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Definition of a physiologic aging autoantigen by using synthetic peptides of membrane protein band 3: Localization of the active antigenic sites

(aging antigenic site/cell membranes/IgG autoantibodies/senescent cell antigen)

MARGUERITE M. B. KAY*†‡§¶, JOHN J. MARCHALONIS‖, JULIE HUGHES§, KIYOTAKA WATANABE*, AND SAMUEL F. SCHLUTER‖

Departments of *Medicine, †Medical Microbiology and Immunology, and ‡Biochemistry and Genetics, and §Olin E. Teague Veterans Center, Texas A&M University, P.O. Box 1887, Temple, TX 76503; and ‖Department of Microbiology and Immunology, University of Arizona, Tucson, AZ 85724

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ABSTRACT **Senescent cell antigen (SCA), an aging antigen, is a protein that appears on old cells and marks them for removal by the immune system in mammals. It is derived from band 3, a ubiquitous membrane transport protein found in diverse cell types and tissues. We have used synthetic peptides to identify aging antigenic sites on band 3, using a competitive inhibition assay and immunoblotting with IgG directed against the aging antigen on old cells. Results indicate that: (*i*) the active antigenic sites of the aging antigen reside on membrane protein band 3 residues that are extracellular regions implicated in anion transport (residues 538–554 and 788–827); (*ii*) a putative ankyrin-binding-region peptide is not involved in SCA activity; and (*iii*) carbohydrate moieties are not required for the antigenicity or recognition of SCA because synthetic peptides alone abolish binding of senescent cell IgG to erythrocytes. One of the putative transport sites that contributes to the aging antigen is located toward the carboxyl terminus. A model of band 3 is presented. Localization of the active antigenic site on the band 3 molecule facilitates definition of the molecular changes occurring during aging that initiate molecular as well as cellular degeneration.**

Senescent cell antigen (SCA), an aging antigen, is a protein that appears on old cells and acts as a specific signal for the termination of that cell by initiating the binding of IgG autoantibody and subsequent removal by phagocytes (1–14). SCA occurs on all cells examined (4). The aging antigen itself is generated by the degradation of an important structural and transport membrane molecule, protein band 3 (5). Besides its role in the removal of senescent and damaged cells, SCA also appears to be involved in the removal of erythrocytes in clinical hemolytic anemias (7, 8) and the removal of malaria-infected erythrocytes (15). Oxidation generates SCA *in situ* (6).

Band 3, the membrane protein from which SCA is derived, is a ubiquitous protein (16–20). It has been found in diverse cell types and tissues besides erythrocytes, including hepatocytes (16), squamous epithelial cells (16), alveolar (lung) cells (16), lymphocytes (16), kidney (21), neurons (16, 17), and fibroblasts (16, 20). Band 3 is also present in nuclear (16), Golgi (18), and mitochondrial membranes (19) as well as in cell membranes. Band 3-like proteins in nucleated cells participate in band 3 antibody-induced cell surface patching and capping (16). Band 3 maintains acid–base balance by mediating the exchange of anions (e.g., chloride and bicarbonate) (22–24). Because of its central role in respiration of CO_2, band 3 is the most heavily used ion-transport system in vertebrate animals. Band 3 is a major transmembrane structural protein (25) that attaches the plasma membrane to the internal cell cytoskeleton by binding to band 2.1 (ankyrin) (26).

We have used synthetic peptides to identify antigenic sites on band 3 recognized by the IgG that binds to old cells. Results indicate that: the active antigenic sites of the aging antigen reside on membrane protein band 3 residues 538–554 and 812–827, which are extracellular. We call the proteins comprising these residues anion 1 and COOH, respectively; the latter peptide is implicated in anion transport and is located toward the carboxyl terminus. Carbohydrate moieties are not required for the antigenicity or recognition of SCA, since synthetic peptides alone abolish binding of senescent cell IgG to erythrocytes.

MATERIALS AND METHODS

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis and Immunostaining of Peptides. Peptides were analyzed on 6–25% or 12–25% linear gels (6, 7, 27–29). Immunoautoradiography was performed by immunoblotting as described (6, 7, 28–30). Immunoblots were exposed to Kodak Ortho G (OG-1) film at −70°C by our standard procedures (29, 30).

IgG Binding and Inhibition Assay. IgG was isolated from senescent erythrocytes from 50 liters of blood and purified with protein A-Sepharose as described (2, 4). IgG eluted from senescent cells (SCIgG), rather than serum IgG, was used because normal serum contains antibodies to spectrin, actin, band 2.1, etc. (28). Competitive inhibition studies were performed with synthetic peptides to absorb the IgG isolated from senescent erythrocytes. Intact dimeric, SCIgG containing the Fc portion binds to senescent cells *in situ* and initiates their removal (1–7, 30). Fab fragments were not used because we were simulating the physiological situation. IgG isolated from aged erythrocytes binds specifically to senescent cells. For example, IgG eluted from young control erythrocytes does not bind to senescent cells (2, 30). Moreover, the specific binding capacity of the autoantibody was eliminated by absorption with purified SCA (SCA; 7). SCIgG (3 μg) was absorbed with synthetic peptides at the concentrations indicated or purified SCA, as a control, for 60 min at room temperature and was incubated with stored erythrocytes for 60 min at room temperature (1, 2, 4, 30). Storage mimics normal aging *in situ* immunologically and biochemically (1–7, 30). The number of erythrocyte-bound IgG molecules was quantitated before and after absorption by using equilibrium

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Abbreviations: SCA, senescent cell antigen; SCIgG, senescent cell IgG; DIDS, diisothiocyanodihydrostilbene disulfonate.

¶To whom reprint requests should be addressed at: P.O. Box 1887, Temple, TX 76503.

binding kinetics (7, 30, 31). Details of the methods are given elsewhere (27). Percent inhibition was calculated from the following formula: $100[1-(x-b/T-b)]$, where x = molecules of IgG autoantibody bound per cell, T = total number of IgG antibody molecules bound in the absence of inhibitor, and b = background protein A binding.

Peptides. Peptides were prepared by solid-phase synthesis with an Applied Biosystems 430A automatic peptide synthesizer. They were analyzed by amino acid analysis, HPLC, sequencing, and/or FABS to determine purity. For details, see ref. 27.

Computer Analysis. Sequence and protein structural analyses were performed by using programs of the Genetics Computer Group (GCG), University of Wisconsin, Sequence Analysis Software Package (33).

RESULTS AND DISCUSSION

Identification of the Aging Antigenic Site(s). We concluded from previous studies that SCA is a degradation product of band 3 that includes most of the ≈35-kDa carboxyl-terminal segment and the ≈17-kDa anion-transport region (5). Both immunoblotting studies with IgG isolated from senescent cells and peptide mapping studies of SCA indicated that SCA lacks an ≈40-kDa cytoplasmic segment that contains the amino terminus and, possibly, additional peptides of band 3 (5–7). Peptide-mapping studies and anion-transport studies suggested that a cleavage of band 3 occurs in the anion-transport region (5). Furthermore, breakdown products of band 3 are observed in the oldest cell fractions but not in young or middle-aged cell fractions, and anion transport is impaired in old cells (5–7, 30).

Based on these structural, biochemical, and immunological data (1, 2, 4–7), we deduced that cleavage of old band 3 occurred approximately one-third of the way into the transmembrane anion-transport region from the carboxyl-terminal end. Therefore, we synthesized peptides of erythrocyte band 3 with putative or suspected anion-transport activity. We selected one anion-transport segment that appears to be exposed to the outside of the cell and one that is further along the molecule towards the amino terminus and outside the region that we speculated was included in SCA (5). The first peptide (anion 1, residues 538–554) includes two important amino acids. (For sequences of all peptides, see Tables 1 and 2.) Lys-539 is a covalent binding site for the anion transport inhibitor, diisothiocyanodihydrostilbene disulfonate (DIDS), and Tyr-553 is radioiodinated by extracellular lactoperoxidase (34). The second peptide (anion 2, residues 588–602) is situated toward the end of the region and is probably intracellular because the potential N-glycosylation site at ASN-593 is not glycosylated (34). This peptide would be predicted to lack inhibitory activity because it would not be presented as an antigen on native band 3. The last peptide from the carboxyl-terminal end ("COOH", residues 812–827) contains both hydrophobic and hydrophilic regions (see Table 1). The lysines found in this region may comprise another binding site for DIDS (35). As a control, we used a peptide from the cytoplasmic segment of band 3 within the region of the putative ankyrin-binding site (36) (CYTO, residues 129–144). As an additional control, a peptide containing the N-glycosylation site that is glycosylated (GLYCOS, residues 630–648) was included because it is extracellular. Peptides were synthesized based on the human sequence data from the paper by Tanner et al. (34).

Competitive inhibition studies were performed by using synthetic peptides to absorb the IgG isolated from senescent erythrocytes. IgG binding and inhibition were determined with a protein A binding assay. This biological assay measures the fate of erythrocytes *in vitro* and *in vivo* (1, 2, 4). Results of these studies suggest that SCIgG recognizes antigenic determinants that lie within a putative anion-transport region, residues 538–554 (anion 1), and a transport site containing a cluster of lysines toward the carboxyl terminus, residues 812–827 (peptide COOH) (Table 1). Anion 2 was only weakly inhibitory and CYTO did not inhibit. The competition inhibition data illustrated in Fig. 1 show that peptides anion 1 and COOH are inhibitory over a range of 3 to 100 μg (Fig. 1*A*), whereas the internal peptide from the anion-transport region (anion 2) and a peptide from an external glycosylated site (GLYCOS) were only weakly inhibitory (Fig. 1*B*), and the putative ankyrin-binding peptide did not react with the antibody (Fig. 1*A*).

For two reasons, we decided to mix the two inhibitory peptides to determine whether they acted synergistically. First, both are inhibitory, but the inhibition is not complete even at 300 μg. Second, the results of our earlier peptide mapping studies with topographically defined segments of band 3 suggested that SCA was composed of peptides from both the anion-transport transmembrane region and the 35- to 38-kDa carboxyl-terminal segment (5). Mixing of these two regions produced a peptide map that closely resembled SCA even though it contained more peptides. The mixture of these two peptides produced ≈50% inhibition at 0.1 μg (i.e., 0.05 μg of each peptide), indicating that anion 1 and COOH interact together to form a three-dimensional structure that functions as an aging antigen (Fig. 1*A*). COOH hexamer (designated N6) consisting of six amino acids on the amino-terminal side of peptide COOH gave significant inhibition (≈50% at 10 μg) but did not synergize as well with anion 1 as did peptide COOH itself, since an ≈10-fold increase in peptide is required to obtain inhibition (≈50% inhibition at 1 μg; Fig. 1*B*). COOH decamer (designated C10) consisting of 10 amino acids on the carboxyl-terminal side of peptide COOH gave 54 ± 3% inhibition at 30 μg. A mixture of anion 2 and peptide COOH did not exhibit synergy (inhibition: 18 ± 4% at 30 μg).

Synthetic peptides would not be expected to be as effective as the native band 3 molecule itself because the short peptide segments do not assume the same tertiary configuration as that of the 911-amino acid band 3 molecule. The synergism of peptides anion 1 and COOH suggests that the conformation of the determinants of these two peptides interacting with each other is similar to that of the intact aging antigen. These results, together with data indicating that DIDS crosslinks these two regions (35), suggest that these peptides lie in close spatial proximity in native, aged band 3. This is consistent with other data indicating that these two sites are in close proximity in native band 3 (24, 36, 37).

Binding of SCIgG to anion 1 and peptide COOH, which is close to the carboxyl terminus of band 3, suggests that these segments are extracellular because the IgG molecule is 150 kDa and too large to enter a cell. This is consistent with the

Table 1. Inhibition of SCIgG binding by synthetic peptides or SCA

Sample	Inhibition, %
SCA	54 ± 7*
Synthetic band 3 fragment (residues; sequence)	
CYTO (129–144; AGVANQLLDRFIFEDQ)	0
Anion 1 (538–554; SKLIKIFQDHPLQKTYN)	88 ± 7*
Anion 2 (588–602; LRKFKNSSYFPGKLR)	30 ± 12*
COOH (812–827; LFKPPKYHPDVPYVKR)	99 ± 1*

Data are presented as the mean ± 1 SD of quadruplicate samples. *$P \leq 0.001$ compared with the control. SCIgG (3 μg) was incubated with peptides (300 μg) or buffer for 90 min at room temperature. Erythrocytes were added to the IgG, and samples were incubated for 90 min at room temperature. IgG on cells was quantitated by using ^{125}I-labeled protein A.

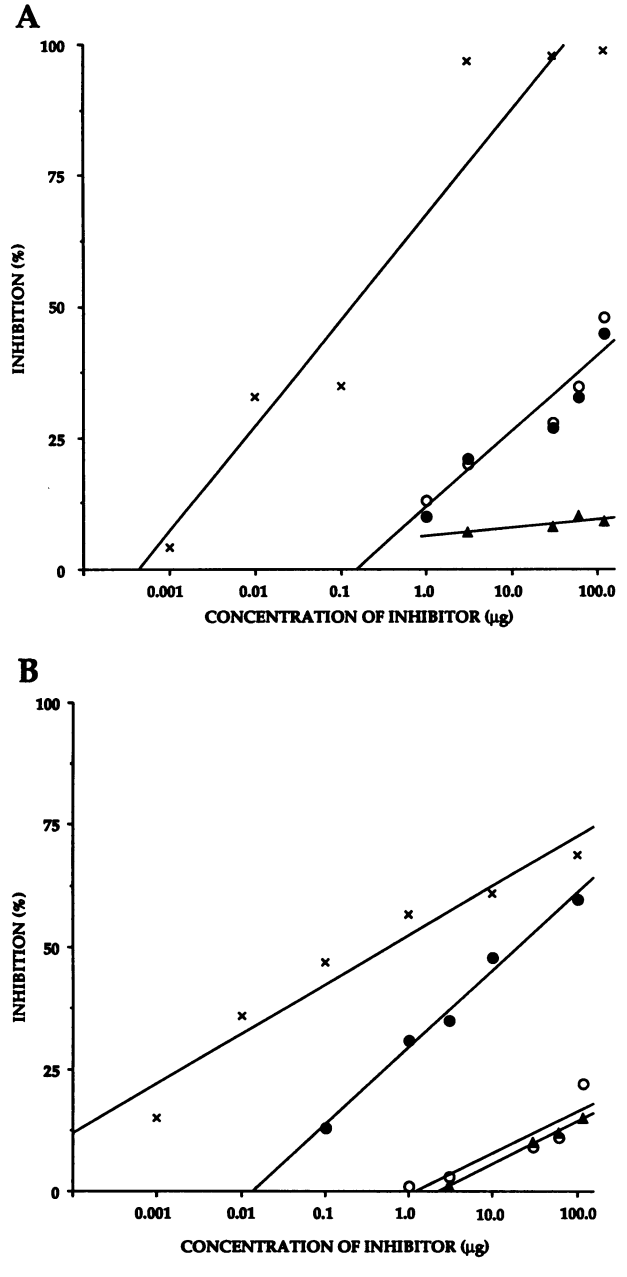


FIG. 1. Inhibition of senescent cell IgG binding to erythrocytes by synthetic peptides or synthetic peptide mixtures. Competitive inhibition studies were performed as described at the concentrations indicated on the graph. For peptide mixtures, the total peptide used was the amount indicated, with each peptide constituting half of that amount. (*A*) ▲, CYTO (residues 139–159); ●, anion 1 (residues 538–554); ○, COOH (residues 812–827); ×, anion 1/COOH mixture. (*B*) ●, COOH hexamer (N6) consisting of 6 amino acids on the amino-terminal side of peptide COOH; ×, COOH hexamer (N6)/anion 1 mixture; ○, GLYCOS (residues 630–648); ▲, anion 2 (residues 588–602). The data for peptides designated anion 1, COOH, and their admixtures are the averages of three separate inhibition experiments, each of which consisted of replicative determinations. Lines were fitted by the method of least squares. The data of anion 1 and COOH could be fitted by a single line in *A*. Other peptide COOH fragments gave the following percent inhibition: residues 818–827 (17 μM, 12.2 μg), 54 ± 7%; residues 818–823 (17 μM, 6.9 μg), 27 ± 4%; residues 822–827 (70.6 μM, 30 μg), 30 ± 2%.

data of Jennings *et al.* (35), which indicate that these regions are extracellular and that a segment located carboxyl-terminal to the trypsin cleavage site at Lys-743 and an S-cyanylation cleavage site ≈7 kDa from the carboxyl terminus is crosslinked by extracellular DIDS (35).

Results of these studies with synthetic peptides are consistent with the physiological data showing that old erythrocytes have impaired anion transport (6, 7, 30), the biochemical and immunological data indicating that band 3 undergoes degradation with loss of a cytoplasmic segment during the aging process (5–7, 30), and the data derived from alterations and/or mutations of band 3 indicating that changes associated with accelerated aging involve changes in the anion-transport region of band 3 (7, 38, 39).

Model of the Membrane-Associated Region of Human Band 3. Based on the above considerations and others, we developed a working model for the membrane-associated region of human band 3 protein, approximately residues 400–870. The model was constructed by using the program PEPPLOT of the GCG package to identify membrane-spanning nonpolar helices (40) and intervening hydrophilic loops (41). The location of the hydrophilic loops as extracellular or intracellular was predicted on the basis of established chemical or biological markers—e.g., the demonstration that residues 814–829 contain a DIDS binding site or the availability of Tyr-553 for external radioiodination (34). These regions are assembled into a model in Fig. 2. Key residues are identified to facilitate their identification within the sequence. This is a two-dimensional representation that does not reflect three-dimensional associations of residues that are separated by long stretches of sequence. Our present results show, however, that close steric association must be maintained by external loops 02 and 04. When these regions are associated on the same band 3 monomer, then band 3 loops back upon itself so that these regions are contiguous.

Alternatively, the functional assembly may be dimers in which close associations of loops 02 and 04 form between separate molecules. Our incorporation of recent human data (34) with the antigenic results presented here allows refinement and development of a model incorporating band 3 designated loop 04 that contains the peptide COOH and its component N6, which are potent inhibitors of the binding of autoantibodies to the aging antigen to erythrocytes. All of the data support the external presentation of this region. The short bilayer spanning segments may represent segments that enter the bilayer and exit again, looping back on themselves, without transversing the bilayer to the other side. These projections are based on hydrophobicity plots and may or may not exist in the membrane.

Amino acids that are exposed to the outside could reside on a hydrophobic helix within a membrane pore and still be accessible to the outside even though they are not on the outermost membrane. This probably applies to some band 3 sites designated as being external, although it is not reflected in the model. We suspect that the tertiary structure of band 3, when finally elucidated, will turn out to be a ring.

Computer search for regions of internal homology within band 3 was performed by using programs COMPARE and DOTPLOT with a window of 30 residues and stringencies of 10.0 (low) and 15.0 (high). Homologies were disclosed among the membrane-spanning nonpolar helical regions. For example, a

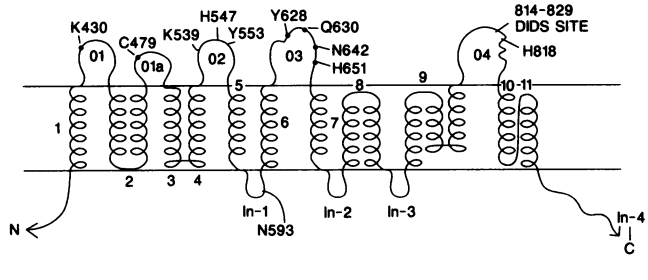
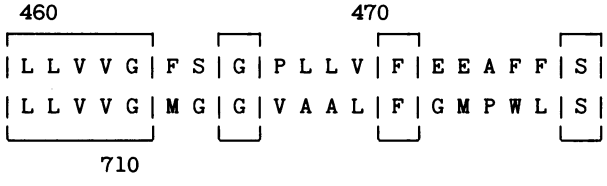


FIG. 2. Model of membrane-associated and external regions of anion-transport protein band 3. The regions are described in the text.

segment of bilayer-spanning helix adjacent to extracellular loop 02 had ≈40% identity to a corresponding segment of bilayer-spanning helix between internal loops 2 and 3:



Such relationships were not obvious among the hydrophilic regions (e.g., external hydrophilic loops 02 and 04 did not show significant sequence homology).

The reader is cautioned that (*i*) models such as the one we have drawn are not accurate representations of the real world, and (*ii*) most, if not all, information on the location of functions on the band 3 molecule is based on circumstantial and indirect evidence. Molecular modeling of band 3 is best described by Plato in the "Allegory of the Cave."

Molecular "Walking" of Band 3 to Define the Antigenic Site. Our previous experiments suggest that the active antigenic sites of SCA reside on the peptides that we have designated anion 1 and COOH. In this series of experiments, we attempted to define the active antigenic site by "walking" the anion-transport domain of band 3 molecule, which our studies indicate contains the antigenic determinants.

By "walking" we mean the antigenic analysis of a series of synthetic overlapping peptides that encompass the entire polypeptide chain adjacent to the active sites we have identified and include all predicted extracellular segments of band 3. The synthetic peptides are 17- to 19-mers and overlap their adjacent neighboring peptides by 6 residues in the overlap regions to optimize the feasibility of synthesis and to expect reasonable resolution of individual antigenic sites.

Peptides used to define the antigenic sites are listed with their sequences in Tables 1 and 2. In addition, we synthesized two predicted external sequences, which are indicated by asterisks in Table 2, the first of which (residues 426–440) is not in a putative transport region. This was done to complete the testing of all predicted external band 3 sequences for SCA, since the antigenic determinants must be external to be accessible to the 150-kDa IgG molecule.

We used immunoblotting of the peptides followed by reaction with IgG eluted from senescent erythrocytes (SCIgG) to localize the aging antigen site (Fig. 3 *A* and *B*). We also used the IgG binding and inhibition assay at a single concentration of peptide and performed Scatchard analysis (Table 2). We selected 30 μm for this "single concentration" assay because our two synergistic peptides, anion 1 and COOH, give ≥95% inhibition at this concentration, whereas "noninhibitory" peptides give ≤20% inhibition. Peptides that were negative by immunoblotting were also negative in the inhibition assay (Fig. 3 and Table 2). We relied on immunoblotting with SCIgG for peptides that were not completely soluble under physiologic conditions (Fig. 3).

Immunoblotting studies with SCIgG showed binding to residues 788–805 and 800–818 in addition to residues 812–827 of peptide COOH. Peptides comprising residues 788–805 and 800–818 are to the amino-terminal side of peptide COOH whereas that comprising residues 822–839, which has no labeling, is to the carboxyl-terminal side. Fragments of residues 800–817 and 822–839 have six amino acids in common with peptide COOH. Binding of antibody to residues 630–648 and trace binding to residues 645–659 was observed.

However, the competitive inhibition assay shows that significant inhibition of senescent cell IgG binding is obtained only with the peptide comprising residues 822–839, which is on the carboxyl-terminal side of peptide COOH and contains six amino acids of COOH. Thus, its inhibitory effect may be due to the six common amino acids. Peptides comprising residues 776–793, 788–805, and 800–839 were not completely soluble and were not tested in the cellular binding assay.

Table 2. "Walking" of band 3 protein to define the antigenic site; inhibition of SCIgG binding to erythrocytes by synthetic peptides of band 3

Synthetic peptide		
Residues	Sequence	Inhibition, %
426–440*	LLGEKTRNQMGVSEL	11 ± 1
515–531	FISRYTQEIFSFLISLI	15 ± 1
526–541	FLISLIFIYETFSKLI	NT
549–566	LQKTYNYNVLMVPKPQGP	NT
561–578	PKPQGPLPNTALLSLVLM	0
573–591	LSLVLMAGTFFFAMMLRKF	NT
597–614	FPGKLRRVIGDFGVPISI	52 ± 4
609–626	GVPISILIMVLVDFFIQD	12 ± 1
620–637	VDFFIQDTYTQKLSVPD	NT
630–648†	QKLSVPDGFKVSNSSARGW	9 ± 0
645–659*	ARGWVIHPLGLRSEF	NT
776–793	MEPILSRIPLAVLFGIFL	NT
788–805	FGIFLYMGVTSLSGIQL	NT
800–818	LSGIQLFDRILLLFKPPKY	NT
822–839	VPYVKRVKTWRMHLFTGI	35 ± 2

Data are presented as the percent inhibition ± SD of the binding of human autoantibody to SCA. All peptides were tested at 30 μg. NT, not tested in the competitive inhibition assay because the peptide was not completely soluble in physiologic solutions even when coupled to bovine serum albumin.
*Predicted external sequences.
†GLYCOS peptide.

In the anion 1 and anion 2 peptide series, trace binding was observed in immunoblots to anion 1 (residues 538–554) and the peptides to its amino (residues 526–541)- and carboxyl (residues 549–566)-terminal side, both of which have a 6-amino acid overlap with anion 1. SCIgG bound to anion 2 (residues 588–602) and bound faintly to the fragment to its carboxyl-terminal side (residues 597–614), which has a 6-amino acid overlap.

The competitive inhibition assay shows that inhibition of 52 ± 4% was obtained with the peptide formed of residues 597–614. Residues 526–543 and 549–566 could not be tested because of low solubility of the peptides. The peptide from the external loop comprising residues 426–440 was negative.

We used two approaches to determine the peptides with which autoantibody to SCA reacts. The direct binding in immunoblot (Western) analysis shows that the antibody bound to a number of peptides, including some that are internal and do not block in the cellular inhibition assay. This probably results from the fact that aged erythrocytes that bind autoantibody are phagocytosed by macrophages, and antigenic peptides are generated for presentation to T cells. These peptides are not exposed in the normal presentation of band 3 and SCA on the erythrocyte surface, however. Our competitive inhibition studies show that the native determinant is formed by interaction of two exposed peptide regions contributed by anion 1 and COOH.

Thus, SCA is localized to a region within residues 538–554 and 788–827. Even though an antigen binding site is only six amino acids in size, these amino acids are probably not adjacent in the primary structure. A minimal number of amino acids is probably required to generate the twists and turns necessary for the active three-dimensional structure. The synergy between anion 1 and peptide COOH supports this. The data from the amino-terminal 6 amino acids of peptide COOH indicate that even though significant inhibition can be obtained with a six-mer peptide, larger quantities of the peptide are required, and synergy is impaired because ≥95% inhibition cannot be obtained at concentrations up to

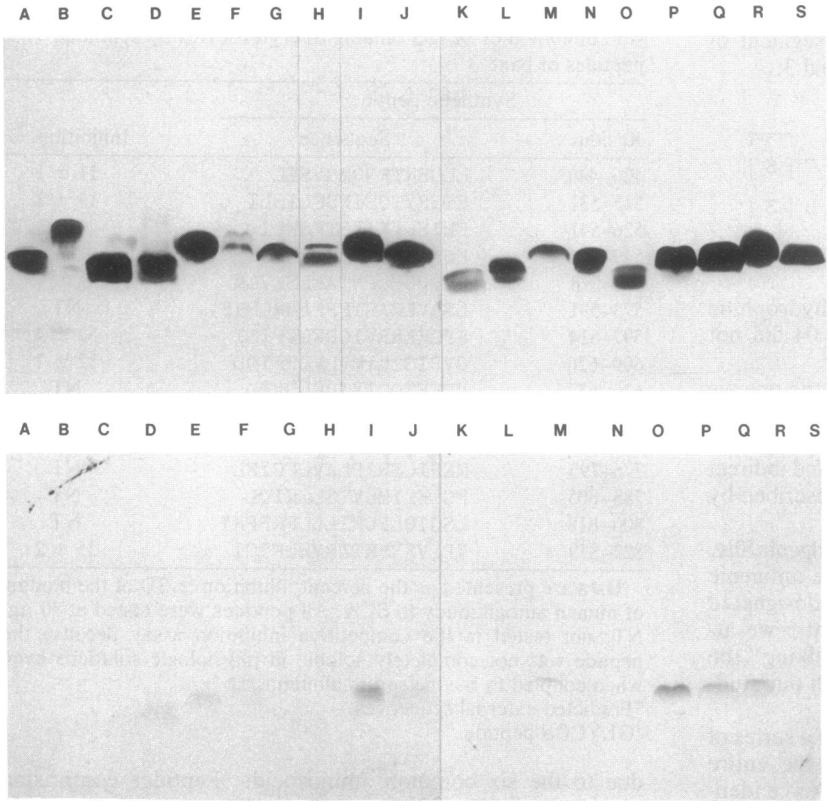


FIG. 3. "Walking" the band 3 molecule to localize the active aging antigenic site by using binding of SCIgG to synthetic peptides as determined by immunolabeling of electrophoretic blots. (*A*) Polyacrylamide gels of peptides stained with Coomassie blue R followed by silver stain. (*B*) Autoradiograph of immunoblot incubated with IgG autoantibody from senescent erythrocytes. The peptides in each lane and their residue numbers are: A, CYTO, 129–144; B, 426–440; C, 515–531; D, 526–541; E, anion 1, 538–554; F, 549–566; G, 561–578; H, 573–591; I, anion 2, 588–602; J, 597–614; K, 609–626; L, 620–637; M, GLYCOS, 630–648; N, 645–659; O, 776–793; P, 788–805; Q, 800–818; R, COOH, 812–827; S, 822–839. The sequences of these peptides are listed in Tables 1 or 2.

100 μg. In contrast, ≥95% inhibition can be obtained with the mixture of anion 1 and peptide COOH at 10 μg, and, in some experiments, at 3 μg.

Studies indicate that the transport region of band 3 is highly conserved across tissues, individuals, and species (21, 27, 34, 42, 43). Since SCA is generated on all cell types, tissues, and species examined (4), the regions that comprise SCA must also be highly conserved both evolutionarily and in various tissues. In this study, we show that active antigenic determinants of SCA reside on peptides anion 1 and COOH within residues 538–554 and 788–827. These peptides reside in highly conserved regions (34, 43).

We have localized SCA, an aging antigen, in terms of primary structure. Localization of the active antigenic site of SCA facilitates the next logical step—namely, definition of the molecular changes occurring during aging that initiate molecular as well as cellular degeneration and regulation of cellular lifespan.

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