β2-Microglobulin Deficient Mice Catabolize IgG More Rapidly Than FcRn-α-Chain Deficient Mice

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FcRn, a nonclassical MHC-I protein bound to β2-microglobulin (β2m), diverts IgG and albumin from an intracellular degradative fate, prolonging the half-lives of both. While knockout mouse strains lacking either FcRn-α-chain (AK) or β2m (BK) show much shorter half-lives of IgG and albumin than normal mice, the plasma IgG half-life in the BK and AK strains is different, being shorter in the BK strain. Since β2m does not affect the IgG production rate, we tested whether an additional β2m-associated mechanism protects IgG from catabolism. First, we compared the fractional disappearance rate in plasma of an intravenous dose of radioiodinated IgG in a mouse strain deficient in both FcRn-α-chain and β2m (ABK), in the two parental knockout strains (AK and BK), and in the background wild-type (WT) strain. We found that IgG survived longer in the β2m-expressing AK strain than in the β2m-lacking ABK and BK strains, whereas the IgG half-lives between the ABK and BK strains were identical. Then we compared endogenous concentrations of four typical plasma proteins among the four strains and found that steady-state plasma concentrations of both IgG and albumin were higher in the AK strain than in either the BK or the ABK strain. These results suggest that a β2m-associated effect other than FcRn prolongs the survival of both IgG and albumin, although leaky gene transcription in the AK strain cannot be ruled out. Exp Biol Med 233:603–609, 2008

Key words: steady-state; albumin; half-life; knockout; Fc receptor

Introduction

FcRn[2] is a heterodimer consisting of a nonclassical MHC-I-related glycoprotein (FcRn-α-chain; gene *fcgrt*) noncovalently bound to β2-microglobulin (β2m). The heterodimer binds IgG and albumin and rescues both from degradation by an intracellular recycling mechanism that operates throughout the life time of the individual (1–6). Because FcRn binding of IgG is saturable, humans and mice show a direct concentration-catabolism relationship; i.e., the fractional catabolic rate of IgG is lower at lower endogenous IgG concentrations, whereas IgG is more rapidly catabolized at higher IgG concentrations (3, 7, 8). In contrast, in FcRn-lacking mice the fractional catabolic rate of IgG is independent of endogenous IgG concentration (4). As well, it is known that both FcRn-lacking humans and mice, despite their hypoalbuminemia, also catabolize albumin, the other ligand for FcRn, at a greater fractional rate compared with the normal (1, 9).

Because both FcRn subunits are required for efficient expression of a functional FcRn (10, 11), mouse strains deficient in either FcRn-α-chain or β2m manifest faster than normal plasma decay of IgG and albumin and lower than normal serum concentrations of both ligands (1, 2, 4). Curiously, the half-lives of IgG in the two strains appear to differ when measured after an intraperitoneal injection of labeled IgG, being somewhat shorter in the β2m-deficient (BK) mouse strain (12). Although prolonged peritoneal absorption of IgG might confound the interpretation of FcRn effects on IgG half-life, we also observed lower plasma concentrations of both FcRn ligands, IgG and albumin, after intravenous infusion in the BK strain compared with the AK strain (1). Since the IgG production

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[2] The term FcRn is ambiguous. It was originally coined to mean the protein heterodimeric receptor (α+β) capable of binding IgG (13), but some have applied the term to the α-chain alone.

rate is the same regardless of β2m expression (14), a difference in the steady-state plasma concentration of IgG between the two strains (β2m deficient and FcRn-α-chain deficient) is likely caused by an altered IgG catabolic rate; thus, one must consider effects of β2m on IgG degradation or recycling other than those manifested by FcRn.

Therefore, in the present study we sought an FcRn-independent effect of β2m on IgG catabolism. To elucidate a β2m-associated effect, we first generated a mouse strain deficient in both the FcRn-α-chain and β2m (ABK). Then we systematically compared IgG half-lives among the four mouse strains (AK, BK, ABK, and WT) after a single intravenous bolus injection of labeled IgG. We found that IgG survived longer in the β2m-expressing AK strain than in the two β2m-lacking ABK and BK strains, whereas the IgG half-lives between the ABK and BK strains were identical. Finally we compared the steady-state plasma concentrations of the two FcRn ligands, IgG and albumin, in the plasma of the four strains and found that endogenous plasma concentrations of both IgG and albumin were higher in the AK strain than in either the BK or the ABK strain. These results suggest that a β2m-associated effect other than FcRn prolongs the survival of both IgG and albumin.

Materials and Methods

Animals. We used four strains of mice including three knockout strains and the relevant wild-type control strain (WT; FcRn-α-chain$^{+/+}$ β2m$^{+/+}$; C57BL/6J). The three knockout strains were FcRn-α-chain knockout (AK; FcRn-α-chain$^{-/-}$ β2m$^{+/+}$; B6.129X1/SvJ*Fcgrt*^{Tm1Dcr}; (12)), β2-microglobulin (β2m) knockout (BK; FcRn-α-chain$^{+/+}$ β2m$^{-/-}$; B6.129P2-*B2m*^{tm1Unc}; Ref. 15), and the double-knockout of both FcRn-α-chain and β2m (ABK; FcRn-α-chain$^{-/-}$ β2m$^{-/-}$; breeding details below). Breeders of the BK strain and their corresponding WT control strain were obtained from Jackson Laboratories while AK strain breeders were kindly provided by Dr. Roopenian of The Jackson Laboratory (Bar Harbor, ME). Previously the breeders of two knockout strains (AK and BK) had been backcrossed to the background strain (WT) >11 times. Mice used in these experiments were all male and 8-wk old. The Ohio State University Institutional Laboratory Animal Care and Use Committee approved all animal studies.

Breeding. In order to produce the four homozygous stains (WT, AK, BK, and ABK) from the same origin, we first established double-heterozygous breeders (FcRn-α-chain$^{+/-}$ β2m$^{+/-}$) by mating AK × BK strains. Then, male and female mice with the FcRn-α-chain$^{+/-}$ β2m$^{+/-}$ genotype were mated to produce the mouse strains with 16 different genotypes. After confirming their genotypes (details below), the four pure homozygous strains (FcRn-α-chain$^{+/+}$ β2m$^{+/+}$, FcRn-α-chain$^{-/-}$ β2m$^{+/+}$, FcRn-α-chain$^{+/+}$ β2m$^{-/-}$, and FcRn-α-chain$^{-/-}$ β2m$^{-/-}$) were obtained. These strains were used for further intra-strain breeding to produce the experimental mice of four different strains (WT, AK, BK, and ABK).

Genotyping. The DNA was isolated from 0.5 cm tail tips using DNAeasy Tissue kit (Qiagen, Valencia, CA). Each reaction contained 100 ng DNA in a 50-μL PCR reaction containing 200 μM dNTPs (Invitrogen, Carlsbad, CA), 1× PCR buffer with 1.5 mM $MgCl_2$ (Qiagen), and 1 unit of Taq (Qiagen). For FcRn-α-chain genotyping, the oligo pair, o393F-GGGATGCCACTGCCCTG and o394R-CGAGCCTGAGATTGTCGAGTG, gave a 248-bp WT allele; whereas the targeting vector oligo, o395F-GGAATTCCCAGTGAAGGGC, *vs* o394R gave a 378-bp targeted allele. The thermocycler was run at 95°C for 10.00 min; followed by 40 cycles of 94°C for 45 sec, 60°C for 1 min, 72°C for 1 min; and ended with 72°C for 5 min.

For β2m genotyping, primer pair IMR0185-CTGAGCTCTGTTTTCGTCTG *vs* IMR0184-TAT-CAGTCTCAGTGGGGGTG gave a 280-bp WT allele; the oligo IMR0160-TCTGGACGAAGAGCATCAGGG *vs* IMR0184 gave a 400-bp targeted allele. Thermocycler conditions were 94°C for 3.00 min; 12 cycles of 94°C for 1 min, 64°C for 45 sec, 72°C for 45 sec; followed by 25 additional cycles of 94°C for 30 sec, 58°C for 45 sec, 72°C for 45 sec with a final extension cycle at 72°C for 2 min.

Proteins and Radioiodination. Mouse IgG1 myeloma protein MOPC21 (catalogue # 64335) and mouse IgA myeloma protein TEPC15 (catalogue # 50326) were obtained from ICN (ICN Pharmaceuticals, Aurora, OH). These proteins were dialyzed against pH 7.2 buffer containing 50 mM NaCl and 100 mM phosphate, and were radioiodinated by the chloroglycouril (CGU) method (16) using 5–10 μg CGU (Iodogen, Pierce, St. Louis, MO) per 100 μL protein solution (1). IgG was labeled with ^{125}I and IgA with ^{131}I at molar substitution ratios of 0.2 atoms of ^{125}I or ^{131}I per molecule of protein.

Catabolism of IgG. Mice, male and 8-wk old, were injected *via* tail veins with 100 μL of a mixture of ^{125}I-labeled IgG and ^{131}I-labeled IgA diluted in phosphate buffered saline containing 10% normal mouse serum (ICN, Aurora, OH). Within 2 min of injection (time zero) and daily through 120 h, 30 μL blood was sampled *via* heparinized tubes from the retro-orbital plexus (1). Plasma was harvested; the proteins in 10 μL plasma were precipitated in 12.5% TCA; radiolabeled proteins in the pelleted precipitates were counted in a γ-counter and were always >95% of the total radioactivity. The plasma radioactivity, normalized for dose, was plotted on a log scale *vs* time, and the half-lives were calculated from the terminal- or elimination-phase decay using the formula $t_{1/2} = \ln(2)/\beta$ where β was the slope of a line fitted by least squares regression analysis (WinNonlin, Pharsight®, ver. 4.0, Mountain View, CA) to a plot of the log concentration-time values that appeared to fall along a straight line (17). Day 2 after injection was chosen to be the start of the terminal phase for both IgG and IgA since it has shown to provide better linearity than day 1 (1). The harmonic mean

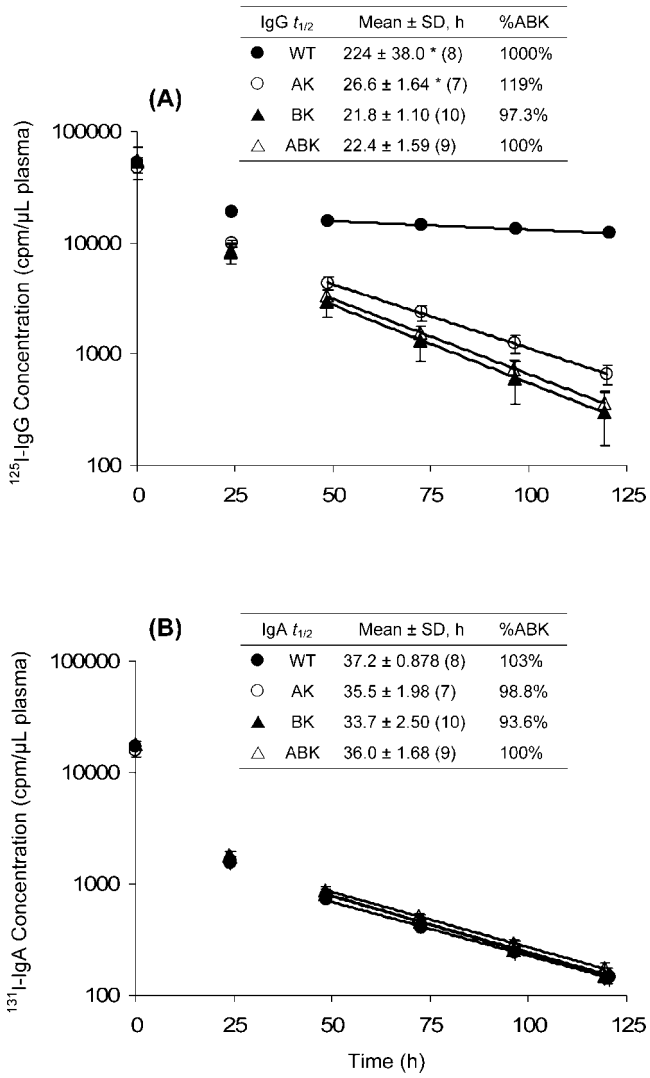


Figure 1. Plasma radioactivity-time profiles of IgG and IgA in the four mouse strains. The plasma radioactivities of ^{125}I-IgG (Panel A) and ^{131}I-IgA (Panel B) remaining at indicated times were determined by TCA precipitation after intravenous injection of WT (filled circles), AK (open circles), BK (filled triangles), and ABK (open triangles) strains. Mean half-lives ($t_{1/2}$) of terminal-phase decay (beginning from Day 2) in hours are shown with one SD (error bar) and the number of mice (N). The %ABK represents the percent ratio of the half-life in each mouse strain to the half-life in the ABK strain. * $P <$ 0.05 compared with the ABK strain.

$t_{1/2}$ and its standard deviation (SD) were calculated using the jackknife technique as described by others (18).

Steady-State Plasma Protein Concentrations. A set of mice (8-wk and male) separate from that used for the decay studies was used for the measurement of endogenous concentrations of plasma proteins. Mice were exsanguinated *via* the inferior vena cava using heparin-coated syringes, and plasma was harvested. Steady-state plasma concentrations of endogenous mouse IgG, IgA, albumin, and transferrin were measured using the sandwich ELISA (Bethyl Laboratory, Montgomery, TX) (1, 2, 17). Albumin and transferrin were chosen as the additional ligand for FcRn and control (not a ligand) protein, respectively (2).

Effects of β2m. To compare the extent of the FcRn-mediated effect on prolonging IgG half-life with the unidentified β2m-associated effect beyond the FcRn effect, first a ratio of IgG half-life in the WT strain to that in the AK strain (i.e., WT/AK) was calculated. This is the recycling effect of IgG by FcRn. Second, a half-life ratio between the AK and ABK strains (i.e., AK/ABK) was calculated, which represents the recycling effect by β2m other than FcRn. Then a relative effect of β2m to FcRn on prolongation of IgG survival was computed by dividing β2m-effect (AK/ABK) by FcRn-effect (WT/AK).

Statistical Analyses. A two-way ANOVA and the Tukey's multiple comparison tests were performed with the aid of SPSS (SPSS Inc., ver. 14.0, Chicago, IL). Differences among strains were considered significant at $P <$ 0.05. The main purpose of the current study was to ascertain whether IgG decay rate in the ABK strain resembled that in the AK or the BK strain. Therefore, we compared the percent ratio of the value (either half-life or plasma steady-state concentration) in each mouse strain to the value in the ABK strain (%ABK). Data were presented as means ± SD.

Results

To assess the fractional rate of plasma IgG disappearance in the presence and absence of FcRn-α-chain and β2m proteins, we injected radioiodinated IgG intravenously into mice of four strains: AK (deficient in FcRn-α-chain), BK (deficient in β2m), ABK (deficient in both FcRn-α-chain and β2m), and WT (wild-type strain); and we measured its plasma decay over the course of 5 d. IgG terminal-phase decay assessed on a semilogarithmic scale was linear (first-order decay) beginning at day 2 after injection in all four strains. IgG disappeared more slowly in the AK strain compared with the BK strain: IgG half-life in the AK strain (26.6 h) was significantly longer by 22% compared with BK strain (21.8 h; $P < 0.001$), as was also observed in another study (12). Furthermore, in the AK strain IgG also survived significantly longer by 19% compared with the ABK strain (22.4 h; $P < 0.001$). However, the IgG half-lives in the ABK and BK strains were not different ($P = 0.685$; Figure 1A and inset).

The WT strain showed a much longer IgG half-life (224 h) than the three KO strains; specifically, the half-life of the WT strain was 7.4-, 9-, and 9-fold greater than the AK, BK, and ABK strains, respectively (Fig. 1A), suggesting a large capacity of FcRn-mediated rescue of IgG from a catabolic fate. To determine the relative magnitude of IgG recycling by unidentified β2m-associated effects to FcRn-mediated effects, we compared the half-life ratio between AK and ABK with that between WT and AK. The unidentified β2m-

associated effect accounted for 14.1% of the extent of FcRn-mediated IgG recycling (defined in Materials and Methods).

The fractional rates of IgA decay in the three KO strains were not different from one another (Figure 1B). However, the decay of IgA was somewhat faster (10%; $P = 0.044$) in the BK strain compared with the WT strain (Figure 1B insert) but not when compared with the AK or ABK strain.

We also measured by ELISA the plasma steady-state concentrations of endogenous IgG in the same four strains (Figure 2A), and we found that the AK strain maintained a higher IgG concentration by 84% ($P < 0.05$) and 141% ($P < 0.01$) than the BK and ABK strains, respectively, whereas the BK and ABK strains did not differ in IgG concentration ($P = 0.308$). The plasma IgG was much higher in the WT strain compared with the three KO strains; namely, the IgG concentration in the WT strain was 4.7, 9, and 13 times higher than in the AK, BK, and ABK strains, respectively. Plasma IgA concentrations, in contrast, were not different among the four mouse strains (Fig. 2B). As anticipated, plasma albumin concentrations (Fig. 2C) showed a similar pattern among the strains to what was seen with the IgG concentrations: The AK strain maintained a moderately but statistically significantly higher concentration of albumin (17.3 mg/mL) compared with both BK and ABK strains ($P < 0.001$, both strains) and there was no difference in physiological albumin concentrations between the two β2m-deficient mouse strains (15.4 vs. 15.5 mg/mL; $P = 0.998$). The WT strain showed the highest albumin concentration (34.1 mg/mL). The plasma concentration of transferrin, which is not an FcRn ligand (2), was significantly higher in the AK strain compared with WT, BK, and ABK strains (Fig. 2D). The higher transferrin concentration in the AK strain than in the WT and BK strains, resulting from hypoalbuminemia-induced transferrin upregulation, was consistently seen in our previous studies (2, 17).

Discussion

The results of this protein turnover study on IgG survival using four different mouse strains on the same genetic background supported the possibility that other undefined β2m-associated effects extend IgG half-life. Based on the mass balance relationship considering the catabolic rate and steady-state concentrations of IgG, it has been shown that the estimated IgG production rates are unchanged regardless of the presence of β2m (14), from which fact we presume that the IgG production rates are not different among the four strains of our study. Therefore, because the steady-state IgG concentration in plasma is maintained by a balance of production with catabolism, the higher IgG concentration in the AK strain than in the BK or ABK strains must result from a β2m-mediated decrease in catabolism. We found no difference in half-life and steady-state concentrations of IgA among the three knockout strains, affirming the ligand specificity of the β2m-associated but FcRn-independent effect.

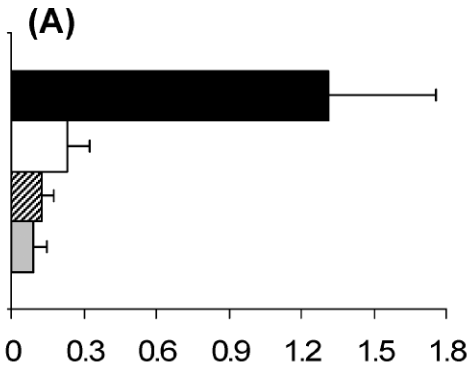
Likely, these conclusions about IgG can be extended to albumin, although limited numbers of animals prevented our measuring albumin decay directly. We previously showed that FcRn binds and protects albumin from degradation as it does IgG (1, 3, 9, 19), and we quantified the FcRn-mediated recycling effects of both ligands in mice (2) and humans (20). Further, the AK strain shows a longer albumin half-life (1) and higher albumin plasma concentration (present study) compared with the BK strain, suggesting that the BK strain catabolizes albumin more rapidly than the AK strain given that the albumin production rates are the same between the two strains (discussed in detail below).

Unlike IgG, the AK strain produces both albumin (FcRn-ligand) and transferrin (FcRn-non-ligand) more rapidly than the WT strain as a result of compensatory upregulation in response to hypoalbuminemia induced by an increase in albumin catabolism (2, 17). By the same mechanism it is anticipated, although not measured, that the albumin production rate in the hypoalbuminemic BK and ABK strains would also be enhanced compared with normal mice. Furthermore, since the two β2m-deficient strains have lower albumin concentrations than the AK strain, the compensatory upregulation of albumin production in both BK and ABK strains could be slightly greater than, or at least equal to, that in the AK strain. Taken together, our results demonstrate that the β2m-associated FcRn-independent recycling extends the half-lives of both IgG and albumin.

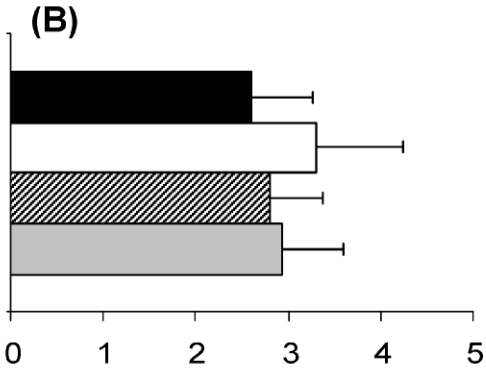
The IgA half-lives in the present study, 33–37 h, are much longer than those reported previously (23–25 h; Ref. 1). Different beginning points of the terminal phase decay (day 2 for the present study and day 1 for the previous study; Ref. 1) could contribute to this difference. In a more recent study we reported IgA half-lives (29–36 h) similar to the present study; these were determined from the terminal phase decay beginning at day 2 in mice of the same ages as the present study (17). We also noted a modest difference in IgA half-life between the WT and BK strains ($P = 0.044$). Since this difference was of borderline statistical significance and was not seen in the ABK and BK strains of the previous studies (1, 17), we are unable to endow the observation with any biological meaning.

Higher transferrin plasma concentration in the AK strain compared with the WT and BK strains was consistently seen in the previous studies (2, 17); as described above, the difference results from a compensatory upregulation of the production rate of transferrin as well as albumin, induced by hypoalbuminemia. In contrast, the hypoalbuminemic BK strain (1, 17) catabolizes transferrin more rapidly than the WT strain as a result of a direct effect of iron overload (17), which is induced by deficiency in HFE, another nonclassical MHC-I molecule associated with β2m. Therefore, these two β2m-deficient strains appear to maintain transferrin concentrations identical to the WT strain due to two canceling effects of the same magnitude: i.e., increases in both the production (through compensatory

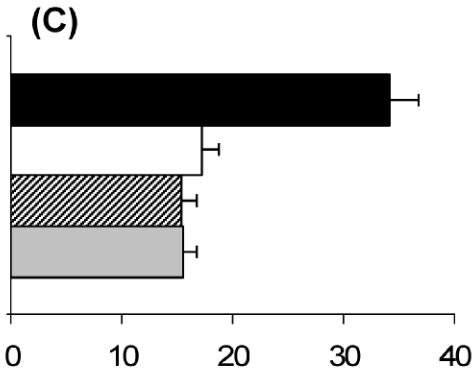
IgG	Mean ± SD, mg/mL	%ABK
WT	1.31 ± 0.447 * (14)	1380%
AK	0.230 ± 0.0982 * (11)	241%
BK	0.125 ± 0.0540 (20)	131%
ABK	0.0953 ± 0.0509 (28)	100%



IgA	Mean ± SD, μg/mL	%ABK
WT	2.62 ± 0.659 (14)	89.1%
AK	3.30 ± 0.946 (11)	112%
BK	2.81 ± 0.567 (20)	95.8%
ABK	2.93 ± 0.659 (28)	100%



Albumin	Mean ± SD, mg/mL	%ABK
WT	34.1 ± 2.64 * (14)	220%
AK	17.3 ± 1.36 * (11)	112%
BK	15.4 ± 1.41 (20)	100%
ABK	15.5 ± 1.22 (28)	100%



Transferrin	Mean ± SD, mg/mL	%ABK
WT	7.36 ± 0.443 (14)	97.6%
AK	8.95 ± 0.783 * (11)	119%
BK	7.89 ± 0.670 (20)	105%
ABK	7.54 ± 0.479 (28)	100%

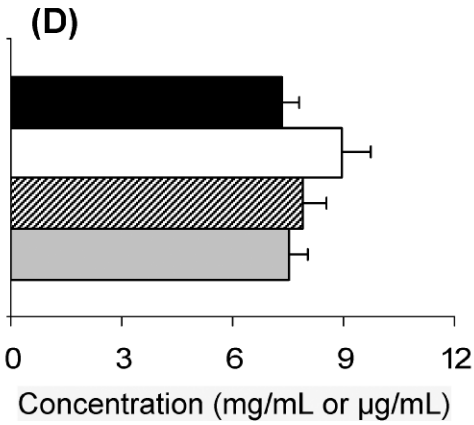


Figure 2. Plasma concentrations of endogenous proteins in the four mouse strains. Steady-state plasma concentrations of IgG (Panel A), IgA (Panel B), albumin (Panel C), and transferrin (Panel D) were determined by ELISA using the plasma of WT (solid bars), AK (empty bars), BK (hatched bars), and ABK (gray bars) strains. Mean concentrations in mg/mL (IgG, albumin, and transferrin) or μg/mL (IgA) are shown with one SD (error bar) and the number of mice (N). The %ABK represents the percent ratio of the concentration in each mouse strain to the concentration in the ABK strain. * $P < 0.05$ compared with the ABK strain.

upregulation) and catabolism (by iron overload) of transferrin.

Our breeding program for these studies has uncovered a β2m-associated sex-linked deficiency in fecundity. Double heterozygous individuals were produced from two types of trios (two females and one male) of which either the females were AK and the males were BK or the females were BK and the males were AK. While all offspring were genetically identical heterozygotes for both genes, the trios with AK females showed a greater likelihood of weaning pups (13 of 18 *vs* 3 of 17 trios), weaned more pups per trio (15.1 *vs* 10.5 pups/trio; $P = 0.046$), and weaned a greater percentage of pups (60.5% *vs* 23.6%; $P = 0.039$). While the production of double heterozygous individuals appeared skewed towards females (60 females to 46 males), the sex ratios of individual litters did not deviate from the 50% expectation (54.4%). We conclude that BK females mated with AK males appear to be less fecund than AK females mated with BK males. Most likely, β2m is required for normal fecundity, as others have recently suggested (21).

What FcRn-independent β2m-associated mechanism can be proposed for extending the half-lives of both FcRn ligands? The β2m-associated molecules other than FcRn, including CD1, HFE, MHC-I, are not known to protect IgG and albumin from degradation although their functions are not fully understood (22, 23). Other mechanisms are possible: First, there could be another β2m-associated molecule that is responsible for 'direct' recycling of both IgG and albumin, in the fashion of FcRn. This direct recycling process includes any recirculation of IgG from an 'irreversible' loss of IgG such as excretion or secretion through the body. While the kidney is one of the major sites for processing many plasma proteins (24), there is no direct evidence to date that FcRn-unrelated β2m-associated molecules recycle IgG or albumin in the kidney. The second possibility is that the FcRn-independent β2m-associated effect is 'indirect' or secondary to other important β2m functions. An example was seen with HFE, a regulatory protein in iron homeostasis: As discussed above, mice deficient in HFE catabolize transferrin more rapidly by 14% than normal mice, a consequence of higher serum iron levels induced by the uncontrolled iron uptake in the absence of HFE rather than the absence of a direct HFE-mediated recycling of transferrin (17).

We cannot exclude the possibility that the AK strain is incompletely deficient in FcRn-α-chain because of leakiness of the FcRn-α-chain gene transcription mechanism (12). While our finding that both FcRn ligands (IgG and albumin) were saved from degradation by the β2m-associated effect would appear compatible with this mechanism, direct measurements of FcRn expression in the neonatal gut of the AK strain showed the level to be nil (1). Since the site of tissue expression of FcRn that is functionally recycling IgG is not known, a definitive measurement of leakiness is currently impossible.

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