1-3-2005

Secretion of Recombinant Human fibrinogen by the Murine Mammary Gland

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Abstract

Transgenic animals secreting individual chains and assembled fibrinogen were produced to evaluate the capacity of the mammary gland for maximizing assembly, glycosylation and secretion of recombinant human fibrinogen (rhfib). Transgenes were constructed from the 4.1 kbp murine Whey Acidic Protein promoter (m**WAP**) and the three **cDNA**s coding for the Aα,Bβ and γ fibrinogen chains. Transgenic mice secreted fully assembled fibrinogen into milk at concentrations between 10 and 200 μg/ml, with total secretion of subunits approaching 700 μg/ml in milk. Partially purified fibrinogen was shown to form a visible and stable clot after treatment with human thrombin and factor **XIII**. The level of assembled fibrinogen was proportional to the lowest amount of subunit produced where both the Bβ and γ chains were rate limiting. Both the Bβ and γ chains were glycosylated when co-expressed and the degree of saccharide maturation was dependent on expression level, with processing preferred for γ chains over chains. Also, the subunit complexes $A_{\alpha\gamma 2,}$ γ_2 and the individual subunits Aα, Bβ and γ were found as secretion products. When the Bβ was secreted individually, the glycosylation profile of the molecule was of a mature complex saccharide indicating recognition of the molecule by the glycosylation pathway without association with other fibrinogen chains. To date secretion of Bβ chain has been not observed in any cell type, suggesting that the secretion pathway in mammary epithelia is less restrictive than that occurring in hepatocytes and other cells previously used to study fibrinogen assembly.

Key words: mammary gland expression, recombinant human fibrinogen, transgenic mouse WAP promoter.

Comment: This paper was originally published in the Journal of "Transgenic Research" Vol. 13 Pages 437-450, 2004.

1.0 Introduction

Fibrinogen $(A\alpha B\beta\gamma)_2$ is a complex plasma glyco-protein that participates in the blood coagulation cascade. It is synthesized in the liver as a dimeric molecule that is comprised of two subunits linked by three disulfide bonds, each containing three polypeptide chains for a combined molecular weight of 340 kDa. The three polypeptides, Aα (66 kDa), Bβ (54 kDa), and γ (48 kDa) are linked by disulfide rings to form a coiled spiral of α-helices (Doolittle, 1984). N-linked polysaccharides are present on the Bβ and γ chains of both subunits and glycosylations of these chains are required for fibrin polymerization by thrombin. Applications for fibrin sealant have been developed including use as a hemostatic agent and as a carrier matrix for the delivery of drugs and biologics (Greco et al., 1991; Alving et al., 1995; MacPhee et al., 1996a, b; Lasa et al., 1996). Thus, there is considerable economic incentive to develop recombinant versions of human fibrinogen (rhfib), which would be specific-pathogen-free and cost effectively abundant (Velander et al., 1996).

The cDNA sequences coding for each fibrinogen chain have been cloned (Rixon et al., 1983; Chung et al., 1983a, b), and the synthesis and secretion of fibrinogen have been extensively studied in cultured hepatocytes and a variety of transfected cell types (Yu et al., 1984; Danishefsky et al., 1990, Roy et al., 1990, 1991; Hartwig & Danishefsky, 1991; Huang et al., 1993, 1996: Zhang & Redman, 1996). Pulse chase experiments in hepatocytes have determined that the rates of new chain synthesis for each peptide are identical (Roy et al., 1994), and newly synthesized Bβ-chain associates rapidly with already present intracellular pools of Aα and γ chains, suggesting β-chain incorporation in chain assembly is the rate limiting step in fibrinogen production (Yu et al., 1984). Regulation of the intracellular pools of free chains appears to be modulated by retrotranslocation, where association of the free chains with translocon component Sec61b leads to their eventual degradation in the proteasome (Hui & Redman, 2001). Secretion into cell culture media is not limited to fully assembled fibrinogen, but includes free chains of Aα and γ , Aα – γ complex and Aα Bβ γ half molecules (Hartwig & Danishefsky, 1991; Roy et al., 1991). In contrast, secretion of significant amounts of Bβ -chain has not been observed by any cell type and is thought to be retained and degraded when the other fibrinogen chains are not present (Danishefsky et al., 1990).

Currently, fully assembled fibrinogen has been secreted into the milk of both mice and sheep carrying the genomic fibrinogen coding sequences linked to the ovine β lactoglobulin (oBLG) milk protein promoter (Prunkard et al., 1996; Cottingham et al., 1997). The concentration of fibrinogen subunits in mouse milk, as determined by reduced Western analysis, varied from 30 to 2000 μg/ml depending upon the transgenic line studied. It was also reported that when all three subunits were expressed in a balanced ratio, 100% of the subunit chains were incorporated into fully assembled fibrinogen. The glycosylation status of the Bβ and γ subunits was not reported, but purified rhfib was shown to undergo thrombin induced polymerization and γ-chain cross-linking by Factor XIII.

The objective of this study was to evaluate the capacity of the mammary gland for secreting each separate fibrinogen subunit and to then characterize the maturity of the glycosylation. We confirm the assembly of functional fibrinogen using the whey acidic protein promoter to direct synthesis of each of the subunit chains and then assess the secretion and glycosylation of individual chains, along with the glycosylation state of both the Bβ and γ subunits of secreted assembled fibrinogen. In the context of the normal physiology of the mammary epithelial cell as it occurs in a transgenic mouse, we show for the first time that the Bβ chain having mature glycosylation can be efficiently secreted by a mammalian cell.

2.0 Materials and methods

2.1 Transgene construction

The 4.1 kbp murine whey acidic protein (mWAP) promoter was obtained by digestion of p227.6 (American Red Cross, Rockville, MD) with *Not***I**, *Kpn***I** and *Hind***III.** The fragment containing the 30 UTR and pUC *Not***I** + vector was obtained by partial digestion of pUCWAP5 (Virginia Polytechnic Institute and State University, Blacksburg, VA) with *Not***I** and a full digestion with Kpn**I**. The resulting fragments were then ligated to make the cassette vector pUCWAP6. After size and restriction site verification (Not**I** and Not**I**/ Kpn**I** digest), large scale purification was performed. The cDNAs for cloning were obtained from Susan Lord (University of North Carolina, Chapel Hill, NC) and end modified by polymerase chain reaction (PCR) to produce KpnI sites directly 5'and 3' of the coding start and stop sequences, respectively. The vector pUCWAP6 was linearized with KpnI, combined with each cDNA and ligated. The following restriction endonuclease enzymes were used for verifying the cDNA orientation: pUCWAP6FibAα1/*Pst***I** yielding expected fragments of 2.1, 2.4 and 5.9 kbp;

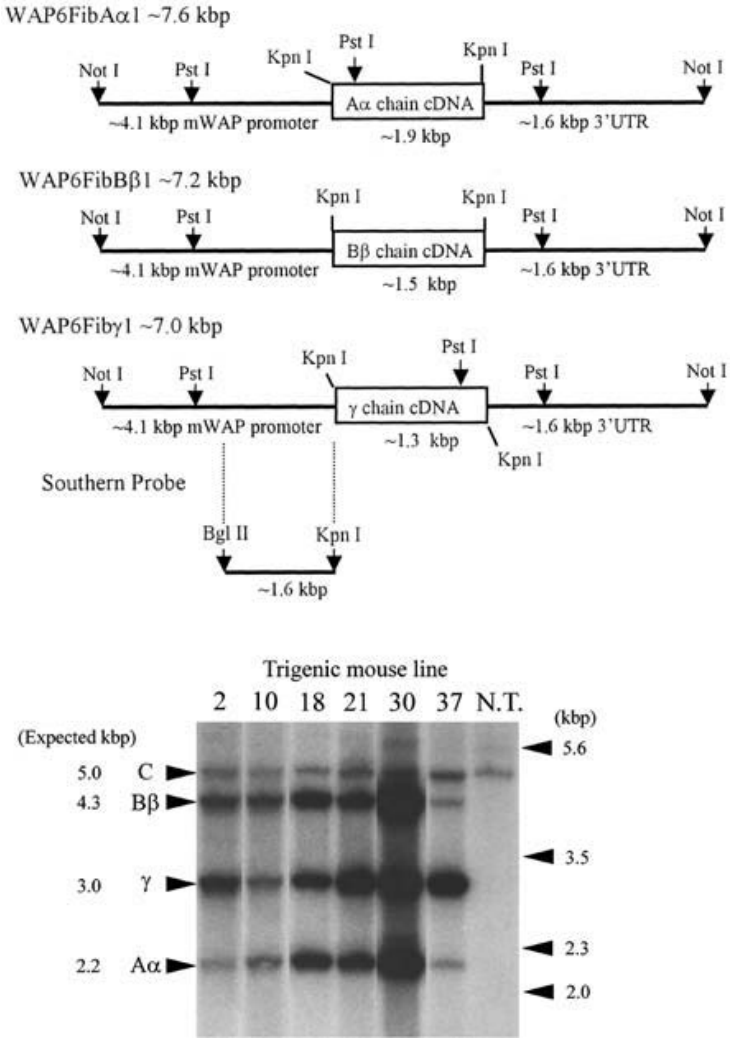

Figure 1. *Transgene design and Southern analysis of established transgenic lines. The three fibrinogen transgenes, WAP6FibAa, WAP6FiBβ and WAP6Fib1 were produced by introducing the cDNA for each chain between the 4.1 kbp promoter and 1.6 kbp30UTR elements of mWAP, upper panel. These constructs, in turn, were used to produce founder animals through microinjection. Offspring from these founders were screened by Southern analysis with one example from each line presented in lower panel. N.T. = non-transgenic,°C= band produced by endogenous WAP. Bands Aa,Bβ and γ are produced by their corresponding transgenic constructs.*

pUCWAP6FiBβ b1/*Bam***HI** yielding fragments of 750 bp and 9.5 kbp; and pUCWAP6Fibc1/*Pst***I** yielding fragments of 980 bp, 3.2 and 5.8 kbp (Figure 1). After verifying the sequences across the promoter/cDNA and cDNA/3' UTR junctions, plasmids were cut with *Not***I**/*Pvu***I** and constructs purified by ultracentrifugation through a NaCl step gradient (7.5, 12.5, 17.5, and 20%) by the method of Fink (1991). Construct DNA was diluted to 5 μg/ml in microinjection buffer (10 mM

Tris–HCl, 0.1 mM EDTA) to produce monogenic lines or mixed in equal molar amounts for co-microinjection at a final concentration of 5 μg/ml.

2.2 Generation of transgenic animals

Generation of transgenic mice followed the general method of Hogan et al. (1986) for embryo collection and Canseco et al. (1994) for embryo microinjection. Experiments on animals in this study were approved by the University Animal Care Committee and followed the National Research Council's guide for the care and use of laboratory animals. Briefly, female CD-1 mice approximately 25–29 days of age were superovulated with intraperitoneal injections of 10 IU Pregnant Equine Serum Gonadotropin followed by 5 IU human Chorionic Gonadotropin (hCG) 48 h later and placed with males. Zygotes from mated mice were collected in M2 medium 21 h after hCG and microinjected with 1–4 pl of DNA solution at 5 μg/ml. Surviving embryos were transferred to oviducts of pseudopregnant females (30–100 embryos per recipient) with the number transferred reflective of survivability studies done in culture for each preparation of DNA.

2.3 Identification of transgenic animals

DNA samples were isolated from murine tail biopsies by the method of Velander et al. (1992) and subjected to PCR analysis by general method of Saiki et al. (1989). PCR positive animals were further screened by Southern analysis. Southern analysis was performed on 10 lgof tail biopsy DNA digested with endonuclease *Pst**I*** and probed with a radiolabelled [a-32P]dATPPstI/KpnI promoter fragment. Digestion of trigenic mouse DNA with endonuclease PstI, which cuts each construct at the same site in the promoter region but varies the 3' cut, produced fragment sizes of 4.3 kbp for WAP6FiBβ b1, 3.0 kbp for WAP6Fibc1 and 2.2 for WAP6FibA1 (Figure 1). The WAP probe hybridized with each fragment identically and also hybridized with a 5.0 kbp fragment produced by the endogenous WAP gene. Relative copy numbers for each construct within a given line were determined by comparing each lane's internal WAP control (2 copies) to the signal intensity produced for each construct. Signal intensity (by volume determination) from autoradiographic film was performed using a densitometer and the corresponding Image Quant software (Molecular Dynamics, Sunnyvale, CA).

2.4 Collection and processing of mouse milk

Milk from transgenic and control mice was directly collected into 1.7 ml centrifuge tubes by vacuum aspiration, with 200 µl of milk removed, added to 200 µl of milk buffer (50 mM Tris and 200 mM EDTA, pH 8.5) and frozen at -90 °C. Milk samples were defatted by centrifugation at 15,000 x g in a bench top centrifuge for 20 min at 4°C. After skimming, the resulting clarified milk (prepared whey) was stored at- 90 °C until analysis.

2.5 Western analysis

Gel electrophoresis was carried out on polyacrylamide gradient gels from Novex (San Diego, CA), 8–16% for reducing conditions and 4–12% for non-reducing conditions using Tris–glycine buffer (**25 mM Tris, 0.1% SDS and 200 mM** glycine). Unless otherwise noted, 1 µl of prepared whey was loaded per sample and electrophoresis was conducted until the 7.0 kDa marker reached the end of the gel or ran off. Gels were transferred to PVDF membranes (BioRad) in Towbin buffer (10% methanol, 200 mM glycine, 25 mM Tris, 0.1% SDS) at 4°C, at 200 mA overnight using a BioRad Trans-blot cell. Membranes were blocked using TBSTC (20 mM Tris, 50 mM NaCl, 0.05% Tween 20, 0.5% bovine casein, pH 7.2) for 3 h to overnight with gentle agitation. Blots were probed with a primary antibody for 1–3 h at 37 °C with gentle agitation. Primary antibodies and the concentrations used were: polyclonal raBβ it anti-Aα-chain (AB784), anti-Bβ -chain (AB785), anti-γ-chain (AB786) from Chemicon International Inc. (Temecula, CA) and used at 1:5000; ADI (1:1000), raBβ it polyclonal for all three fibrinogen chains (American Diagnostic Inc.); Y18 mAb (1:1000), mouse monoclonal for Aα-chain; Shainoff mAb(1:300), mouse monoclonal for Bβ chain; and NS15 pAb (1:150), raBβ it polyclonal for γ-chain . The Y18 mAb, Shainoff mAb and NS15 pAb were kindly provided by Susan Lord (University of North Carolina; Chapel Hill, NC). After incubation, the blots were rinsed in deionized water for 5 min. Horseradish peroxidase conjugated antibodies used include raBβ it anti-hfib (Dako A/S, Denmark), goat anti-raBβ it IgG and goat anti-mouse IgG (Sigma A9169, and A4416), respectively. After incubation the blots were rinsed three times in deionized water and developed according to manufacturer's instructions for 1–10 min using a metal enhanced Diaminobenzidine kit (Pierce, Rockford, IL). Deglycosylation experiments were performed using Endoglycosidase-H or PNGase (New England Biolabs, Beverly, MA) with 150 U/mg of protein, per manufacturer's instructions. Samples were separated by electrophoresis and blotted as above

and probed with either AB785 or AB786 (1:5000) polyclonal raBβ it antibody (Chemicon International, Inc.). Hfib standard was provided by the American Red Cross.

2.6 Detection of rhfib by ELISA

The level of rhfib in the prepared whey samples was determined by polyclonal ELISA. Plasma derived hfib was used as a reference standard. Immulon II microtiter plates were coated with 5 mg/ml of raBβ it anti-hfib diluted in 0.1 M NaHCO3, pH 9.6. Wells were washed with TBST and 100 ng of hfib reference, transgenic prepared whey or nontransgenic prepared whey dilutions were added to the plate and incubated for 30min at 37°C. Wells were washed with TBS/ Tween and 1:10,000 diluted horseradish peroxidase-conjugated raBβ it anti-hfib (Dako, Carpinteria, CA) was added to all wells and incubated for 30min at 37°C. Upon completion of incubation the wells were washed and 100 llof OPD (O-phenylenediamine; ABβ ott Labs, ABβ ott Park, IL) solution was added to each well. The reaction was stopped after 3–4 min by the addition of 100 llof3N H2SO4 and the absorbance was measured at 490 nm.

Table 1. Summary of transgene mouse production*

Construct	WAP6FibT.C.	WAP6FibAα1	WAP6FibBβ1	WAP6Fibγ1
Number of mice	39	37	31	17
Number of founders	9	11	9	8
Percent transgenic	23	30	29	47
Founders transmitting	6	6	6	2
Percent transmitting	66	54	66	25

* See text for construct descriptions.

2.7 Partial purification of the transgenic fibrinogen

A whey pool sample was prepared from mouse milk collected from different trigenic fibrinogen mice. Frozen EDTA-treated prepared whey samples (derived as described above) were defrosted at 10oC and mixed in a vortex mixer to help resolubilize cryoprecipitated fibrinogen. Three ml of each prepared whey pool was applied to a 2 ml Fast-flow DEAE-Sepharose chromatography column, the column was then washed with 125 mM NaCl, 50 mM Tris and 200 mM EDTA, pH 8.5 buffer, and then eluted with 250 mM NaCl, 50 mM Tris and 200 mM EDTA, pH 8.5 buffer. The column eluate was concentrated to 0.5 ml by lyophilization and rendered with 8 mM zinc acetate, pH 7. The mixture was centrifuged at 10,000 x g for 3 min at room temperature and the supernatant and pellets were evaluated by SDS-PAGE and Western Blot

analysis. Both the supernatant and pellets of the control mouse and transgenic whey purification products were retained for clotting analysis. Pellets were reconstituted in 50 mM Tris, 100 mM CaCl, pH 8.5 buffer at room temperature.

2.8 Functional activity of transgenic fibrinogen

The clotting activity of reference and purification products derived from control whey and transgenic samples was evaluated by the visual formation of a clot precipitate by the addition of 200 U/ml bovine thrombin. Reference treatment samples consisting of 300 μg/ml hfib (gift of the American Red Cross) dissolved in 50 mM Tris, 100 mM CaCl, 7 and 8 mM zinc acetate, pH 8.5 buffer. A similarly treated control whey superna- tant product was also evaluated. The thrombin reaction mixtures were allowed to incubate for 15 min at 21°C. All samples were centrifuged for 2 min at 10,000 x g and examined for a clot. The samples containing evidence of a clot precipitate were then resuspended and adjusted to 200 μg/ml Factor XIII derived from human plasma(gift of the American Red Cross).The samples were again incubated for 15 min at 21C and then centrifuged at 10,000 x g and then treated with 10 M Urea, 1% sodium dodecyl sulfate, 1% mercaptoethanol by gentle heating for 2 min at 100 °C in a capped 1 ml Eppendorf tube. The dissolved clot–pellet solutions were evaluated by SDS-PAGE and Western analysis using polyclonal anti-era against hfib γ-chain.

3.0 Results:

3.1 Expression of individual fibrinogen chains by the transgenic mammary gland.

Individual lines of transgenic mice were established by out breeding founder animals that carried one of the mammary specific expression vectors WAP6FibAα1 WAP6FibBβ1 or WAP6Fibγ1.Production efficiencies are summarized in Table 1 for each individual construct where transgene integration and transmission fell in the typical range for transgenic mouse production. Upon establishing lines for each construct Southern analysis was performed to judge transmissible copy numbers for each line,where total transgene copies varied from 1 to greater than 50 among the different lines (Table 2).Evaluation of individual fibrinogen chain expression was conducted on prepared whey from naturally lactating females (minimum n=3) of a given line during the course of lactation (days 5,10 ,15) and over successive lactations.Prepared whey samples were subjected to Western analysis under reducing conditions and compared hfib standards.

Table 2. Summary of established monogenic mouse lines

Mouse line	Construct	Copy number*
14	WAP6FibAα1	> 50
26		6–8
27		2
33		2
43		1–2
45		6
8	WAP6FibBβ1	7
14		4
16		13
21		14
25		6
28		10
1	WAP6Fibγ1	> 50
6		3–4

* Copy number was determined from a minimum of three F^1 offspring for each line.

Only one line (43) of the generated WAP6FibAα1 mice produced a sufficient quantity of Aα-chain under non-reducing conditions that migrated identical to human standard(Figure 2a),suggesting this chain is properly processed and secreted in monomer form by memmary ephithelia. In contrast to the Aα-chain and Bβ chain was not secreted in monomer form by WAP6FibBβ1 mice and no chain complexes were detected under non reducing conditions using several different antibodies .However ,secreted Bβ-chain was detected under reducing conditions with mobility similar to hfib(figure 2b) in three of the six established lines. The Presence of this band only under reducing conditions indicates that Bβ-chain is either secreted in association with other proteins or secreted in a conformation that prevents epitope recognition by variety of antibodies .Western analysis of milk from WAP6Fibγ1 transgenic mice under reducing conditions produced a band which migrated identically to human standard (figure 2c),suggesting the γ-chain was able to be secreted into milk by the mammary epithelia of these animals. These animals represented one of the two established lines.

3.2 Expression of rhfib and chain complexes by the transgenic animal mammary gland.

Transgenic mice carrying all three fibrinogen chain constructs were produced by co-microinjection of individual construct at an equal molar concentration. Overall nine triple transgenic(WAP6FibT.C.) founder mice were generated and upon breeding with control animals ,six lines of transgenic mice wee established. Since the three constructs were not physically linked before microinjection the transgene locus for a given line may vary in composition by the ratio and copy numbers of the gene constructs within the locus .Southern

analysis of each established line was performed to verify PCR results and to quantify the number of transgenes within the integration site.The four bands produced by Sothern analysis of a transgenic mouse DNA are indicated in figure 1.The fragment produced by the endogenous WAP genes (~50 kbps) acts as amount of DNA loaded for gel electrophoresis,due to the presence of endogenous WAP at two copies per diploid genome (GeneBank ,NIH).By comparing the signal intensities for a given lane the relative copy numbers of each transgene within the integration site were determined and presented in table 3.Variations of both the molecular ratio of transgenes and number of copies present within the given locus were observed similar to that previously reported when using three separate transgenes (Punkard etal.,1996)

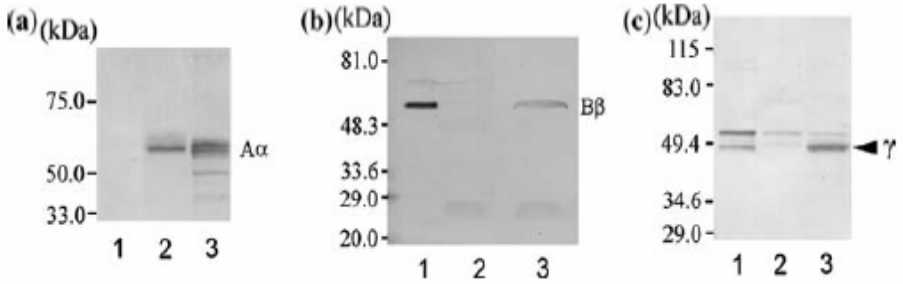

Figure 2. Western analysis of prepared whey from monogenic mice under reducing conditions. Panel a, prepared whey from a mouse containing only the WAP6FibAα transgene was separated by SDS-PAGE, transferred and probed with the ADI polyclonal antibody producing the band Aα. Lane 1, 1 μl prepared whey from non-transgenic; lane 2, 1 μl of prepared transgenic mouse whey: lane 3, 120 ng of hfib. Panel b, prepared whey from a mouse containing only the WAP6FibBβ1 transgene was separated by SDS-PAGE, transferred and probed with the Shainoff monoclonal antibody producing the band Bβ. Lane 1, 500 ng of hfib; lane 2, 1 μl of prepared negative control mouse whey; lane 3, 3 μl of prepared transgenic mouse whey. Panel c, prepared whey from a mouse containing only the WAP6Fibγ1 transgene was separated by SDS-PAGE, transferred and probed with a polyclonal γ-chain antibody producing the band γ. Lane 1, 156 ng of hfib; lane 2, 1 μl of prepared negative control mouse whey; lane 3, 1 μl of prepared transgenic mouse whey.

Prepared milk samples from several animals of a given line over the course of first and second lactation were subjected to ELISA to determine which lines were expressing rhfib, to what level, and if expression was stable during the course of lactation. ELISA results identified that three of the six trigenic lines secreted detectable amounts of rhfib and/or rhfib chain complexes into their milk (Table 3). Upon analysis of these lines over the course of lactation, variations in expression were observed. Previous research has indicated that murine WAP-based transgenes are influenced by chromosomal DNA elements flanking the integration site by modulating the hormonal response of the promoter elements (Burdon et al., 1991). In order to verify that the lack of secreted individual chain and chain complexes is not due to the processing activity of mammary epithelia, but instead due to promoter activity, we conducted Northern analysis and have found a direct correlation between absence of mRNA and absence of protein.

Samples with the strongest ELISA signals were subjected to Western analysis in order to determine if each chain was correctly processed and to identify the secretion products composing the signal.

Table 3. Summary of established trigenic lines

Mouse line	Transgene copy number[a]			Transgene ratio Aα: Bβ:γ	Concentration of subunits[b] (μg/ml)
	Aα	*Bβ*	γ		
2	1	4	6	1:4:6	0
10	6	2	4	3:1:2	60–450
18	8	8	8	1:1:1	0
21	8	6	12	4:3:6	0–125
30	8	10	8	4:5:4	0
37	1	1	8	1:1:8	170–700

[a] Copy number was determined from a minimum of three F^1 offspring for each line.
[b] A minimum of three F^1 offspring were screened per line on days 5,10 and 15 of lactation.

Prepared whey samples from the three expressing lines were subjected to Western analysis under reducing conditions using a polyclonal 3 chain antibody (Figure 3b). All three chains were detected in transgenic prepared whey with the migration of the rhfib Aαand Bβ chains being identical to human standard (lane 1), but the γ-chain having a slightly faster mobility. Also present are several higher molecular weight species which most likely represent incomplete reduction and separation of fibrinogen and/or fibrinogen chain complexes, as these are not found in non-transgenic prepared whey. In order to identify the degree of secreted subunit assembly, identical samples were run under non-reducing conditions and probed with the same ADI antibody, (Figure 3a). The antibody recognizes only a single band in the human standard (lane 1), but also binds to mouse fibrinogen with a low affinity in lane 2. Trigenic milk samples have several bands present, indicating secretion of individual chains and/or chain complexes. There are two prominent bands (Aαand AG) with apparent sizes of 66 and 135–150 kDa, respectively, which correspond in molecular weight to Aachain monomers and an Aac2chain complex. The monomer form is found secreted in all lines studied, suggesting its ability for free secretion similar to monogenic a-chain mice. The Aαmonomer signal produced by prepared whey from line 10 is more intense than that produced by milk from mice of line 37 and 21, but in contrast, band AG is very weak in line 10 and very strong in the other two lines. It is important to note the trans-gene ratio with this line where the a transgene ratio is higher than the other two

lines. Upon mRNA analysis by Northern a higher ratio of a message was observed in line 10 as compared to the other lines, confirming the behavior of the integration site to be copy number dependent as seen with other mWAP driven transgenes (Burdon et al., 1991). These observations along with the absences of bands below 198 kDa in the non-transgenic whey sample indicate individual chains and partial chain complexes can be secreted by the mammary gland. The other minor bands

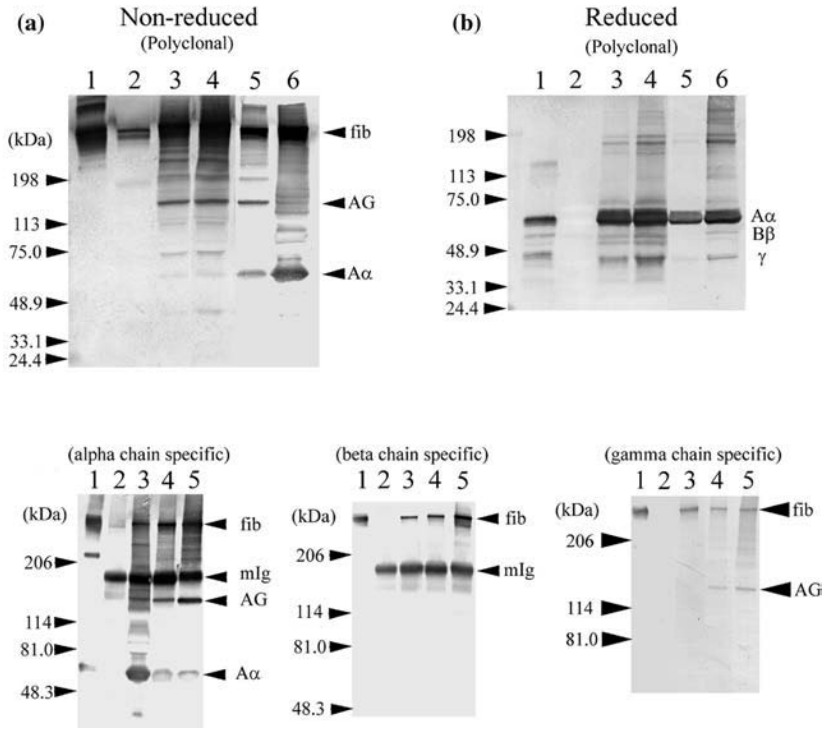

Figure 3. *Western analysis of prepared whey from triple transgenic mice under reduced and non-reduced conditions. Upper panel, prepared whey from triple transgenic mice was separated by SDS-PAGE under non-reducing (a) or reducing conditions (b) and probed with the ADI polyclonal antibody. Lane 1, hfib 120 ng; lane 2, 1 ll of non-transgenic prepared whey; lane 3, 1 ll of line 37 prepared whey; lane 4, 1 ll of prepared whey from line 37 homozygous animal; lane 5, 1 ll of line 21 prepared whey; and lane 6, 1 ll of line 10 prepared whey. Lower panel, prepared whey from triple transgenic mice was separated by SDS-PAGE under non-reducing conditions and probed by a chain specific antibody; Y18, Shainoff, NC15 for Aa,Bβ and°Cchains, respectively. Lane 1, 200 ng hfib; lane 2, 1 ll of non-transgenic prepared whey; lane 3, 1 ll of line 10 prepared whey; lane 4, 1 ll of line 21 prepared whey; and lane 5, 1 ll of line 37 prepared whey. Band designations are: A, fibrinogen Aa-chain; AG, Aα□°Ccomplex; mIg, mouse c immunoglobulin; and fib, assembled fibrinogen.*

present in the transgenic milk samples may be the association of free chains or chain complexes with naturally occurring milk proteins such as the a caseins which are known to interact among themselves via cystine bridging.

In order to identify whether the A band is free Aa-chain and the AG band contains the Aasubunit, a non-reduced blot from trigenic lines was probed with a Aa-chain-specific monoclonal antibody (Figure 3, lower panel). There are four major bands identified: Aα at~ 66 kDa, AG at ~130–150 kDa, mIg at 180 kDa and fib ~340 kDa. The concentration of the Aαband is found most abundantly in the line 10 sample with lower levels found in samples from lines 21 and 37. In contrast, the AG band was found most intense in milk samples from line 21 and 37. These observations indicate that free Aαhain is readily secreted by the mammary gland and a higher molecular weight complex containing at least the Aα-subunit is also secreted under conditions when all three chains are present. The mIg band produced in all mouse samples was due to recognition of secreted IgG by the secondary conjugated antibody (A4416, anti-mouse) used during blot development.

To evaluate whether Bβ -chain was being secreted by these mice as free chain or as a complex, a blot using a monoclonal Bβ -subunit antibody was performed and is shown in Figure 3, lower panel. Only two significant bands were identified, the mIg band previous discussed and the rhfib band migrating identically to the human standard. These observations indicate that the β-chain is being produced at a level where it is incorporated into fully assembled rhfib. Since this antibody does not crossreact with mouse fibrinogen, the relative amounts of rhfib secreted among different mice can be observed. To follow the pattern of secretion for the γ-chain and to rule out the possibility that the AG band was not an oligomer of Aa-subunits, a c-subunit blot was performed using a γ-chain specific polyclonal antibody (Figure 3, lower panel). Two bands were produced: AG at 130–150 kDa and rhfib at 340 kDa. The fibrinogen band was found in all lines and in the human standard. The AG band was not detected in line 10 milk, but was present in milk samples from mice of line 21 and 37. Thus, the high molecular weight species that we have observed does contain all of the fibrinogen subunits.

3.3 Glycosylation state of rhfib chains

The glycosylation state of secreted rhfib and chain complexes was investigated in the highest expressing mice in order to evaluate if the mammary gland can perform necessary posttranslational modifications without limitation. The Aa-subunit of hfib has a punitive N-linked glycosylation site Asn269 and Asn400 (Rixon et al., 1983), but it is not glycosylated in fully

assembled hfib. Deglycosylation experiments were performed to identify if Aa-subunit remains deglycosylated when secreted by both monogenic and trigenic mice with results presented in Figure 4. No band shifting was observed when human standard was digested with the enzymes Endoglycosidase H (high mannose, Endo-H) or Glycopeptidase (high mannose or complex, PNGase) that cleave N-linked saccharides. Upon treatment of trigenic prepared whey and monogenic prepared whey with these enzymes, shifting of the Aa-subunit was not observed. This suggests that the punitive site is not recognized by mammary epithelia following a similar pattern to that of the liver.

The Bβ -chain of hfib is glycosylated with the carbohydrate side chain being in the complexed form. Glycosylation of the Bβ -subunit is not required for secretion of assembled fibrinogen, as cells treated with tunicamycin still can secrete fibrinogen (Yu, 1983). To evaluate if the secreted Bβ -subunit is glycosylated when no other fibrinogen chains are present, deglycosylation experiments were performed on monogenic prepared whey with results shown in Figure 5a. As expected, no band shifting is observed with human standard when treated with Endo-H, but

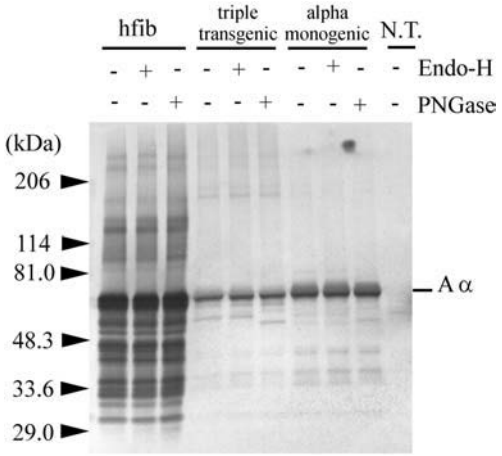

Figure 4. *Glycosylation analysis of Aa-chain from WAP6FibAaand WAP6FibT.C. mouse prepared whey. Prepared whey (l µl) from both monogenic and trigenic lines and hfib standard (230 ng) was subjected to deglycosylation with either Endoglycosidase H (Endo-H, recognizing high mannose) or Glycopeptidase (PNGase, cleaving both high mannose and complex structures) with resulting products run on SDS -PAGE and probed with the Aa-chain AB784 polyclonal antibody. Aα= fibrinogen Aα-chain, N.T. = non-transgenic prepared whey (l µl).*

fibrinogen standard does shift with PNGase treatment. Some shifting of the secreted Bβ subunit is observed with Endo-H treatment, suggesting that some portions of the secreted Bβ chains are of the high mannose type. Unfortunately, exact quantification can not be performed as the Bβ subunit of murine fibrinogen migrates at the same rate, but it was observed that all of the rhfib Bβ

-subunit is glycosylated as shown by shifting of all material by PNGase. This suggests that mammary epithelial cells can recognize the glycosylation site of rhfib Bβ chains when produced without the other fibrinogen chains and core glycosylation does occur. In this case, the processing ability of the mammary epithelial cell appears to be limiting, resulting in secretion of both high mannose and complex saccharide glycosylated Bβ subunits. Limitations in Bβ chain glycosylation are not restricted to mammary epithelia as it has been observed with transfected COS-1 cells that a fractional amount of Bβ subunits in secreted rhfib was Endo-H sensitive. To evaluate the amount of Endo-H sensitive Bβ subunits that are present in mammary epithelia-derived rhfib, similar deglycosylation experiments were performed on trigenic prepared whey. Treatment of prepared whey derived from the

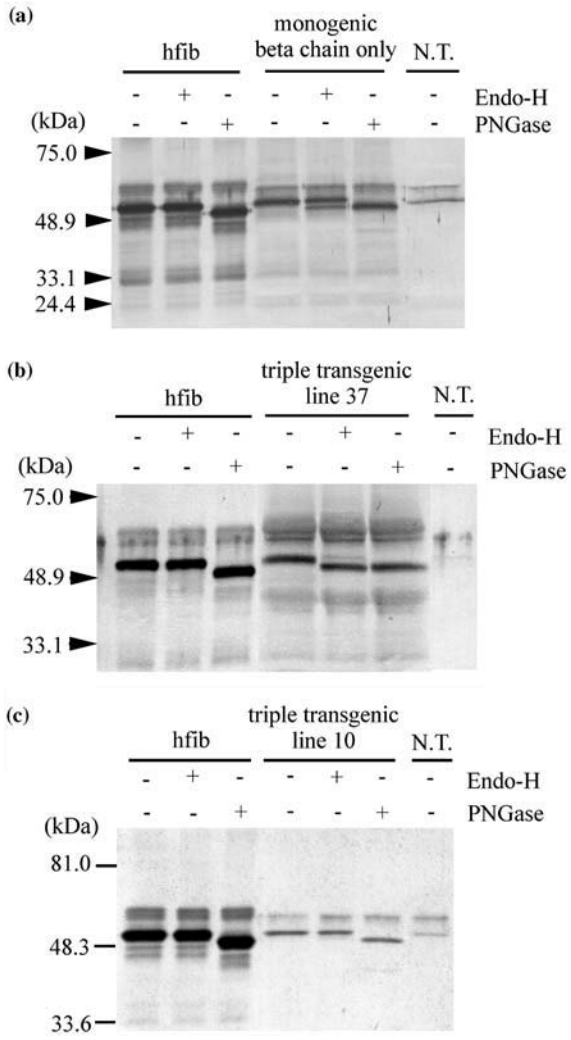

Figure 5. *Glycosylation analysis of Bβ -chain from WAP6Fib Bβ 1 and WAP6T.C. prepared mouse whey. Panel a, prepared whey (1 μ l) from a WAP6FiBβ b1 monogenic line and hfib standard (200 ng) was subjected to deglycosylation with either Endoglycosidase H (Endo-H) or Glycopeptidase (PNGase) with resulting products run on SDS-PAGE and probed with the Bβ -chain AB 785 polyclonal antibody. Note the*

partial shifting of the Bβ -chain produced by Endo-H digestion of monogenic whey. Panel b, prepared whey (1 µl) from a high expressing WAP6FibT.C. (line 37, homozygous) mouse and hfib standard (300 ng) was subjected to deglycosylation with Endo-H and PNGase, separation and probing with AB 785 polyclonal antibody. Note the majority of Bβ -chain is sensitive to Endo-H in the high expression whey. Panel c, prepared whey (1µ l) from a medium expressing WAP6FibT.C. (line 10) mouse which is similar in expression to the monogenic in panel a, and hfib standard (230 ng) was subjected to deglycosylation with Endo-H and PNGase, separation and probing with AB 785 polyclonal antibody. Note the lack of sensitivity to Endo-H in the expressed whey. N.T. ¼ non-transgenic.

highest expressing line with Endo-H (Figure 5b) resulted in shifting of all recombinant Bβ chains to the deglycosylated state indicating that all assembled Bβ -chain was in the immature form. In contrast, when rhfib was secreted at lower levels in milk as with line 10 mice, the Bβ -chain was not susceptible to Endo-H treatment (Figure 5c). These observations suggest a rate limitation for β-chain glycosylation when it is incorporated into fully assembled fibrinogen.

Several cell types have demonstrated the ability to N-glycosylate the°Cchains of assembled hfib with saccharides complex in structure. To determine the glycosylation state of the γ-chain when expressed without the a-or β-chain , prepared whey from WAP6Fibc1 mice was subjected to deglycosylation experiments (Figure 6). Hfib standard did not shift when treated with Endo-H, but did show a faster mobility when treated with PNGase. Mammary epithelia derived°Cchains were not affected by Endo-H treatment, but did shift under PNGase treatment. This observation indicates that the γ-chain can be recognized by glycosylation pathways in mammary epithelia when only the γ-chain is present and does not require association with the other chains for glycosylation to occur. When the same experiment is performed on trigenic mouse prepared whey derived from line 37, a small fraction of the°Cchains became deglycosylated under Endo-H treatment, suggesting a subpopulation of immature°Cchains is able to be secreted. Thus, when total fibrinogen and fibrinogen chain complexes are secreted by mammary epithelia into milk at concentrations approaching 700 µg/ml, the ability of mammary epithelia to glycosylate properly is compromised.

The functionality of assembled fibrinogen was assessed by first partially purifying the recombinant fibrinogen away from the endogenous mouse fibrinogen which typically occurs in milk and can be variable due to plasma passover from injury that can occur during milking. We adapted aZn2+-dependent solubility based purification process used for isolating proteins from transgenic milk (Degener et al., 1998) that discriminates between mouse and hfib (Butler et al.,

1997). After removal of the bulk of the caseins by ion exchange chromatography, the ion exchange eluate was rendered with ZnCl (8 mM) to precipitate mouse fibrinogen. After treatment

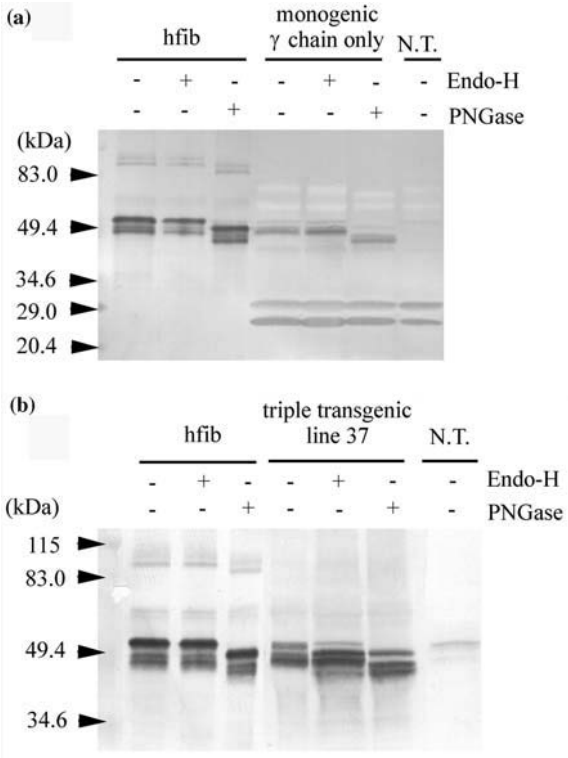

Figure 6. *Glycosylation analysis of γ-chain from WAP6Fibc1 and WAP6T.C. prepared mouse whey. Panel a, prepared whey (1 µl) from a WAP6Fibc1 monogenic line and hfib standard (230 ng) was subjected to deglycosylation with either Endoglycosidase H (Endo-H) or Glycopeptidase (PNGase) with resulting products run on SDS -PAGE and probed with the Chemicon γ-chain antibody. Note the lack of shifting under Endo-H conditions with the monogenic whey. Panel b, prepared whey from a WAP6FibT.C. (line 37, homozygous) mouse and hfib (230 ng) was subjected to deglycosylation treatment by either Endoglycosidase H (Endo-H) or Glycopeptidase (PNGase) and separated by SDS-PAGE under reducing conditions then probed with the γ-chain Chemicon antibody. Note the partial shifting of the°Cband upon Endo-H treatment of WAP6FibT.C. mouse whey. N.T. ¼ non-transgenic mouse whey (1 µl).*

with thrombin, the supernatant containing the soluble human transgenic hfib was shown to form a visible clot-like precipitate similar to that of the human reference sample. The clot density of the precipitate was found to increase after treatment with Factor XIII. No clots were formed with supernatants analogously derived from control mouse milk. Western analysis of these reaction mixtures containing human plasma derived fibrinogen (Figure 7a) and transgenic hfib (Figure 7b) showed the disappearance of the γ-chain (lane 3 of panel a and panel b; human

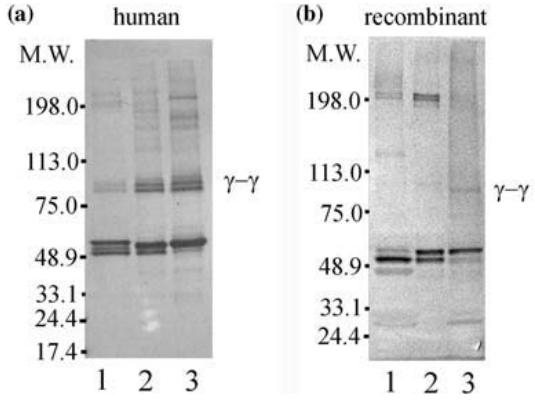

Figure 7*. Functional activity of recombinant fibrinogen. Recombinant fibrinogen was purified as listed in materials and methods and subjected to thrombin activation and factor XIII cross linking along with human standard. Products were denatured then separated by SDS-PAGE followed by probing with polyclonal AB786 antibody. Panel a: lane 1, hfib without thrombin or factor XIII treatment; lane 2, hfib treated with thrombin; lane 3, hfib treated with thrombin and factor XIII. Panel b: lane 1, purified recombinant without thrombin or factor XIII; lane 2 recombinant with thrombin; lane 3, recombinant treated with both thrombin and factor XIII.°C□°Crepresents the γ-chain –γ-chain crosslink.*

and transgenic hfib, respectively) and the appearance of a species at an Mr of approximately 90 KDa after treatment with thrombin and Factor XIII. Western blot analysis specific to γ-chain confirmed the 90 KDa species to be a c–c crosslinked product arising from the thrombin-Factor XIII enzymatic reaction (data not shown). As reported previously (Prunkard et al., 1996), we found evidence of c-glutamyl transferase activity in the milk mixtures leading to the formation of c–c cross-linked chains even without the presence of human Factor XIII after thrombin treatment.

4.0 Discussion

The use of transgenic livestock as bioreactors to produce pharmaceutical biologics is maturing with several derived proteins in or about to enter clinical trials (Drohan and Clark, 2000). New biologics are being discovered and brought forward as potential candidates for being produced by the transgenic mammary gland, requiring rapid evaluation of both synthesis rate and functionality. Transgenic mice offer this platform for evaluation due to their short generation intervals and ease of genetic manipulation, thus allowing production of a biologic before investigation in larger animals. In contrast to previous studies using immortalized cell lines, we have sought greater understanding of the relationship between the extent of glycosylation and assembly of individual chains in the context of the healthy native physiology of the transgenic mammary gland. Previous studies with fibrinogen secreted into the milk of transgenic mice (Prunkard et al., 1996) did not focus upon glycosylation or secretion limits of individual chains in relationship to assembly of functional mature fibrinogen.

In this report, we established lines of transgenic mice to study the genetic stability and transmission characteristics of each transgene integration site and to develop both protein and

RNA expression profiles for each unique locus. When embryos were microinjected with an equal molar ratio of DNA constructs, the resulting offspring had both balanced and unbalanced ratios incorporated into the genome. This observation is similar to a previous report (Burdon et al., 1991) where three different transgenes consisting of slightly modified mouse WAP genomic sequences were co-injected resulting in highly imbalanced loci with regards to the three mouse lines analyzed. We performed a detailed analysis of mRNA expression for each fibrinogen gene in the WAP6FibT.C. loci (data not presented) and observed concert expression of each gene with regards to copy number within the locus and that expression of the locus varied over the course of lactation depending upon the line studied. This suggests that the 4.1 kbp mWAP promoter is heavily influenced by elements flanking the integration site similar to the 2.4 mWAP promoter and that the additional promoter sequences do not afford insulation from these outlying elements (McKnight et al., 1995). However, when the 4.1 kbp promoter is not inhibited by outlying repressive elements, it can drive expression of fibrinogen cDNA to sufficient levels where protein expression is approximately the same as when fibrinogen genomic coding elements are used such as with the 4.2 kbp BLG promoter (Prunkard et al., 1996). Indeed, we show here that the 4.1 kbp mWAP promoter can be used to independently express each fibrinogen subunit so as to result in the assembly of fully functionally fibrinogen capable of forming a cross-linked clot.

While our primary goal is to evaluate the capacity of transgenic livestock as bioreactors to produce rhfib in milk, the mammary gland provides a novel system from which to study intracellular processing, assembly and secretion in a pathway which differs from most transformed cell lines. For example, the expression of rhfib in mammary epithelial cells of transgenic mice should provide a naturally less restrictive trafficking environment due to the nutritional role of most milk proteins. This was especially reflected by the unassembled b-rhfib chain as well as other partially assembled rhfib complexes which were secreted into the milk. We have previously shown that mammary epithelial tissue of transgenic mice can perform complex post-translational processing at cell synthesis rates of greater than about 1–10 pg/cell/day (Velander et al., 1992) and these recombinant protein synthesis rates are similar to that found in previous rhfib cell culture studies (Yu et al., 1984; Danishefsky et al., 1990; Hartwig & Danishefsky, 1991; Roy et al., 1994). Due to the high cell density of the mammary tissue relative to cell culture, the expression levels in milk resulting from cDNA rhfib constructs under the control of the 4.1 kb mWAP promoter were about 100–700 μg/ml total rhfib species. Fully

assembled rhfib was about 200 μg/ml of the total secreted rhfib species, indicating secretory permissiveness in the case of assembly limitations. From this standpoint, the study of secreted rhfib chains and complexes is more easily done in milk than in cell culture media.

Perhaps the biggest difference between mammary gland and cell culture expression was with respect to secretion of free Bβ -chain; the milk of Bβ -monogenic mice showed free Bβ -chain, while previously no cell line has been shown to secrete Bβ -chain alone. For example, the secretion of immature rhfib and free hfib chains in COS-1, CHO, BHK, and HepG2 cell lines showed a lack of detectable, free Bβ -chain (Danishefsky et al., 1990; Roy et al., 1994). This occurred in both Bβ -chain only and Aa,Bβ ,γ-chain transfected cell lines indicating a higher retention selectivity for Bβ -chain by the secretory pathway in these cells. Xia and Readman (2001) have demonstrated in HepG2 and COS cells that the intracellular levels of each fibrinogen chain are modulated by chain association to the degradation pathway proteins Sec61b (Bβ -chain) and Hsp70 (Aαand γ-chain s). Their observations coupled with those we observed with the Bβ chain secretion by Bβ -monogenic mice have allowed us to postulate that mammary epithelia has a less restrictive secretory pathway as compared to cell culture lines studied so far.

We observed that the secretion of free β-chain could occur at 100 μg/ml in monogenic mice, which is similar to the levels of the secreted free a (150 μg/ml) and°C(50 μg/ml) hfib chains in the respective monogenic mouse lineages. The glycosylation of the secreted free β-chain occurring in monogenic β-chain mouse milk was estimated to be about 30% high mannose core. However, when secreted at similar levels in assembled hfib, the β-chain contained about 95% Endo-H sensitive carbohydrate which is similar to that found in assembled rhfib from COS-1 cells. It is possible that the co-expression of a and c-rhfib chains with β-chain and/or the assembly process occurring in trigenic mice can lower the efficiency of glycosylation processing of the nascent β-chain . Since there was no free β-chain species detected in the milk of trigenic rhfib mice, it is unlikely that β-chain s having high mannose core were kinetically unsuitable for rhfib assembly.

Free a-chain was expressed in milk at the highest levels of the monogenic lineages and represented as much as 10% of the total hfib species present in the milk of trigenic lineages. In addition, Aα–c complexes were found at significant levels in the milks of trigenic lineages and represented as much as about 30% of the rhfib species as detected by Western analysis. Thus, the trafficking leading to secretion of Aα-only and Aα- chain containing complexes including mature rhfib was efficient in mammary tissue and similar to other non-epithelial cell types (Danishefsky

et al., 1990; Roy et al., 1994). In contrast to β-chain , γ-chain was found to have no Endo-H sensitivity in freely secreted γ-chain from monogenic or assembled γ-chain in rhfib from trigenic lineages. Thus, in mammary epithelial cells the trafficking and γ-chain assembly into rhfib appears to be largely independent of glycosylation. The relative molecular weight of about 150 kDa of the a–c containing complexes would be consistent with an a–c–c complex as has been postulated to occur in other cell types. Similarly to a-chain, no free γ-chain was detected in the milk of trigenic lineages indicating that intracellular γ-chain was not pooled in excess relative to the a-chain.

In summary, we have used the less restrictive secretory pathway of the mouse mammary epithelia to express individual chains of a complex multimeric protein to be secreted to enable glycosylation patterns to be studied.

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