **Senescent cell antigen is a glycosylated polypeptide, migrating in the band 4.5 region of NaDodSO$_4$/polyacrylamide gels, that appears on the surface of senescent and damaged cells. Appearance of the senescent cell antigen initiates specific binding of IgG autoantibodies to it and the removal of erythrocytes (RBCs). Previous experiments suggested that the senescent cell antigen may be immunologically related to an integral membrane protein designated band 3 that is involved in anion transport across the RBC membrane. In the present studies, senescent cell antigen was mapped along the band 3 molecule by using topographically defined fragments of band 3. Both binding of IgG eluted from senescent RBCs ("senescent cell IgG") to defined proteolytic fragments of band 3 in immunoblots and two-dimensional peptide mapping of senescent cell antigen, band 3, and defined proteolytic fragments of band 3 were used to localize senescent cell antigen along the band 3 molecule. The data suggest that the antigenic determinants of the senescent cell antigen that are recognized by physiologic IgG autoantibodies reside on an external portion of a naturally occurring transmembrane fragment of band 3 that has lost a M_r ≈40,000 cytoplasmic (NH_2-terminal) segment and part of the anion-transport region. A critical cell-age-specific cleavage of band 3 appears to occur in the transmembrane, anion-transport region of band 3.**

The senescent cell antigen is a glycosylated polypeptide that appears on the surface of senescent and damaged erythrocytes (RBCs) (1–14). It is recognized by the antigen-binding Fab region (3, 4) of a specific IgG autoantibody in serum, which attaches to it and initiates the removal of cells by macrophages (5). Although the senescent cell antigen was first demonstrated on the surface of senescent human erythrocytes (1, 2), it has since been demonstrated on the surface of lymphocytes, polymorphonuclear leukocytes, platelets, embryonic kidney cells, and adult liver cells (4).

It was postulated that the senescent cell antigen was a component of the band 4.5 region on NaDodSO$_4$/polyacrylamide gels that was derived from the transmembrane glycoprotein designated band 3 (6) that is involved in anion transport across the RBC membrane. (The "4.5 region" consists of polypeptides migrating between band 4.2 and band 5 (actin) in polyacrylamide gels that have not been structurally and functionally characterized.) Experiments designed to test this hypothesis revealed that the senescent cell antigen is immunologically related to band 3 (9–11). Both band 3 and senescent cell antigen abolished the phagocytosis-inducing ability of IgG eluted from senescent cells, which binds specifically to the senescent cell antigen. Spectrin, bands 2.1, 4.1, actin, glycophorin A, periodic acid/Schiff's reagent (PAS)-staining bands 1–4, and desialylated glycophorin A and PAS-staining bands 1–4 did not alter the phagocytosis-inducing ability of senescent cell IgG. In addition, monospecific rabbit antibodies to both purified band 3 and the senescent cell antigen reacted with band 3 and its proteolytic products of M_rs ≈60,000, 40,000, and 18,000–26,000 as determined by immunoautoradiography of RBC membranes, indicating that these molecules share common antigenic determinants. These lower molecular weight band 3-related polypeptides are thought to represent proteolytic fragments generated *in vivo* because they are observed in membranes from old cells prepared with the protease inhibitors diisopropylfluorophosphate (iPr_2P-F), EDTA, EGTA, and phenylmethylsulfonyl fluoride but not in membranes from young cells (11).

In the present study, binding of IgG eluted from senescent RBCs ("senescent cells IgG") to proteolytic fragments of band 3 that presumably are generated *in vivo* (11) and to those produced *in vitro* by protease treatment (15–18) was examined to locate the position of the senescent cell antigenic determinants on the band 3 molecule. In addition, these fragments of band 3 and senescent cell antigen were analyzed by two-dimensional peptide mapping.

MATERIALS AND METHODS

Cell Separation. RBC were separated into young, middle-aged, and old populations on Percoll (Pharmacia) gradients as described (5, 9, 10). RBC were washed three times in 20 vol of phosphate-buffered saline by centrifugation at 3000 × *g* for 10 min and lysed, and the membranes were washed with 5 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 5 mM iPr_2P-F, and 100 μg of phenylmethylsulfonyl fluoride per ml as protease inhibitors.

Isolation of IgG from Senescent RBC and Senescent Cell Antigen. IgG was isolated as described (3, 4). Senescent cell antigen was isolated by affinity chromatography with senescent cell IgG conjugated to Sepharose 4B as described (4, 6).

Enzymatic Treatment of RBCs. Fragments are referred to by the nomenclature of Steck *et al.* (15, 16, 18). Washed RBCs were incubated with phosphate-buffered saline containing 1 mM ATP and 200 μg of α-chymotrypsin per ml overnight at 24°C. α-Chymotrypsin specifically digests band 3, yielding fragments of M_rs ≈55,000 and ≈38,000 (15, 16), designated CH-55 and CH-38, respectively (15). The CH-55 fragment appears to have a molecular weight closer to 60,000 in our experiments. Digestion was terminated by the addition of 5 mM iPr_2P-F. Cells were washed four times with phosphate-buffered saline and processed in the same manner as described for intact erythrocytes.

The M_r ≈41,000 (TR-41) cytoplasmic segment of band 3 was produced by mild trypsin digestion of spectrin-depleted, NaOH-stripped inverted vesicles (17). Digestion was terminated by the addition of 5 mM iPr_2P-F and 200 μg of phenylmethylsulfonyl fluoride. Aliquots were removed for electrophoresis. Vesicles were removed by centrifugation, and the cytoplasmic fragments of band 3 were collected by direct ad-

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Abbreviations: RBC, erythrocytes; PAS, periodic acid/Schiff's reagent; iPr_2P-F, diisopropylfluorophosphate.

dition of DEAE-cellulose to the supernate. Fragments were eluted with a salt gradient of 0.2–0.5 M KCl (17). The complementary fragment of TR-41 remained with the membrane and contained CH-38 and CH-17.

The M_r ≈17,000 (CH-17) intramembranous fragment of band 3 was generated by α-chymotrypsin treatment at both sides of the membrane (15, 17, 18). Proteolysis was terminated by the addition of 5 mM iPr_2P-F. Membranes were washed twice with 5 mM sodium phosphate (pH 8.0) containing 1 mM EDTA, 1 mM EGTA, and 1 mM iPr_2P-F. The M_r ≈19,000 intramembranous fragment (CH-TR-19), which includes CH-17, was produced by α-chymotrypsin treatment of intact RBCs followed by treatment of spectrin-depleted inverted vesicles with trypsin (19).

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Proteins were analyzed on three different gel systems: 7% NaDodSO$_4$/polyacrylamide gels and 6–25% and 12–25% linear NaDodSO$_4$/polyacrylamide gradient gels using the discontinuous buffer system of Laemmli (20).

Immunostaining of Membrane Proteins. Immunoautoradiography was performed by the immunoblotting technique of Towbin *et al.* (21) with the modifications described previously (10) or by the gel overlay method (10). Transfer of polypeptides was monitored by loss of Coomassie blue-staining bands from the gel and by the appearance of amido black staining bands on the nitrocellulose paper. Transfer of polypeptides was >90% efficient. Neither serum obtained prior to immunization (preimmune serum) nor protein A bind to RBC proteins under the conditions employed (10). Absorption of antibodies to purified band 3 abolished binding to band 3 and its lower molecular weight proteolytic products in immunoblots.

Two-Dimensional Peptide Mapping. Two-dimensional peptide maps were obtained by the method of Elder *et al.* (22).

RESULTS

Band 3 Degradation Products Increase with Cell Age. Young, middle-aged, and old cell membrane proteins were separated by polyacrylamide gel electrophoresis. Antibodies to band 3 and IgG eluted from senescent cells were used to determine the relative amount of band 3 breakdown products in the membranes of young and old cells by the immunoblotting technique. Antibodies to band 3 and IgG eluted from senescent cells bound to band 3 in immunoblots of young, middle-aged, and old RBCs (Fig. 1). In addition, antibodies to band 3 bound to two lower molecular weight band 3 polypeptides, M_rs ≈62,000 and ≈40,000, in the membranes of old but not young RBCs. Binding to M_rs ≈62,000 and ≈40,000 band 3 polypeptides was not demonstrated in membranes from young cells even though the amount of young RBC membranes loaded on polyacrylamide gels was greater than that of old RBC membranes (Fig. 1). Senescent cell IgG bound to band 3 and the M_r ≈62,000 degradation product of band 3 (Fig. 1). The M_r ≈62,000 breakdown product of band 3 was detected with senescent cell IgG in membranes of old but not young or middle-aged cells. Thus, breakdown products of band 3 appear to increase as cells age.

Binding of IgG Eluted from Senescent Cells to Band 3 and Its Proteolytic Products. As an approach to determining which segment of band 3 carries the antigenic determinants of the senescent cell antigen, binding of senescent cell IgG to band 3 fragments that appeared to be generated *in vivo* in the RBC membrane ("naturally occurring") and those generated by α-chymotrypsin treatment *in vitro* was investigated. Immunoblots revealed binding of senescent cell IgG to band 3 and a polypeptide migrating at M_r ≈62,000 (±3%; range, 60,000–64,000) in membranes from untreated cells (Fig. 2, lane a). The M_r ≈62,000 polypeptide also was labeled with monospecific antibodies to band 3. Senescent cell IgG bound

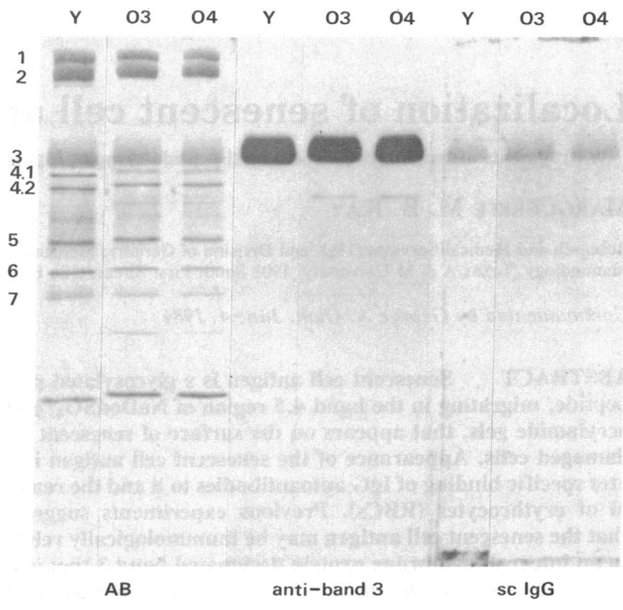

FIG. 1. Binding of antisera to band 3 and IgG eluted from senescent cells to band 3 and its breakdown products. AB, amido black; anti-band 3, antibodies to band 3; sc IgG, "senescent cell IgG" eluted from senescent RBCs; Y, young cells; O3, old cells, fraction 3; O4, old cells, fraction 4. Cell populations were separated on Percoll gradients. Old cells are separated into four bands, old fraction 4 being the densest and old fraction 3 being the second densest. Old cells used for these studies represented <0.6% of the total cells. Cells were washed, and membranes were prepared. Polypeptides were transferred from 6–25% polyacrylamide gradient gels to nitrocellulose paper and were incubated with antibodies to band 3 or IgG eluted from senescent RBC followed by ^{125}I-labeled protein A. Polypeptides were stained with amido black. Both autoradiographs were exposed for the same length of time.

to both the M_r ≈55,000 transmembrane fragment of band 3 (CH-55) generated by mild α-chymotrypsin treatment of intact RBC and its complementary M_r ≈38,000 fragment (CH-38) (Fig. 2, lane b) and to the M_r ≈17,000 transmembrane segment of band 3 (CH-17) (Fig. 2, lane c). However, it did not bind to the M_r ≈41,000 cytoplasmic segment of band 3, which is released from inverted vesicles after trypsin or α-chymotrypsin treatment (TR-41) (Fig. 2, lane e), although it did bind to a complementary M_r ≈60,000 fragment that remained with the membrane (Fig. 2, lane d). The same results were obtained when the gel-overlay rather than immunoblotting technique was used (results not presented).

Peptide Mapping Analysis of Senescent Cell Antigen, Band 3, and Defined Proteolytic Products of Band 3. The extent of homology between senescent cell antigen and band 3 and its proteolytic products was evaluated by comparing peptide maps of these polypeptides (Fig. 3). Senescent cell antigen (Fig. 3*C*) shares peptide homology with band 3 (Fig. 3*A*), the M_r ≈60,000 fragment remaining with the membrane after removal of the TR-41 cytoplasmic segment (Fig. 3*B*) and the CH-38 COOH-terminal segment of band 3 (Fig. 3*D*). The peptides present in the peptide map of senescent cell antigen are present in the maps of band 3 and the M_r ≈60,000 COOH-terminal segment.

The peptide map of CH-38 does not contain all of the peptides present in the map of senescent cell antigen, and it contains additional peptides not found in the map of senescent cell antigen. Approximately 30% of the peptides in the chymotryptic map of the CH-17 anion-transport segment (Fig. 3*E*) are found in peptide maps of senescent cell antigen. This suggests that proteolytic cleavage of band 3 may occur within the anion-transport region.

Since peptides of both CH-38 and CH-17 appeared in the

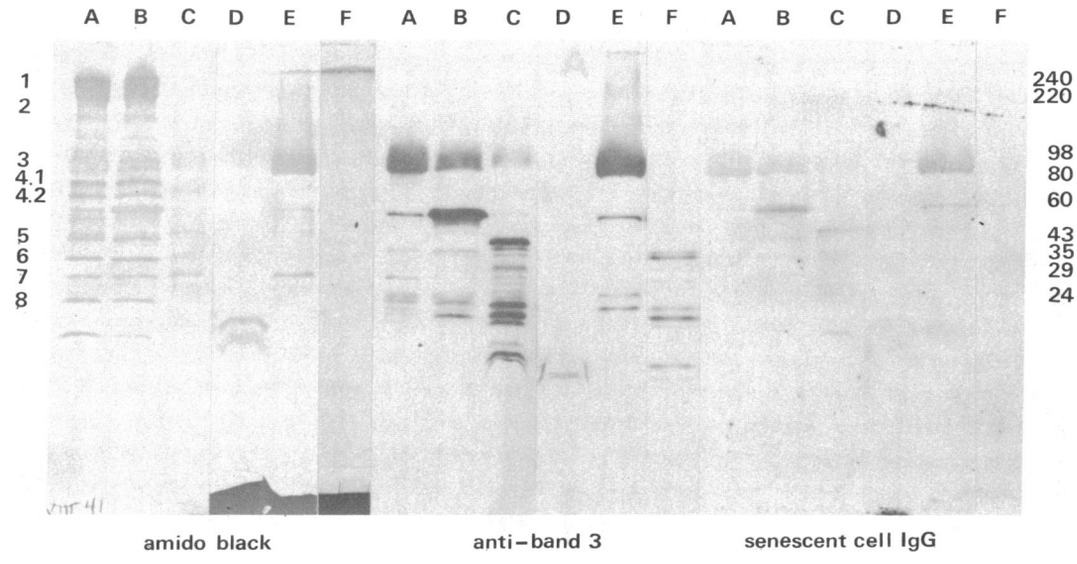

FIG. 2. Binding of anti-band 3 and IgG eluted from senescent RBCs to band 3 and its proteolytic degradation products. Relative molecular weights of membrane proteins ($\times 10^{-3}$) are indicated to the right of the figure. Lanes: A, RBC membrane proteins without enzyme treatment; B, RBC membrane proteins from intact cells treated with α-chymotrypsin overnight to generate CH-55 and CH-38; C, CH-TR-19; D, CH-17 after alkali treatment of membranes to remove peripheral membrane proteins; E, washed membrane pellet of alkali-treated inverted vesicles after removal of TR-41; F, supernatant from alkali-treated inverted vesicles containing TR-41. RBC membrane proteins were separated on NaDodSO$_4$/polyacrylamide gradient gels (6–25%) and transferred to nitrocellulose paper. The paper was incubated with anti-band 3 or IgG eluted from senescent RBCs, washed, and incubated with ^{125}I-labeled protein A. The paper was dried and exposed to Kodak X-Omat RP film for 5 days at −80°C in a Cronex cassette with intensifying screens.

map of senescent cell antigen, an attempt was made to generate a map similar to that of senescent cell antigen by mixing equal amounts of CH-38 and CH-17 on the same map (Fig. 3*F*). The resulting map closely resembles that of senescent cell antigen, although it contains more peptides than are found in the map of senescent cell antigen. These results suggest that peptides of the senescent cell antigen reside on an extracellular, COOH-terminal segment of band 3 that includes most of CH-38 and part of CH-17.

Partial overlap between the CH-55 and senescent cell antigen was observed (Fig. 4). This is an expected result because CH-55 contains the CH-17 anion-transport region as well as the unrelated TR-41 cytoplasmic portion of band 3.

DISCUSSION

IgG eluted from senescent RBCs binds to band 3 and its naturally occurring $M_r \approx 62{,}000$ breakdown product observed in membranes of old RBCs prepared with the protease inhibitors iPr_2P-F, EDTA, and EGTA to avoid artifactual proteolysis. These degradation products of band 3 have not been observed in membranes of young cells (11).

IgG eluted from senescent RBCs also binds to both the CH-55 and CH-38 transmembrane fragments of band 3 produced *in vitro* by α-chymotrypsin treatment of intact RBC and to the CH-17 intramembranous, anion-transport segment of band 3. However, it does not bind to the TR-41 cytoplasmic fragment of band 3. Since IgG eluted from senescent cells binds specifically to the senescent cell antigen (2–4), the senescent cell antigen appears to reside on a transmembrane segment of band 3 that lacks the TR-41 cytoplasmic segment. This segment includes at least part of the CH-17 intramembranous fragment containing the anion-transport site (18) and segments on the COOH-terminal side of that site.

Peptide mapping revealed peptide homology between senescent cell antigen, band 3, and the CH-38 and CH-17 fragments of band 3 and between senescent cell antigen and the $M_r \approx 60{,}000$ outer-surface band 3 fragment generated by removal of TR-41. Peptide homology between senescent cell antigen and TR-41 could not be demonstrated. Thus, results of these studies indicate that both the critical antigenic determinants of the senescent cell antigen and the peptides that comprise the antigen reside on an outer surface segment of band 3 that includes CH-38 and part of the CH-17 anion-transport region.

Differences among the peptide maps of band 3, senescent cell antigen, and defined proteolytic products of band 3 may be due to several technical factors, including (*i*) differences in iodination produced by differences in folding of polypeptides of different sizes, (*ii*) an additional peptide at each end of a fragment where it was clipped from band 3, (*iii*) exaggeration of visualization of peptides because equal cpm were spotted on the TLC plates, allowing peptides that are not detected during the exposure of band 3 to be visualized on the map of the smaller fragment (e.g., 5 cpm/Da were "spotted" for band 3, whereas 8 cpm/Da were spotted for senescent cell antigen), (*iv*) shielding of smaller "spots" in the larger peptides by large, intensely labeled peptides, (*v*) fewer tyrosine-containing peptides in the smaller fragments of band 3, and (*vi*) shifts in migration in either the electrophoretic or chromatographic dimension caused by a disproportionate amount of carbohydrate of those polypeptides that are glycosylated.

It was necessary to underexpose peptide maps of band 3 relative to those of the other polypeptides for purposes of reproduction. When this is not done, the peptides of band 3 converge, rendering identification of single peptides impractical. Unfortunately, underexposure results in the loss of some band 3 peptides that are apparent in the original autoradiographs from exposure times equal to those of other polypeptides. However, we have analyzed every major Coomassie blue-staining polypeptide in the RBC membrane by two-dimensional peptide mapping. Our maps resemble those for which published maps are available (e.g., spectrin, 2.1, and 4.1a and 4.1b). The major peptides present in the map of band 3 and senescent cell antigen and the related maps of defined proteolytic fragments of band 3 do not appear in the maps of the other RBC membrane proteins. The central five peptides in senescent cell antigen form a striking easily identifiable pattern.

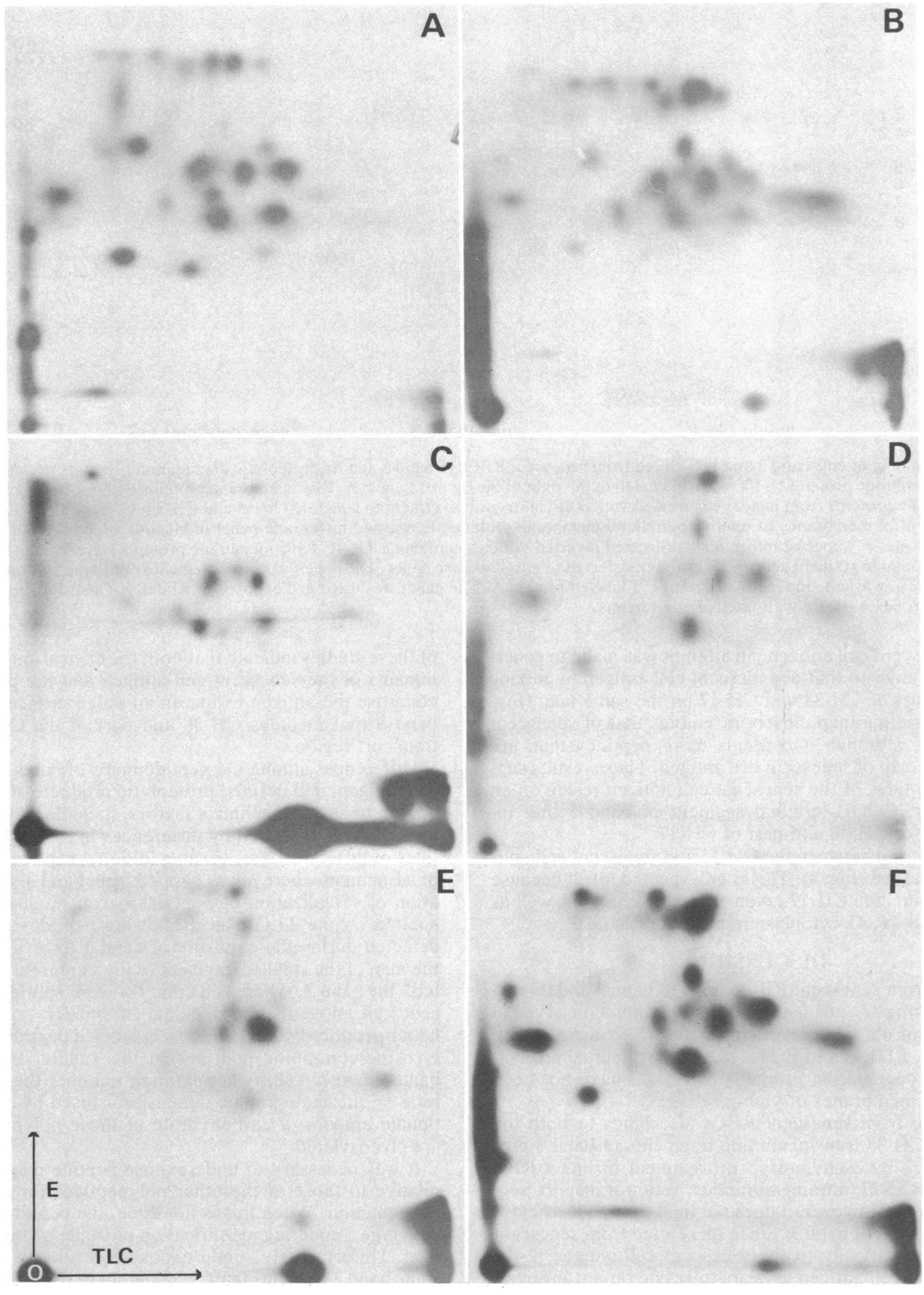

FIG. 3. Two-dimensional peptide maps of band 3, its proteolytic fragments, and the senescent cell antigen. (*A*) Band 3. (*B*) M_r ≈60,000 COOH-terminal polypeptide complementary to TR-41. (*C*) Senescent cell antigen. (*D*) CH-38. (*E*) CH-17. (*F*) CH-38 mixed with equal amounts of CH-17. O, origin; E, electrophoresis. Iodinated gel slices were incubated in two changes of 25 μg of α-chymotrypsin in 25 mM ammonium bicarbonate buffer for 20 hr at 37°C. The supernatants were pooled, lyophilized, and dissolved in acetic acid/formic acid/H_2O, 15:5:80 (vol/vol), so that there were 5×10^5 cpm/μl. Samples (0.5 μl) were applied to cellulose-coated TLC plates. Electrophoresis was carried out in a Pharmacia electrophoresis chamber at 1 kV and 0°C. After the plates were dried, TLC in the second dimension was conducted in 1-butanol/pyridine/acetic acid/H_2O, 32.5:25:5:20 (vol/vol). Chromatographs were dried and exposed to Kodak X-Omat film in Dupont cassettes with Cronex Lightning Plus intensifying screens for 8 hr (band 3 in *A*) to 3 days at −80°C. Band 3 was deliberately underexposed in order to improve resolution. Spots were lost because of underexposure, but an exposure time equal to that of the other maps resulted in peptides in the band 3 map becoming confluent. This experiment was repeated nine times on three different sample preparations.

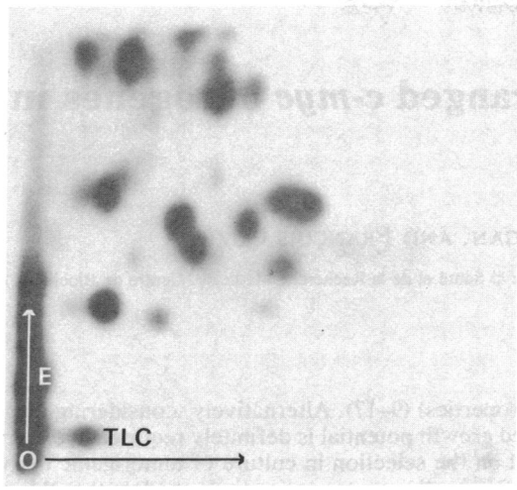

FIG. 4. Two-dimensional peptide map of CH-55.

Senescent cell antigen appears to be a M_r ≈62,000 polypeptide on NaDodSO$_4$ gel electrophoresis; yet, peptide mapping analysis indicates that it contains most of CH-38 and ≈30% of CH-17. Thus, the calculated molecular weight of senescent cell antigen from the peptide mapping data is ≈43,000. The discrepancy between the apparent and calculated molecular weights could result from the following. Band 3 molecule could contain redundant peptide sequences so that identical peptides in different positions on the band 3 molecule "map" in the same or similar position in peptide maps. The autoradiographic intensity of peptides in the band 3 molecule and its larger fragments as compared to peptides from its smaller fragments support this possibility. Senescent cell antigen contains carbohydrates that add to its relative molecular weight but are not detectable in peptide maps because the iodination procedure labels proteins (tyrosine groups) and not carbohydrates. Alternatively, senescent cell IgG, which has two binding sites, could bind to antigenic groups near the COOH terminus of band 3 and to antigenic sites in the transmembrane, anion-transport region. If this occurred, it is possible, although unlikely, that some of the sequences of the molecule outside of the antigenic region could be lost during the isolation procedures.

In another set of experiments, we have shown that polypeptides in the 4.5 region appear to represent several families of peptides, three of which are related to band 3 based on two-dimensional peptide mapping analysis (23). However, in that study, we only analyzed polypeptides that were present in significant quantities (i.e., the total number of copies of three band 3 polypeptides together was ≈100,000 copies per ghost). Senescent cell antigen, which is present in far smaller quantities, was not one of the bands that we analyzed because it is not visible on Coomassie blue staining of RBC ghosts.

Senescent cell IgG binds to band 3 denatured by NaDodSO$_4$ but does not bind to intact band 3 *in situ* in RBCs (2, 3). This suggests that a change in the tertiary structure of band 3 is required to initiate senescent cell IgG binding and removal of cells. Breakdown of band 3 *in situ* could initiate such conformational changes.

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