

Variations in the cytoplasmic region account for the heterogeneity of the chicken MHC class I (B–F) molecules

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Abstract. Molecular variation among major histocompatibility complex (MHC) class I (B–F) proteins from *B*-homozygous chickens is apparently caused by C-terminal variation. Analysis of the total B–F protein pool revealed substantial heterogeneity with two or three molecular mass constituents, each being comprised by several isoelectric focusing variants. This heterogeneity could not be reduced by enzymatic deglycosylation. By contrast, proteolytic removal of a small (M_r 1000–4000) fragment from the α chain resulted in the generation of a M_r 36 000 fragment, common to all the molecular mass variants. Unlike the parent proteins, the M_r 36 000 fragment derived from isolated variants yielded identical, simple patterns in two-dimensional gel electrophoresis and identical finger prints in peptide mapping. This, together with N-terminal amino acid sequencing, as well as comparison of hydrophobicity properties of fragments obtained by gradual proteolytic digestion, indicated that the small peptide responsible for the major B–F heterogeneity was situated in the intracellular, C-terminal part.

Introduction

The *B*-complex is the group of genes comprising the major histocompatibility complex (MHC) in the chicken, that encodes classical class I (B–F) antigens with functions apparently similar to those of the mammalian counterparts (Longenecker and Mosmann 1981; Vainio et al. 1984; Maccubbin and Schierman 1983).

Among mammals, the number of different class I molecules varies considerably between species, and even between individuals within a species. For example, there are around 40 class I-like genes in some strains of mice that differ in genetic location, polymorphism, tissue expression, and apparent function. In contrast, there are only three genes in the Syrian hamster (Klein 1986). Among nonmammalian vertebrates, only the chicken and the clawed toad *Xenopus* have been extensively investigated. There is apparently only one class I alloantigen expressed on *Xenopus* erythrocytes (Flajnik et al. 1984).

The number of chicken class I gene products is unknown. Guillemot and co-workers (1988) identified six apparent chicken class I α chain genes, but demonstrated only one as being transcribed. On the other hand, sequential immunoprecipitation studies using monoclonal antibodies (mAbs) with differential reactivity, distinguished two populations of B–F in *B*-homozygous chickens (Crone et al. 1985). This finding suggested the presence of at least two structurally different B–F antigens, and therefore, possibly, the expression of two or more B–F genes in the *B*-complex. However, such experiments do not rule out factors such as post-translational modifications or varying stages of denaturation as being responsible for a difference in reactivity with certain antibodies.

The present study was undertaken in order to investigate whether multiple molecular forms of B–F, observed within a single haplotype, were likely to represent different class I gene products.

Materials and methods

Animals. Chickens homozygous for the B^{12} , B^{15} , B^{19} , B^{H3} , B^{H4} , B^{126} , or B^{127} haplotypes were inbred White Leghorns kept at the Institute for Experimental Immunology, Copenhagen and the Basel Institute for Immunology, Basel. The *B*-complex nomenclature used follows Briles and co-workers (1982).

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Antibodies. The B-F specific monoclonal antibodies (mAbs) F21-2 (α chain-specific) and F21-21 (β_2 -microglobulin (β_2 m)-specific) have been described (Crone and Simonsen 1987; Skjødt et al. 1986).

Biochemical and immunochemical methods. Isolation of chicken erythrocyte membranes (CEM), detergent extraction of CEM, iodination of detergent extract and western blot procedure were essentially as described (Salomonsen et al. 1987). Cell surface labeling, detergent lysis of cells, immunoprecipitation using protein A-sepharose, enzymatic deglycosylation with a glycosidase preparation containing mostly peptide: N-glycosidase F and with purified endoglycosidase H, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; with or without reducing agents), and fluorography were performed essentially as described (Kaufman et al. 1985a, 1989, 1990a, b).

Purification and fractionation of B-F. Affinity purification and gel filtration of B-F using proteinase inhibitors in all steps were performed essentially as described (Kaufman et al. 1989; Salomonsen et al. 1987). Fractionation of B-F molecular mass variants was performed by fast pressure liquid chromatography (FPLC) anion exchange chromatography (Mono-Q HR-5 S, Pharmacia, Allerød, Denmark), eluted with a linear gradient from 0-500 mM NaCl in 20 mM Tris HCl, pH 8, 0.1% NP-40.

The α polypeptide chains from the individual, Mono Q-fractionated B-F variants were isolated by incubation of B-F containing samples in 0.1% trifluoroacetic acid (TFA) in water, followed by reversed phase chromatography on a Pro RPC 5/10 column (Pharmacia). The column was eluted with a linear gradient from 0-100% acetonitrile containing 0.1% trifluoroacetic acid (TFA).

N-terminal amino acid sequencing. Automatic amino acid sequencing of isolated B-F α chain was carried out on an Applied Biosystems protein sequencer (Applied Biosystems, Copenhagen, Denmark), model 470A equipped with an on-line model 120A phenylthiohydantion (PTH)-analyzer.

One-dimensional slab gel isoelectric focusing (IEF). IEF gels contained 2% NP-40, 8 M urea, 3.78% acrylamide, 0.22% N, N'-methylene-bisacrylamide and 2% ampholines (LKB), mixed in proportions of 40% of the pH 4-6.5 interval, 40% of the pH 5-8 interval, and 20% of the pH 3-10 interval. The gel was run for 7500 V h (max 20 mA, max 1000 V) plus 1500 V h (max 1500 V) on an LKB multiphor 2117 horizontal gel apparatus (LKB Instruments, Bromma, Sweden) at 12 °C. The gel was fixed with 10% acetic acid, 25% 2-propanol overnight, then washed for 1-2 h in 25% ethanol, 2% glycerol.

Two-dimensional PAGE. Intact B-F molecules and proteolytic fragments were focused in tube gels using pH 3-10 ampholines, and then reduced and denatured for SDS-PAGE, as described (O'Farrell 1975). SDS-PAGE of deglycosylated B-F molecules was followed by IEF in flat-bed gels using 40% pH 3.5-10, 20% pH 3.5-5, 20% pH 4-6, 20% pH 5-7 ampholines as described (Shackelford and Strominger 1980; Kaufman et al. 1985b).

Trypsin and papain treatment of B-F under non-denaturing conditions. To radiolabeled B-F a freshly prepared trypsin solution (1 mg/ml trypsin (Sigma, Copenhagen, Denmark), 40 mM Tris HCl, 150 mM NaCl, 10 mM CaCl₂, pH 8), or a solution of activated papain (100 µg/ml) papain (Sigma), 40 mM Tris HCl, 0.1 mM ethylenediaminetetraacetate (EDTA), 1 mM dithiothreitol (DTT), pH 8) was added. After incubation at 37 °C for 2 h, the reactions were stopped by addition of phenylmethylsulfonyl fluoride (PMSF) to 1 mM (trypsin samples) or iodoacetamide to 10 mM (papain samples).

Fingerprinting. The method was modified according to Cleveland and coworkers (1977). In short, a fresh solution of pronase P (Serva, Copenhagen, Denmark) in 62.5 mM Tris HCl, 3% SDS, 10% w/v

glycerol, 8 M urea, 0.0012% bromphenolblue, pH 6.7 was added to samples containing Mono Q-purified B-F variants or to immunoprecipitates. The samples were immediately applied to an 7.5%-17.5% SDS polyacrylamide gel. When the front was half-way through the stracking gel, the electrophoresis was stopped for 20 min.

Phase separation of hydrophobic and hydrophilic proteins in Triton X-114 solution. Buffer change was performed by gel filtration on a PD-10 column (Pharmacia) eluted with 10 mM Tris HCl, 150 mM NaCl, 0.75% Triton X-114, 0.1% ovalbumin, pH 7.4. Temperature-dependent detergent phase separation was then performed essentially as described (Bordier 1981).

Results

Each chicken MHC haplotype has a number of B-F molecular variants. Cell surface iodinated erythrocyte B-F molecules from individual chickens with different B-haplotypes were immunoprecipitated with mAb F21-2. All show multiple α chain bands of M_r 38 000-41 000 on SDS gels (Fig. 1A). These bands were investigated in detail for B-F15 and B-F19 (which are apparently quite similarly based on mAb reactivity; Crone et al. 1985). Just like the immunoprecipitated B-F15 and B-F19 bands (Fig. 1A and data not shown), three B-F15 variants are clearly evident when erythrocyte membrane detergent extracts were analyzed by western blotting using F21-2 (Fig. 1B). The same three variants were also found after immunoaffinity purification of B-F15 molecules from erythrocyte membrane detergent extracts. The high molecular mass variant of M_r 40 000 could be separated by FPLC Mono Q chromatography from the two low molecular mass variants of M_r 39 000 and M_r 37 000 (Fig. 1C).

Immunoprecipitated B-F15 and B-F19 molecules were further analyzed by two-dimensional PAGE. Each of the three distinct B-F molecular mass variants is composed of many IEF variants (Fig. 2A and 3B), in fact too many to be adequately resolved.

A more distinct focusing pattern was obtained using one-dimensional IEF with a narrow pH range. At least 11 sharp bands were observed with B-F15 molecules immunoprecipitated from erythrocyte membrane detergent extract with mAb F21-2 directed to B-F α chains (Fig. 2b, lane a). Exactly the same pattern by SDS gel (data not shown) and IEF (Fig. 2B, lane b) were found with B-F15 molecules immunoprecipitated from erythrocyte membrane detergent extract with mAb F21-21 directed to β_2 m. This demonstrates that all the major proteins associated with β_2 m are detected in the present analysis.

One-dimensional IEF of the two separated B-F15 fractions showed that the high molecular mass variant and the low molecular mass variants have different charge patterns (Fig. 2B, lanes c and d). However, the sum of the two individual patterns corresponds to the patterns obtained with the unfractionated B-F15 (Fig. 2B, lanes a and b).

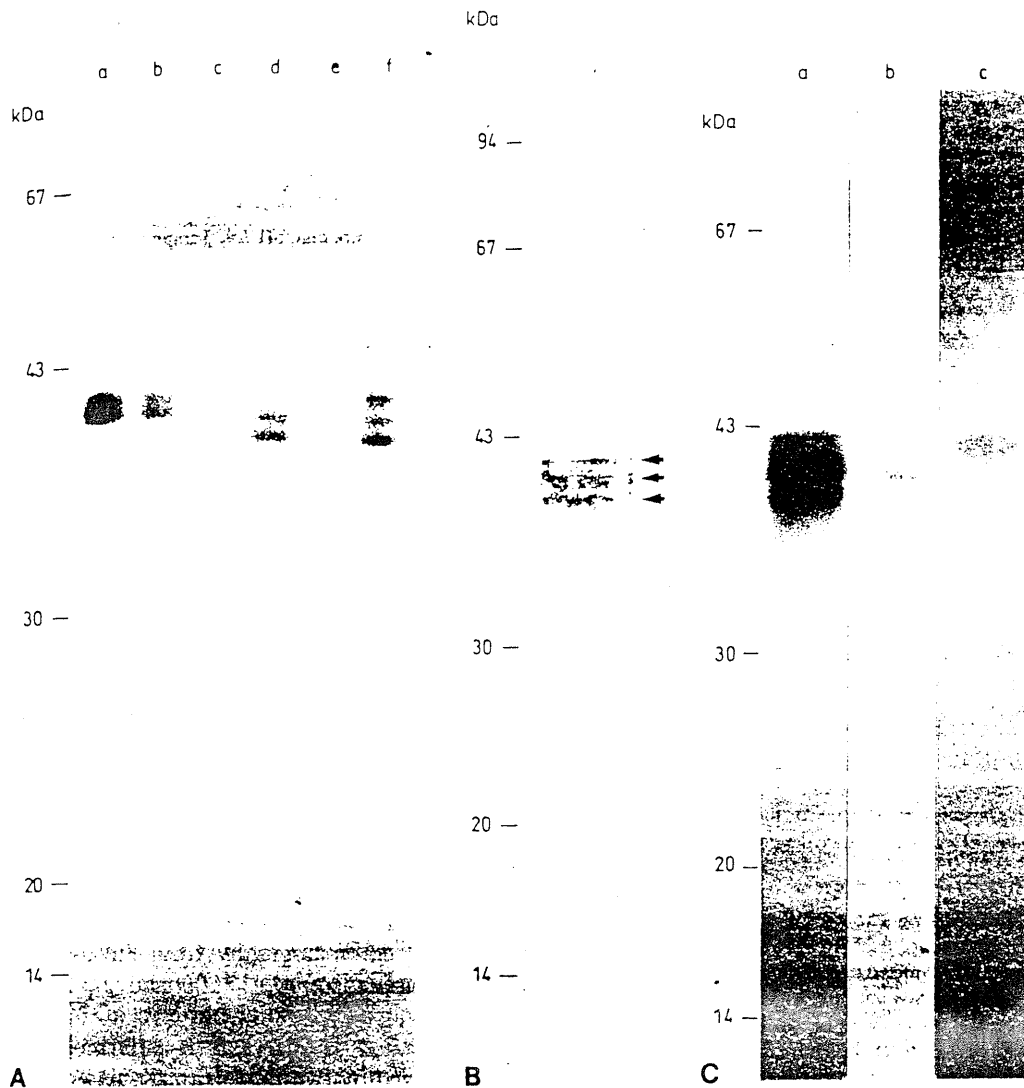


Fig. 1A-C. Isotypic heterogeneity in the B-F antigen pool. A Immunoprecipitation of B-F molecular mass variants from various haplotypes. B-F antigens were immunoprecipitated from surface labeled erythrocytes of individual animals, using the antibody F21-2. B-F from the following B-haplotypes were analyzed by SDS-PAGE: lane a, B^{W3} ; lane b, B^{W4} ; lane c, B^{12} (CB strain); lane d, B^{19} ; lane e, B^{26} ; lane f, B^{27} . B western blotting, showing B-F molecular variants in the CEM extract from birds of the B^{15} haplotype. CEM extract was separated by SDS-PAGE. B-F α chain variants were visualized by western blotting, using the B-F α chain-specific, mAb F21-2 as the primary antibody. The position of molecular mass standards are indicated on the left. C Separation of B-F15 molecular variants. A pool of immunoaffinity- and Sephacryl S300 chromatography-purified B-F15 antigens was separated by means of anion exchange FPLC, using a Mono Q column. SDS-PAGE of starting material (i. e., immunoaffinity-purified B-F; lane a) and selected peak fractions are shown. Lane b: Fractions containing the two lower molecular mass variants (eluting at 0.15 M NaCl); lane c: Fractions containing the higher molecular mass variant (eluting at 0.25 M NaCl). Proteins were visualized by coomassie staining.

Neither the observed molecular mass variation nor the isoelectric point (pI) variation in B-F was due to variation between individuals within a single inbred strain, since it could be observed also in B-F isolated from a single animal (Fig. 1A, Fig. 3A and 3B). Furthermore, the variation was not restricted to B-F on erythrocytes, since B-F isolated from white blood cells showed very similar electrophoretic patterns (Fig. 3B).

Carbohydrate does not contribute to the observed heterogeneity. In order to investigate whether any of the observed heterogeneity could be ascribed to differences in N-linked glycosylation, ^{125}I -immunoprecipitated B-F19 molecules were treated with Endo- β -N-acetylglucosaminidase H (Endo H) or with peptide: N-glycosidase F, in order to distinguish between the main types of N-linked glycosylation.

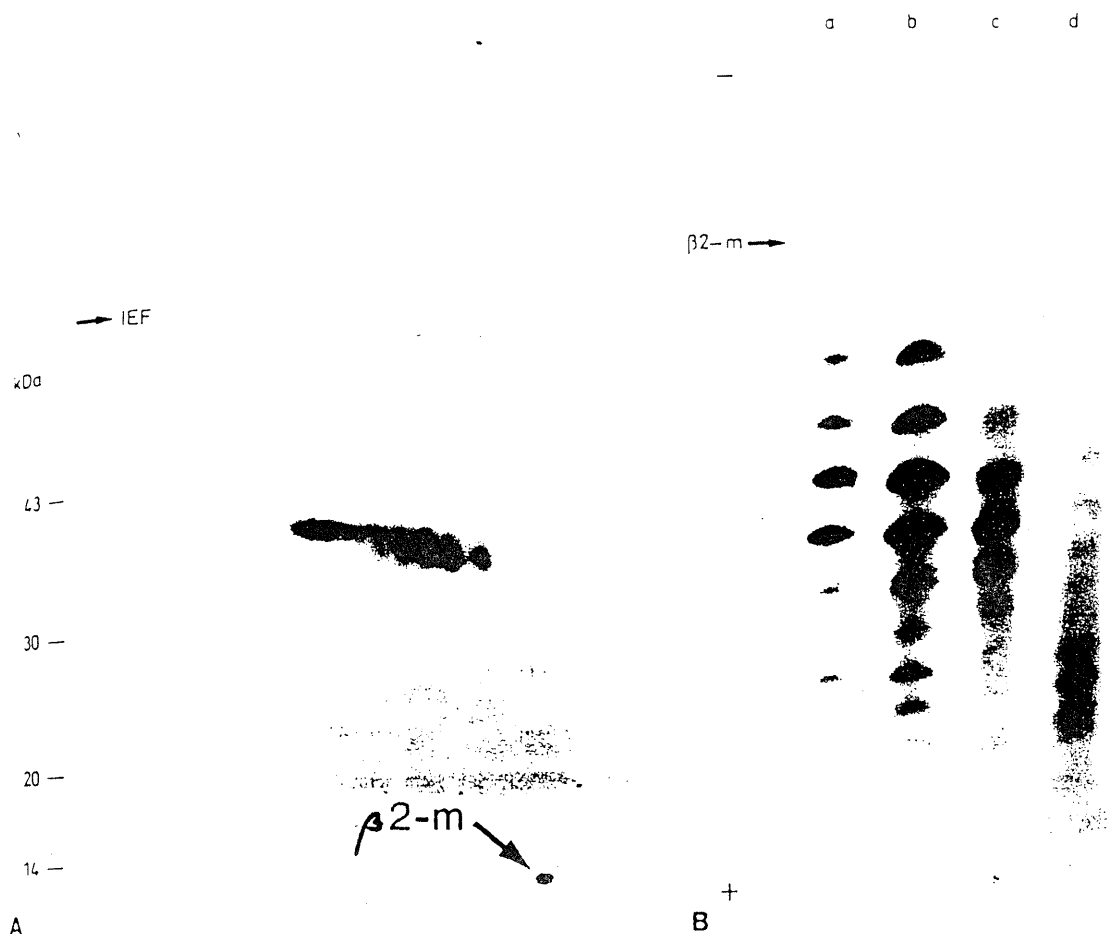


Fig. 2A, B. Isoelectric focusing of B-F15 variants. **A** Two dimensional PAGE. B-F15 antigens were immunoprecipitated from ^{125}I -labeled CEM extract using the mAb F21-2, and the resulting immunoprecipitate was analyzed by two dimensional gel electrophoresis with IEF as the first and SDS-PAGE as the second dimension, followed by autoradiography. The position of $\beta_2\text{m}$ is marked. **B** One dimensional IEF patterns. B-F antigens were immunoprecipitated from ^{125}I -labeled B^{15} CEM extract by α chain-specific antibody (F21-2; lane a) or by antibody to $\beta_2\text{m}$ (F21-21; lane b) and analyzed by slab gel IEF, followed by autoradiography. Additional samples included the FPLC fractionated molecular mass variants (see Fig. 1C): lane c, low molecular mass variants; lane d, high molecular mass variant.

Analyses by simple SDS-PAGE indicated that treatment with the two enzymes did indeed lead to deglycosylation as reflected by a decrease in apparent molecular mass. However, there was no reduction in the molecular mass heterogeneity (Fig. 3A). Thus, after treatment with Endo H, we observed a M_r 3 000 decrease in apparent molecular mass of each of the three B-F variants (Fig. 3A, lane b), while after treatment with peptide: N-glycosidase F, a decrease of M_r 6 000 was observed for each molecular variant (Fig. 3A, lane c). Analysis by two-dimensional gel electrophoresis of B-F19 molecules treated with peptide: N-glycosidase F showed that the number of IEF variants was also retained (Fig. 3B).

This data shows that B-F19 (and B-F15, data not shown) molecules each bear two N-linked glycans: an Endo H-resistant complex glycan and an Endo H-sensitive high mannose glycan. The sequence of the B-F19 cDNA

clone has two N-linked glycosylation sites, in agreement with this data (J. Kaufman, R. Andersen, J. Salomonsen, J. Engberg, K. G. Welinder, and K. Skjødt, submitted). B-F molecules are apparently devoid of extensive O-linked carbohydrate, since treatment with trifluoromethanesulfonic acid, which removes N-linked as well as O-linked glycans also led to a M_r 6 000 decrease in apparent molecular mass (Salomonsen et al. 1987).

Immunoprecipitated ^{125}I -labeled B-F was treated with neuraminidase which removes sialic acid from N-linked as well as O-linked glycans, which simplifies the complex IEF patterns found for mammalian major histocompatibility complex (MHC) molecules (Ploegh et al. 1981; Shackelford and Strominger 1980). Neuraminidase shifted the pattern towards the basic end with apparently two charges, but substantial heterogeneity remained (Fig. 3C). Subsequent treatment with peptide:

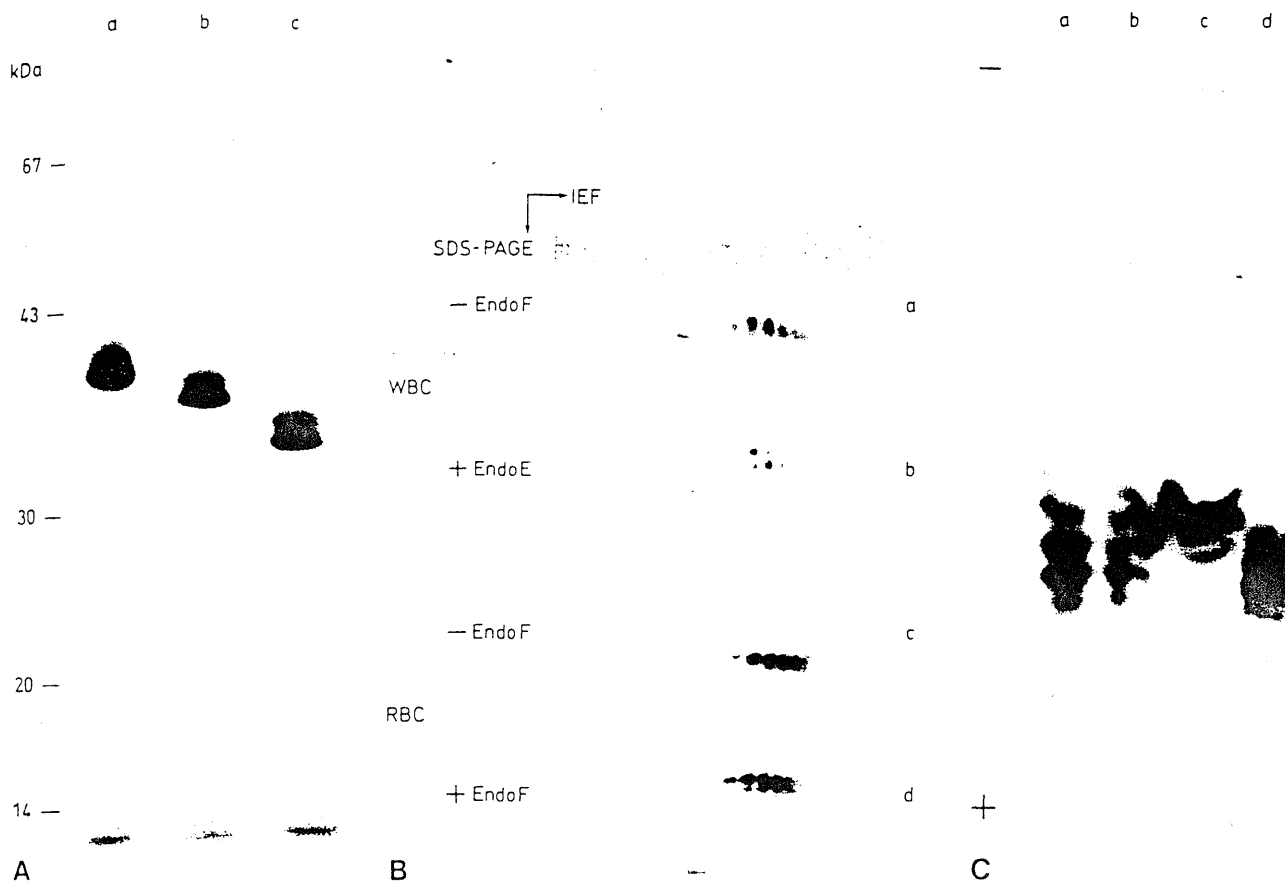


Fig. 3A-C. Deglycosylation studies of B-F. A Deglycosylation studies of B-F19, using glycosidases of different specificities. B-F19 was immunoprecipitated by the mAb F21-2 from lysate of ^{125}I surface labeled erythrocytes obtained from blood from a single animal. The immunoprecipitated material was analyzed directly, or after treatment with deglycosylating enzymes. Analysis was performed by SDS-PAGE under reducing conditions followed by autoradiography. Lane a, untreated; lane b, sample treated with Endo H; lane c, sample treated with peptide: N-glycosidase F. B 2-dimensional PAGE of deglycosylated B-F antigens. B-F was immunoprecipitated from cell surface B^{19} extract using F21-2. Immunoprecipitate was performed from detergent extracts of white blood cells (a, b) or red blood cells (c, d). All the cells were derived from a single bleed. The samples were analyzed directly (a, c) or after treatment with peptide: N-glycosidase F (labeled Endo F in the Figure; b, d). Analysis was performed by 2-dimensional PAGE (SDS-PAGE in first, IEF in second dimension), followed by autoradiography. C Desialylation of B-F15. ^{125}I -labeled low molecular mass FPLC fractions were treated with Neuraminidase and peptide: N-glycosidase F alone or in combination. The samples were analyzed by one dimensional IEF and autoradiography. a: Sample treated with Neuraminidase followed by peptide: N-glycosidase F; b: Sample treated only with peptide: N-glycosidase F; c: Sample treated only with Neuraminidase; d: Untreated.

N-glycosidase F restored the previous pattern by generating aspartic acid at the attachment sites. This means that each and every B-F19 molecule bears two sialic acids, being shifted two charges in the basic direction by the removal of these sialic acids by neuraminidase, and being shifted two charges back in the acetic direction by the generation of aspartic acids in the removal of two N-linked glycans by peptide: N-glycosidase F.

Proteolytic degradation reveals a common, major fragment in each of the three α chains. B^{15} erythrocyte membrane extracts or the separated high and low molecular mass variant fractions were treated with trypsin under non-denaturing conditions, and the resulting B-F fragments were immunoprecipitated and analyzed by

SDS-PAGE (Fig. 4). After treatment, no material was left at the electrophoretic mobilities corresponding to the three intact molecular mass variants. Thus, cleavage had occurred in all the variants. However, only a single limit cleavage product, a M_r 36 000 component, was observed. This degradation product clearly yielded a single, sharp band, in contrast to the parent material. Thus, trypsin had apparently removed a fragment of only M_r 1 000, M_r 3 000, or M_r 4 000, respectively, from the three variant proteins and left the major part as a common fragment with the same electrophoretic mobility in SDS-PAGE.

The trypsin degradation products from the B-F fractions were further analyzed by two-dimensional PAGE (Fig. 5A). In this high-resolution system, the M_r 36 000

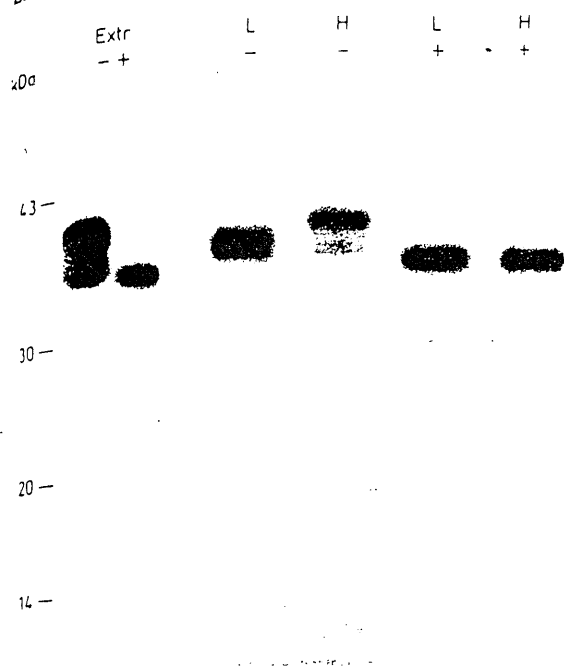


Fig. 4. Trypsin treatment of B-F antigens under non-denaturing conditions. Trypsin treated (+) and non treated (-) ^{125}I -labeled B-F containing samples were included: CEM extract (Extr); Mono Q fractions containing high (H) or low (L) molecular mass variants. The immunoprecipitated material was analyzed by SDS-PAGE and autoradiography.

fragments obtained from the two fractions appeared to be also identical. Surprisingly, the pattern consisted of only three spots, in contrast to the complicated patterns of the parent material (see Figs. 2A, B, and 3B). Since all the three spots occurred in the degradation mixtures from both fractions, none of the spots was unique to a single parent molecular mass variant.

In a similar experiment, degradation with papain was studied. Again, the results found with the two fractions were identical. After papain treatment, each fraction gave rise to two bands in SDS-PAGE, corresponding to fragments of M_r 34 000 and M_r 36 000. However, the pattern obtained was dependent on the enzyme concentration used, since after treatment with papain in a low concentration (below 10 $\mu\text{g}/\text{ml}$), only the M_r 36 000 fragment and the intact α chain occurred (not shown).

When the papain digests were analyzed by two-dimensional PAGE (Fig. 5B), identical patterns were found for the two fractions. Thus, in both cases, the M_r 36 000 fragment showed a series of three spots as was the case after cleavage with trypsin. Also the M_r 34 000 fragment yielded for both fractions a series of three spots. This series showed a minor shift towards the acid area, compared to the spots observed for the M_r 36 000 fragment.

Removal of the small papain fragments from the α chain variants creates identical peptide fingerprints. The

separated molecular mass variants and the corresponding papain fragments were digested with pronase under denaturing conditions (Cleveland et al. 1977) to generate partial peptide maps.

The pronase fingerprints from the two Mono Q fractions differed strikingly from each other (Fig. 6A). The lower molecular mass fraction yielded the more complicated pattern, probably reflecting the presence of two protein variants in this fraction. In several cases, however, the main bands in the fingerprints of this fraction occurred as doublets, formed by components differing in molecular mass by M_r 1–2000. In those cases, a single counterpart band could be observed in the high molecular mass fraction, at a M_r 1–3000 higher molecular mass (indicated by arrows in the Figure).

When papain treatment of the fractions under non-denaturing conditions was performed before the peptide mapping with pronase, the pattern changed markedly (Fig. 6B). The fingerprints from the two fractions became strictly identical.

These findings indicate that each of the three molecular mass variants contained a major, identical fragment. This fragment was liberated by the papain treatment, but its presence was reflected also in the band pattern found after direct peptide mapping.

The small peptides responsible for the major heterogeneity are located in the C-terminal cytoplasmic region. The observations above indicated that the major heterogeneity was caused by a small (M_r 1–4000) fragment, situated at one end of the B-F α polypeptide chain.

Each of the two Mono Q fractions were subjected to Pro-reverse phase chromatography (RPC) in order to isolate the B-F α chain and the purified α chains were analyzed by N-terminal amino acid sequencing (Table 1). The sequences obtained from the two fractions are identical with each other and with the sequence obtained for the total B-F15 α chain pool. This means that, even though a few of the amino acid residues were not identified with certainty, the N-termini of the molecular mass variants are identical in the sense that no stretch of sequence was missing from the molecules in either fraction. This conclusion applies to the first 19 residues, thus covering a range corresponding to approximately M_r 2000 in the N-terminus.

The alternative possibility, i. e., assignment of the heterogenous fragment to the C-terminus, could not be explored directly, since C-terminal sequence data could not be obtained. Instead, this possibility was studied by measuring hydrophobicity of the proteolytic fragments that were devoid of the major heterogeneity.

Immunoabsorbent purified, ^{125}I -labeled B-F was treated with papain as above to yield the two fragments of M_r 36 000 and M_r 34 000, respectively. The digestion mixture was subjected to temperature dependent phase

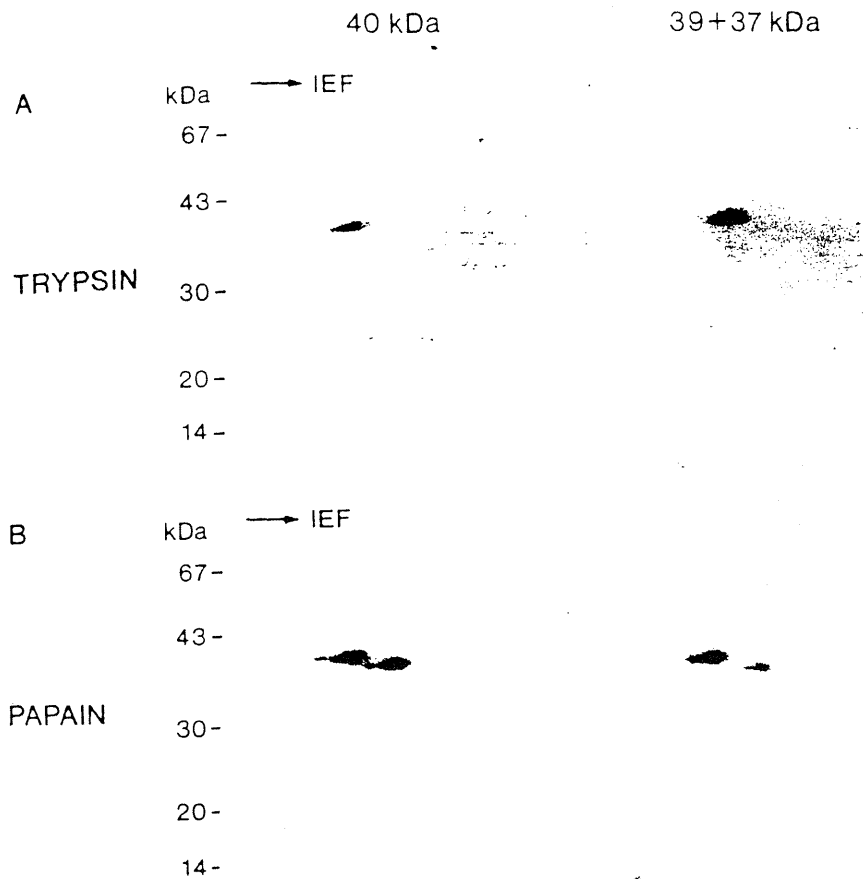


Fig. 5A, B. Two dimensional PAGE analyses of proteolytically cleaved B-F antigens. B-F containing Mono Q fractions were labeled with ^{125}I and treated with trypsin (A) or papain (B) under non-denaturing conditions. After treatment, B-F material was immunoprecipitated from the fractions and analyzed by 2-dimensional PAGE and autoradiography. *Left half* of the Figure: The fraction containing the high molecular mass B-F variant (M_r 40 000); *right half*: The fraction containing the two lower molecular mass variants (M_r 39 000 + 37 000). The basic end of the IEF gels is oriented towards the left. The position of $\beta_2\text{m}$ is seen as a spot in the lower left corner in all the autoradiograms.

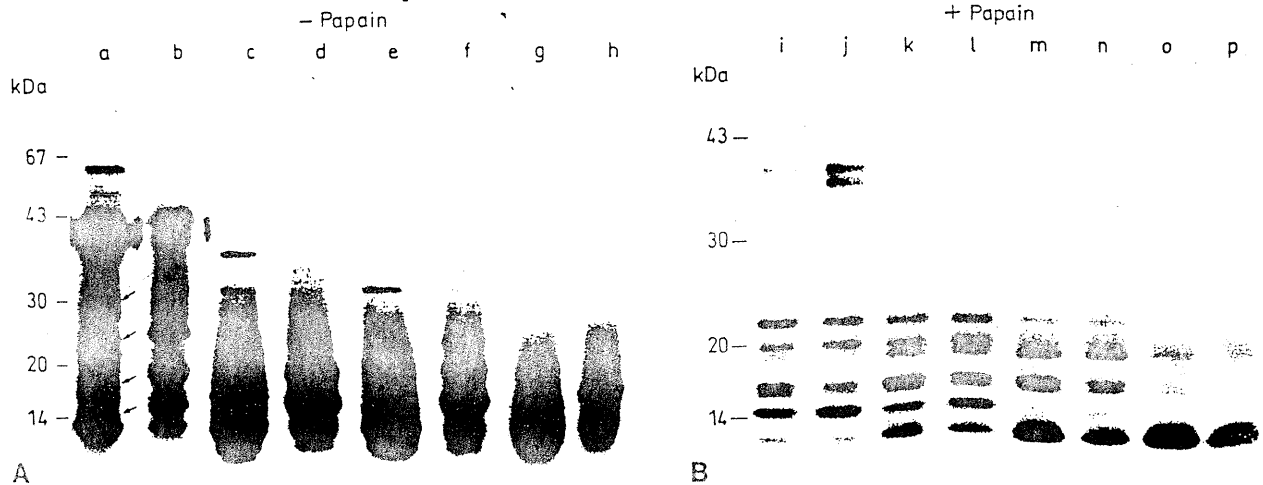


Fig. 6A, B. Peptide mapping of B-F molecular mass variants before and after papain treatment. ^{125}I -labeled B-F material was treated with pronase under denaturing conditions and loaded directly on an SDS-polyacrylamide gel, as described in Materials and methods, followed by electrophoresis and autoradiography. The B-F material used for pronase digestion was the intact antigens (gel A) or material immunoprecipitated from papain-treated B-F fractions (gel B). The samples were derived from the ^{125}I -labeled Mono Q high molecular mass fraction (lanes b, d, f, h, j, l, n, and p) or the low molecular mass fraction (lanes a, c, e, g, i, k, m, and o). The following concentrations of pronase were used: a, b, i, and j: 2.4 ng/ml; c, d, k, l: 24 ng/ml; e, f, m, n: 240 ng/ml; g, h, o, p: 2400 ng/ml. *Arrows* (gel A) indicate sets of 2 bands each in the digest of the low molecular mass fraction which appear to be the counterparts of single, M_r 1 000–3 000 heavier components in the digest of the high molecular mass fraction: see text.

Table 1. N-terminal amino acid sequences of B-F15 α chains isolated from the F21-2 mAb affinity column (B-F15) and the two pools of size variants separated by Mono Q ion exchange chromatography.

	1	5	10	15	21
H. V.	Glu Leu His N.I.	Leu Arg Tyr Ile Ser	Thr Ala Met Thr Asp	Pro Gly Pro Gly Gln	
L. V.	Glu Leu His Thr	Leu Arg Tyr Ile Ser	Thr Ala Met Thr (Asp)	Pro Gly Pro Gly Gln Pro	(Thr) (Tyr) (Val) Asp Val
B-F15	Glu Leu His Thr	Leu N.I. Tyr Ile Ser	Thr Ala Met Thr Asp	Pro Gly Pro Gly Gln Pro	

H. V. indicates high molecular mass variant(s); L. V. indicates low molecular mass variants; *brackets* indicate amino acids not identified with certainty; N.I. indicates not identified at all due to technical problems.

separation in a Triton X-114 solution in order to study the hydrophobicity of the individual fragments (Fig. 7). It is evident that while the intact α chain and the M_r 36 000 fragment were found in the detergent phase, the smaller M_r 34 000 fragment was found in the aqueous phase. As separation into the detergent phase of this system is a feature of integral membrane proteins (Bordier 1981), these findings indicated that while the transmembrane part was still present in the M_r 36 000 fragment, it was lost by further degradation to the M_r 34 000 fragment. This observation localizes the heterogeneous, minor fragments to the C-terminus.

Discussion

The purpose of this work was to determine whether more than one class I gene is responsible for the heterogeneity of B-F molecules observed in a given chicken MHC haplotype. While two mAbs from our laboratory apparently identified separate populations of B-F molecules by sequential immunoprecipitation (Crone et al. 1985),

we subsequently found no difference in these two populations by IEF and cocapping experiments (L. Møller, unpublished observations). In the present report, we use protease and glycosidase digestion to make two points about B-F molecules. First, we show that B-F molecules are structurally similar to mammalian class I molecules. Second, we show that both the size and charge heterogeneity of B-F molecules from B¹⁵ and B¹⁹ erythrocytes is entirely due to variation in the C-terminal cytoplasmic regions. This strongly argues that the different populations of erythrocyte B-F molecules are the products of either a single gene or virtually identical (i. e., recently duplicated) genes, that differ by proteolysis, biosynthetic modification, or alternative splicing in the C-terminal cytoplasmic regions.

Mammalian class I molecules consist of a transmembrane glycoprotein α chain of approximately M_r 44 000 noncovalently associated with an approximately M_r 12 000 protein called β_2 -microglobulin (β_2 m). The β_2 m and a large N-terminal portion of the α chain, including the glycans, are extracellular (Klein 1986). A short hydrophobic transmembrane region leads to the short hydrophilic cytoplasmic region, which is phosphorylated on serines and tyrosines (Guild and Strominger 1984a, b). Proteolysis of native detergent solubilized class I molecules cleaves only the C-terminal cytoplasmic and transmembrane α chain regions, releasing a water soluble complex of β_2 m and the N-terminal extracellular region of the α chain. Trypsin cleaves the cytoplasmic region, while papain releases first the cytoplasmic region and then the transmembrane region (Springer and Strominger 1976; Engelhard et al. 1978).

The biochemical similarity of erythrocyte B-F molecules with mammalian class I molecules is based on sequence homology (Skjødt et al. 1986; K. Welinder et al. 1991; J. Kaufman, R. Andersen, J. Salomonsen, J. Engberg, K. Welinder, and K. Skjødt, submitted) and the following biochemical evidence presented in this paper. The mAb F21-2 to the B-F α chain copurified β_2 m. Treatment of the α chain with glycosidases removed two N-linked glycans, one high mannose and one complex containing a single sialic acid. Treatment of the native B-F molecules with trypsin led to a slightly smaller limit product, while treatment with papain generated first a slightly

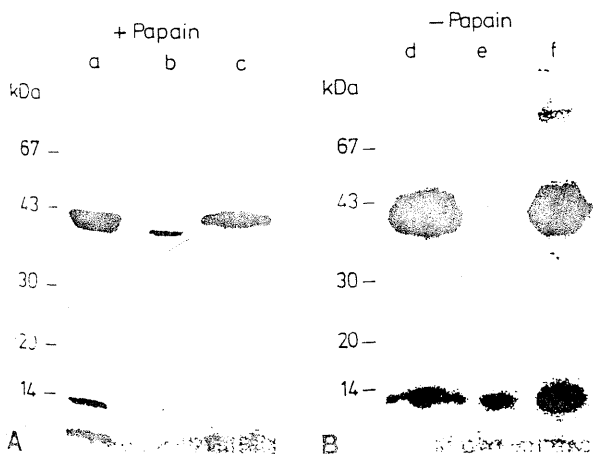


Fig. 7A, B. Analysis of the hydrophobicity of the papain derived B-F fragments. Untreated (-) or a degradation mixture (+), containing the M_r 34 000 and M_r 36 000 fragments obtained after papain treatment of ¹²⁵I-labeled, immunoadsorbent purified B-F, was subjected to temperature-dependent detergent phase separation in a Triton X-114 solution. The two resulting phases, i. e., the aqueous phase and detergent phase, respectively, were analyzed in parallel. a, d: Samples before phase separation; b, e: aqueous phase; c, f: Detergent phase.

smaller product followed by another even smaller limit product. In Triton X-114 experiments, the native B-F molecule and the first large papain fragment partitioned into the detergent phase, while the second large papain fragment partitioned into the aqueous phase. These properties are all in common with mammalian class I molecules.

The various erythrocyte B-F variants have apparently identical N-terminal extracellular regions based on the following evidence. The three B-F15 variants treated with trypsin without denaturation all yielded the same limit products of M_r 36 000 as assessed by two-dimensional gels. Papain proteolysis without denaturation first generated products of M_r 36 000 followed by the same limit products of M_r 34 000 for each of the variants. Such papain and trypsin proteolysis under native conditions also reduced the different complex partial proteolysis fingerprints of the variants to the same simple pattern. The N-terminal sequences of the different variants were identical within the limits of resolution, which means that the molecular variation must occur at the C-terminus. These results also indicate that either a single gene encodes these variants, or that the genes that encode them are virtually identical in the N-terminal region.

The results above also imply that carbohydrate plays no role in the B-F heterogeneity, and this point was demonstrated directly. Treatment of the three B-F variants with glycosidases that remove N-linked glycans led to three smaller molecules, meaning that these N-linked glycans do not contribute to the size heterogeneity. The same size changes were seen with trifluoromethanesulfonic acid treatment (Salomonsen et al. 1987), meaning that these molecules do not contain sizable O-linked glycans. Peptide: N-glycosidase F treatment did not change the IEF pattern; neuraminidase treatment shifted but did not reduce the complexity of the IEF pattern; neuraminidase treatment followed by peptide: N-glycosidase F treatment did not change the original IEF pattern. This means that each and every B-F molecule bears two sialic acids, and that carbohydrate does not contribute to the charge heterogeneity either.

Since the N-terminal portions of the B-F variants are identical, then the differences in size and charge must be located in the C-terminal cytoplasmic regions. There are four clear possibilities for such differences. First, we cannot rule out that proteolysis generates the variants. However, we included many protease inhibitors during purification. Moreover, the same variants were observed whether the analysis was performed directly by western blotting, after immunoprecipitation, or after extensive purification. In addition, there were no clear differences in the B-F molecules immunoprecipitated from metabolically-inactive erythrocytes and metabolically-active leukocytes, meaning that there is not an age-dependent degradation at the C-terminus within the cell.

Second, biosynthetic modification like phosphorylation might contribute to the heterogeneity. Several amino acids located in the cytoplasmic region are phosphorylated in mammalian class I molecules (Poher et al. 1978; Loube et al. 1983; Guild and Strominger 1984a, b), and these amino acids are the only ones in the cytoplasmic region that are invariant in mammalian class I and B-F19 sequences (J. Kaufman, R. Andersen, J. Salomonsen, J. Engberg, K. Welinder, and K. Skjødt, submitted). Different phosphorylation might explain the three charge variants of identical size that still remain after papain and trypsin proteolysis (though this could also be due to proteolysis at adjacent charged amino acids or to chemical modification like deamidation and carbamylation).

The third possibility, alternative splicing at the RNA level, is the likeliest mechanism for size and charge variation in the cytoplasmic region. Comparison of the cDNA clones for B-F12 (Guillemot et al. 1989) and B-F19 (J. Kaufman, R. Andersen, J. Salomonsen, J. Engberg, K. Welinder, and K. Skjødt, submitted) shows the alternative use of an eleven amino acid stretch which includes potential serine phosphorylation sites. This stretch has subsequently been identified as exon 7 in the B-F12 gene (Kroemer et al. 1990). This differential exon usage is reflected in the B-F15 variants, as assessed with antibodies to the peptides encoded by exon 6 and 7 of B-F19 (J. Kaufman, R. Andersen, J. Salomonsen, J. Engberg, K. Welinder, and K. Skjødt, submitted). A similar situation with alternative splicing has been observed for the mouse class I genes where transcripts containing alternatively spliced exons in the H-2K C-terminal encoding region have been cloned and sequenced (Rogers et al. 1986; Lew et al. 1986).

The fourth possibility is that the variants are actually encoded by several genes that have virtually identical extracellular and transmembrane regions, but different cytoplasmic regions. It is in fact not yet clear how many class I-like genes encode cell surface proteins in the chicken. Six strongly hybridizing class I genes were identified in cosmid clusters representing part of the B-F/B-L region, but only one was shown to be transcribed (Guillemot et al. 1988). However, the 8.4 gene also hybridizes strongly with the B-F19 cDNA clone (J. Kaufman, unpublished observations) and other weak hybridizing bands are also observed in Southern blots (Kaufman et al. 1989; Chausse et al. 1989). The B-F12 cDNA clone hybridized with two RNA species in liver cells and only one of the two in lymphocyte cell lines, but the relationship of these transcripts is not clear.

Recently, a B cell specific class I-like molecule identified with the mAb CB3 was described. It is probably encoded by another class I-like gene, but it is clearly not one of the B-F variants described in this paper since it is not recognized by the mAb F21-2 to B-F molecules. It is recognized poorly by the mAb F21-21 to β_2m , and

it has much more carbohydrate than the bursal B-F molecules identified with F21-2 (Pickel et al. 1990). From the IEF experiments presented here, it is clear that the three B-F variants are virtually the only molecules on chicken erythrocytes recognized by the mAb F21-21 to β_2m and are the major molecules on chicken spleen cells recognized by the mAb F21-2.

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