**Biosynthesis of High Molecular Weight Breast Carcinoma Associated Mucin Glycoproteins*\)**

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We have studied the biosynthesis of mucin glycoproteins recognized by monoclonal antibody W1 in MCF7 cells. Proteins of M, 170,000, 185,000, 260,000, and 275,000 were immunoprecipitated from cells pulse-labeled with [3H]threonine and [3H]proline. Evidence suggesting that these proteins were precursors of high molecular weight mucin(s) included: 1) their kinetics of disappearance corresponded with appearance of mature mucin(s) (t1/2 = 30 min); 2) their processing into mature mucin(s) was not blocked by cycloheximide, but was disrupted by monensin, which impairs glycoprotein processing in the Golgi; 3) they were inaccessible to antibody added outside the cells, whereas mature mucin(s) was accessible and appeared at the cell surface with a t1/2 = 45 min; and 4) their mobilities of precursors varied between different cell lines, but generally correlated with mobilities of mature mucin(s). The precursors were sensitive to endoglycosidase H, indicating that they contained high mannose N-linked oligosaccharides. Less than 3% of threonine residues in the precursors, but more than 75% in mature mucin(s), were substituted with O-linked oligosaccharides. Therefore, initiation of N-linked oligosaccharides occurred soon after initiation of core protein synthesis, but initiation of O-linked oligosaccharides occurred much later, just prior to appearance of mature mucin(s) at the cell surface.

High molecular weight (M, > 203,000) mucin glycoproteins are frequently found associated with human breast tumors (1-5). Immunologically related mucins are also found in normal human milk (1-5), although these may be antigenically distinct from mucins made by tumor tissues (6). Monoclonal antibodies directed against tumor associated mucins are used for diagnosis of human tumors (7-18), in particular for serum assays to monitor tumor progression (14-18).

Because of their clinical usefulness as targets for cancer diagnosis, it is desirable to determine structural features of breast carcinoma associated mucins which influence their antigenicity. Although compositional data are not available for tumor-derived mucins, Shimizu and Yamauchi (19) have isolated from human milk a mucin, termed PAS-0, which was determined to contain 50% by weight of carbohydrate, attached to the protein core primarily by O-linkages. The highly glycosylated nature of these mucin(s) makes it likely that both protein and carbohydrate structures contribute to their antigenicity.

Much of what is known about the core protein of these mucin(s) comes from the cDNA cloning studies of Taylor-Papadimitriou and colleagues (20, 21). The core protein of mucin derived from human milk was estimated to be approximately 68,000 kilodaltons following deglycosylation with hydrogen fluoride (20); a product of similar molecular mass was also identified by immune precipitation from in vitro translation products of poly(A)* RNA of MCF7 breast carcinoma cells (20). A partial cDNA clone for the protein core of a mucin from MCF7 cells has been isolated (20). Blot hybridization experiments (21) using this cDNA clone as probe have shown that sizes of restriction fragments containing core protein genes vary in parallel with sizes of their products, suggesting that differences in the length of the protein core are partly responsible for the electrophoretic polymorphism of the mucin(s) (products of the PUM locus).

Much less is known about the carbohydrates of carcinoma associated mucins or the sequence of events involved in glycosylation of the core protein. In general, events during the addition of O-linked carbohydrates to proteins are not as well established as they are for N-linked oligosaccharides (22). The first step in the biosynthesis of O-linked oligosaccharides is the addition of GalNAc to serine or threonine residues on the core protein (22). There is evidence for initiation of O-linked oligosaccharide synthesis occurring early (23-25), late (26-29), and continuously (30) in the biosynthetic pathways of different proteins. Following initiation, chains may be elongated, usually proceeding first with the addition of galactose to give Galβ1-3GalNAc.

Because of their importance for cancer diagnosis and because they represent a model system for studying O-linked glycosylation, we have investigated the biosynthesis of breast carcinoma associated mucin glycoproteins. For these studies, we have used the monoclonal antibody, W1, that recognizes high molecular weight antigens found at elevated levels in sera from breast cancer patients (18). In this paper, we report the characterization of novel biosynthetic intermediates of mucin(s) recognized by this antibody. While these studies were in progress, a preliminary description was published by Hilkens et al. (31) on the biosynthesis of carcinoma associated mucins.

**EXPERIMENTAL PROCEDURES**

**Materials—** EN' HANCE, D-[1,6-3H]glucosamine, and L-[2,3,4,5-3H]proline were purchased from Du Pont-New England Nuclear. L-[55S]Methionine, L-[4,5-3H]leucine, and [35S]sulfate were purchased from Amersham Corp. L-[3,4-3H]Threonine was purchased from ICN Pharmaceuticals, Inc. and L-[3-3H]Threonine from Amersham Corp.

Cycloheximide and monensin were purchased from Sigma. N-Glycanase, O-glycanase, and endoglycosidase H were purchased from Genzyme. Neuraminidase from Vibrio cholerae, ovalbumin, and Pansorbin Adsorbent (formalin-fixed Staphylococcus aureus) were purchased from Behring Diagnostics. Endoproteinase V-8 was purchased from Miles. Affinity purified goat anti-mouse IgG was purchased from Hyclone. Monoclonal antibody W1 was obtained from

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Cetus Corp. Monoclonal antibody HMFG-2 was obtained from Uni-
path Ltd., Bedford, United Kingdom. Antibodies 115D8 and F36/22
were from Dr. J. Hilgers, Netherlands Cancer Institute, and from Dr.
M. Chu, Roswell Park Memorial Institute, respectively.

Cell Culture—MCF7 cells were obtained from Dr. Marc Lippman,
National Institutes of Health, and routinely maintained in Dulbecco's
modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS),
penicillin/streptomycin, and gentamicin, in a humidified atmosphere
containing 5% CO2.

Metabolic Labeling—Cells were labeled with [35S]methionine and
[3H]glucosamine as described previously (33). For [3H]leucine labeling,
2 x 106 cells were collected, washed, and resuspended in 1 ml of
leucine-free DMEM containing 0.25 mCi (147 Ci/mmol) of [3H]
leucine. Labeling was for 1 h at 37°C. For [35S]sulfate labeling, a monolayer
of cells was placed in 2 ml of salt-free medium, and [35S]sulfate (1.25 mCi in 10 ml of salt-free medium,
25% of the total activity) was added after 1 h of labeling (39).

[3H]proline labeling, cells (5-20 x 106) were starved overnight at
37°C in DMEM lacking threonine plus 10% dialyzed FBS. Cells were
resuspended in 1-2 ml of threonine-free DMEM containing 0.5-0.75
mCi of [3H]threonine (19 Ci/mmol) and 0.1-0.2 mCi of [3H]proline
(18 Ci/mmol) and incubated at 37°C for periods noted. For [3H]leucine labeling, cells were treated by centrifugation and washed in medium
lacking the compound used for labeling. For pulse-chase experiments
cells were resuspended in DMEM containing threonine plus 10% FBS
and 2 mM proline and incubated at 37°C for periods noted. For experiments to determine the extent of threonine substitution, [3H]threonine without [3H]proline was used for labeling; in all other experiments, the T-G-h-labeled isotope was used. When cyclohexi-
more was used, it was included at 10 mM in the chase solution. When
methionine was used, it was dissolved in dimethyl sulfoxide and added
to a final concentration of 20 mM for 30 min prior to initiation of
labeling and in the labeling and chase solutions. Control experiments
showed that cells retained >95% viability after this treatment.

Immunoprecipitation Analysis—Radiolabeled cells were lysed and
prepared for immunoprecipitation analysis as described (33). Aliquots
of lysate (50-100 µl) were incubated with 4-6 µg of monoclonal
antibody for 1 h at 4°C, followed by 4 µg of goat anti-mouse IgG
(heavy and light chain specific) for 45 min at 4°C. Immune complexes
were then precipitated with 60 µl of a 10% solution of formalin-fixed
S. aureus cells and washed as described (33). Radiolabeled antigens
were then separated by SDS-PAGE and identified by autoradiogra-
phy.

Electrophoresis—SDS-PAGE was performed on gradient slab gels
(5-15%, with a 4% stacking gel) using the Laemmli system (34).

RESULTS

Incorporation of Radioactive Precursors into Carcinoma As-
associated Mucin—To analyze the biosynthesis of breast car-
cinoma associated mucin(s), we first determined conditions for
radio labeling mucin(s) made by cultured carcinoma cells by
evaluating various radioactive compounds for their ability
to be incorporated into mucins which could be precipitated
by the monoclonal antibody W1 (18). Both the W5-6 (32) and
MCF7 cell lines were used in early experiments, but since we
found fewer nonspecifically immunoprecipitated proteins
with MCF7 cells, we chose this line for most experiments.

As shown by the autoradiograph in Fig. 1, high molecular
weight mucin(s) were specifically precipitated from extracts
of MCF7 cells which had been labeled with [3H]glucosamine,
[35S]sulfate, or with a mixture of the amino acids [3H]threo-
nine and [3H]proline. High molecular weight mucin(s) were
not clearly visible in precipitates from extracts labeled with
[35S]methionine or [3H]leucine. This is consistent with the
amino acid composition of a human milk-derived mucin (19),
which is rich in threonine and proline, but relatively poor in
methionine and leucine.

Additional bands of M, 170,000 and 90,000 were also
immunoprecipitated from extracts labeled with [3H]
glucosamine, the [3H]threonine and [3H]proline mixture, [35S]
methionine or [3H]leucine; these were subsequently identified

1 The abbreviations used are: DMEM, Dulbecco's modified Eagle's
medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel elec-
trophoresis; FBS, fetal bovine serum.

threonine- and [3H]proline-labeled antigens were treated with
N-glycans (peptide-N4-[N-acetyl-β-D-glucosaminyl]asparagine
amidase), as recommended by the supplier, and endoglycosidase H as described previously (33). O-Glycosylation (endo-
α-N-acetylgalactosaminidase) treatment was essentially as described by the supplier, except sodium phosphate buffer, pH 6.0, was substituted for Tris maleate buffer; the serum albumin (1 mg) was added
and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM N-tosyl-l-phenylalanine chloromethyl ketone, and 0.1 mM N-tosyl-l-lysine chloromethyl ketone) were added to the digest buffer. Reactions proceeded for 16 h at
37°C and were stopped by the addition of concentrated SDS-PAGE
sample buffer. Digested antigens were then analyzed by SDS-PAGE
and autoradiography.
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Fig. 1. Incorporation of radioactive precursors into carcinoma associated mucins. MCF7 cells were labeled with the indicated radioactive precursors and subjected to immunoprecipitation analysis as described under "Experimental Procedures." Labeling periods for \(^{35}\)Smethionine, \(^{3}H\)leucine, \(^{3}H\)glucosamine (N\(_H\)Glc), and the \(^{3}H\)threonine and \(^{3}H\)proline mixture were for 1 h at 37°C. Labeling with \(^{35}\)SSulfate was for 16 h at 37°C. Odd numbers, precipitations without added monoclonal antibody; even numbers, precipitations with monoclonal antibody W1. Solid lines indicate the positions of M, 170,000 and 260,000 precursors and the stacking gel (S).

as mucin precursors (see below). Other bands were also precipitated from cells labeled with the \(^{3}H\)threonine and \(^{3}H\)proline mixture, but these were not seen in every experiment (see, for example, Fig. 4) and were also present (although at reduced amounts) in control samples from which antibody had been omitted; we consequently believe these other bands were nonspecifically precipitated. A band of M, \(\sim 20,000\) was also seen with \(^{3}H\)leucine, but was not investigated further. Since \(^{3}H\)threonine and \(^{3}H\)proline were the amino acids tested for labeling high molecular weight mucin(s), these were used for analysis of mucin biosynthesis.

Pulse-chase Analysis of Carcinoma Associated Mucins—To investigate the biosynthesis of high molecular weight mucin(s), we performed pulse-chase analyses on MCF7 cells which had been metabolically labeled with a mixture of \(^{3}H\)threonine and \(^{3}H\)proline (Fig. 2). A densitometric analysis of the autoradiogram shown in Fig. 2A is presented in Fig. 3.

When cells were pulse-labeled for 5 min, washed, and then placed in medium containing an excess of unlabeled threonine and proline, the earliest species detected were two doublets, one of proteins of M, 170,000 and 185,000 and the second of proteins of M, 260,000 and 275,000. Levels of these proteins continued to increase in parallel (except for the M, 185,000 protein which remained constant) for the first 5 min of the chase period. The higher molecular weight bands of each of the doublets (M, 185,000 and 275,000) then disappeared at comparable rates, these species did not behave as precursors of each other. Likewise, since the lower molecular weight bands of each doublet (M, 170,000 and 260,000) disappeared at equivalent rates, these species did not behave as precursors of each other. The kinetics of disappearance of the M, 185,000 and 275,000 species did not correspond with the kinetics of appearance of the high molecular weight mucin(s), suggesting that former species were not direct precursors of the latter species. The simplest explanation of these results is that the upper band of each doublet (M, 185,000 and 275,000) was first processed into the lower molecular weight band of each doublet (M, 170,000 and 260,000) and that the latter species were then processed into high molecular weight mucin(s).

Although in this experiment only one diffuse high molecular weight species was seen with MCF7 cells, in some experiments the high molecular weight species could be resolved into two distinct species. It is therefore likely that the high molecular weight mucin(s) shown in Fig. 2 actually represents two diffusely migrating species which are not always resolved by SDS-PAGE. Most other cell lines having the M, 170,000 and 260,000 precursor proteins also exhibited two high molecular weight proteins (see below).

To test the possibility that the M, 170,000 and 260,000 species formed a disulfide-linked complex, samples from a pulse-chase analysis were subjected to SDS-PAGE under nonreducing conditions (Fig. 2B). Since the same M, 170,000 and 260,000 species were observed under nonreducing conditions, these did not form a disulfide-linked complex.

It was possible that the M, 170,000 and 260,000 proteins were not actual precursors of high molecular weight mucin(s), but instead represented different molecules which fortuitously cross-reacted with antibody W1. If this were true, then blocking protein synthesis after pulse-labeling the M, 170,000 and 260,000 proteins should prevent appearance of the more slowly formed high molecular weight mucin(s). We therefore performed a pulse-chase analysis in which the chase was carried out in the presence of 10 \(\mu M\) cycloheximide (Fig. 2C). During this treatment, protein synthesis was inhibited by 85% (measured by trichloroacetic acid-insoluble radioactivity) during the 1-h chase period. Although the amounts of radioactivity in the M, 170,000 and 260,000 proteins as well as high molecular weight mucin(s) were reduced somewhat by this treatment, high molecular weight mucin(s) were formed with essentially the same kinetics as in untreated cells (Fig. 2A).

When the cycloheximide concentration was increased to 30 \(\mu M\), protein synthesis was inhibited by 90%, but high molecular weight mucin(s) were still formed (data not shown). Thus, cycloheximide treatment blocked protein synthesis by 85–90%, but did not prevent synthesis of high molecular weight mucin(s), suggesting that the appearance of the latter species was not dependent on protein synthesis during the chase period.

To investigate the processing of the M, 170,000 and 260,000 proteins into high molecular weight mucin(s), we tested the effects of the carboxylic ionophore, monensin, on mucin maturation. Monensin blocks post-translational processing in the Golgi apparatus of many proteins (42). As shown in Fig. 2D, treatment of cells with monensin prevented formation of high molecular weight mucin(s), resulting instead in a smear of radioactivity ascending upward from the M, 260,000 precursor protein. The appearance of larger species during monensin treatment, rather than smaller breakdown products, further
Antibodies—Since the structure of the epitope for the W1 proteins is conversion into high molecular weight species. The determinant for this antibody contained carbohydrate residues and therefore would not be present on initial translation products of the core protein gene. This possibility was suggested by our previous work showing that binding of this antibody was reduced by neuraminidase or periodate treatment of its antigen. To determine if earlier, lower molecular weight intermediates were involved in the biosynthesis of carcinoma associated mucins, we compared antigens precipitated by antibody W1 with those precipitated by antibody HMFG-2, which recognizes an epitope on the mucin core protein expressed in bacteria. MCF7 cells were pulse-labeled for 5 min with [3H]threonine and [3H]proline, washed, and resuspended in chase medium as described under "Experimental Procedures." At the time points indicated, aliquots were removed, washed, and subjected to immunoprecipitation analysis with monoclonal antibody W1 (+ lanes) or no monoclonal antibody (− lanes); B, as in A, except that SDS-PAGE was run under nonreducing conditions; C, as in A, except that 10 μM cycloheximide was included in the chase solutions; D, as in A, except that cells were incubated with 20 μM monensin for 30 min before labeling and during the labeling and chase periods. Solid lines indicate the positions of Mr 170,000 and 260,000 mucin precursors and the stacking gel (S).

In summary, the earliest products identified by immune precipitation analysis from extracts of pulse-labeled MCF7 cells were Mr 170,000, 185,000, 260,000, and 275,000 proteins. The Mr 185,000 and 275,000 proteins were not clearly resolved in this experiment, but in other experiments these were visible and were processed as in Fig. 2A.

In summary, the earliest products identified by immune precipitation analysis from extracts of pulse-labeled MCF7 cells were Mr 170,000, 185,000, 260,000, and 275,000 proteins. The Mr 185,000 and 275,000 proteins were turned over more rapidly than the Mr 170,000 and 260,000 proteins which, in turn, showed precursor-product kinetics with the high molecular weight mucin(s). The Mr 170,000 and 260,000 species were not disulfide-linked, and their conversion into high molecular weight antigen(s) was relatively insensitive to inhibition of protein synthesis, but sensitive to disruption by monensin, which blocks protein sorting in the Golgi. Thus, mucin precursors most likely undergo post-translational processing in the Golgi, before the appearance of high molecular weight mucin(s).

Comparison of Mucins from Different Cell Lines—Current evidence suggests that mucins from different individuals may show variation in the size of their core proteins. We were therefore interested to know how the mobilities of mucin precursors varied between different cell lines. We precipitated mucins from a total of seven different cell lines which had been labeled for a period of 1 h with a mixture of [3H]threonine and [3H]proline. All antibodies precipitated proteins of Mr 170,000 and 260,000, as well as a diffuse high molecular weight specie(s), which were identical in size with those precipitated by the W1 antibody (Mr 170,000, 185,000, 260,000, and 275,000), indicating that earlier intermediates were not detected by an antibody that reacts with mucin core protein(s) (data not shown).

Other antibodies were also compared for their abilities to precipitate mucin precursors. We compared antibodies P36/22 (4), 115D8 (3), HMFG-2, and M38 (48), all of which recognize mucin epitopes, from extracts of MCF7 cells which had been labeled for 1 h with a mixture of [3H]threonine and [3H]proline (data not shown). All antibodies precipitated proteins of Mr 170,000 and 260,000, as well as a diffuse high molecular weight specie(s), which were identical in size with those precipitated by antibody W1. In similar experiments, we found that monoclonal antibodies M15, M22, M23, and M27, which are reactive with deglycosylated mucin from human milk (48), also precipitated the Mr 170,000 and 260,000 proteins. These findings provide further evidence that the Mr 170,000 and 260,000 proteins are precursors of high molecular weight mucin(s) made by MCF7 cells.

Comparison of Mucins from Different Cell Lines—Current evidence suggests that mucins from different individuals may show variation in the size of their core proteins.
As shown in Fig. 4, a M₁, 260,000 precursor band was precipitated from all seven lines, and a M₂, 170,000 precursor band was precipitated from four of seven lines. The major precursor bands exhibited by the T47-D and ZR75.1 cell lines migrated slightly faster and slower, respectively, than the M₁, 170,000 band seen with MCF7 cells. W5-6 cells gave only a M₁, 260,000 precursor band. The high molecular weight mucins from these lines showed considerable differences in mobility. Some lines (MCF7, HTB81, and W5-6) gave only single diffuse high molecular weight species, although, as noted above, the MCF7 cell line in other experiments gave two high molecular weight species. Other lines gave two distinct high molecular weight species. It is interesting to note that the W5-6 line also exhibited just one precursor band. Thus, in most cases, variation on migration of mature mucins from different cell lines reflected variation in migration of the mucin precursors. Most cell lines which gave rise to two mucin precursors also gave rise to two high molecular weight mucin(s).

In an experiment to test whether the M₁, 170,000 and 260,000 mucin precursors gave rise to different high molecular weight mucin(s), we performed a pulse-chase analysis identical to that described in Fig. 2A with the ZR75.1 cell line, which yielded two precursor proteins and two high molecular weight mucin(s) (Fig. 4). The results of this experiment (data not shown) were that both high molecular weight mucin(s) rose in parallel, with kinetics inversely related to the kinetics of disappearance of the precursor proteins. This result suggested that each of the precursor proteins made by this cell line were converted into different high molecular weight mucin(s).

Time of Transit of Mature Mucin to the Cell Surface—To obtain further evidence that the early labeled species were mucin precursors, we measured the kinetics of appearance of mature mucin(s) on the cell surface of MCF7 cells, using the cell surface immunoprecipitation technique of Spiro et al. (36). The rationale for this experiment was that mucin precursors should be localized intracellularly and thus would be inaccessible to antibody mixed with intact cells. In contrast, mature mucin(s) are localized at the cell surface (32) and are accessible to antibody added outside the cell.

As shown in Fig. 5, mature mucin was first detected at the cell surface 0.5 h after the pulse-labeling period (15 min). The sample precipitated with no monoclonal antibody was from cells lysed before antibody addition and contains more nonspecific proteins than cell surface immunoprecipitations performed without added antibody but contains high molecular weight mucins. The amount of cell surface accessible mucin reached a maximum at 1 h (half-time of 0.75 h), but at no time was either the M₁, 170,000 or M₂, 260,000 precursor detected at the cell surface.

Analysis of the Carbohydrate Composition of W1 Antigens—The fact that the M₁, 170,000 and 260,000 precursors were labeled with [³H]glucosamine (Fig. 1) indicated that they were glycoproteins. To characterize the carbohydrate residues present on mucin precursors and mature mucin(s), we tested for the presence of N- and O-linked oligosaccharides via chains by determining sensitivities of the various forms to glycosidases of known specificity (Fig. 6, and data not shown).

MCF7 cells were labeled under conditions which give rise to either the precursor doublets (Fig. 6A) or mature mucin(s) (Fig. 6B). Immunoprecipitates were prepared, and labeled proteins were tested for sensitivity to the following enzymes: endoglycosidase H (cleaves high mannose N-linked oligosaccharides (43)), N-glycanase (cleaves most N-linked oligosaccharides (44)), and neuraminidase (cleaves terminal sialic acid residues). Ingredients of some of the digestion buffers altered the protein migration such that the upper band of each doublet was not resolved during SDS-PAGE.

As shown in Fig. 6, the M₁, 170,000 and 260,000 precursors were sensitive to both endoglycosidase H and to N-glycanase, indicating that both precursors contain high mannose-type N-linked oligosaccharides. The upper bands of each doublet were also sensitive to endoglycosidase H. The precursors were insensitive to neuraminidase.
Precursor and mature mucin forms were labeled with [3H]threonine or [3H]proline. By using this technique, background levels of radioactivity eluting with α-amino acid were higher than they were with thin layer chromatography, which made it difficult to discriminate the degree of substitution associated with the mucin precursors. However, in two experiments with the high molecular weight mucin(s), we determined 80.7 and 92% conversion of [3H]threonine to α-[3H] α-amino butyric acid.

We conclude that initiation of most threonine-linked oligosaccharide chains occurred concomitant with mucin maturation. O-Linked oligosaccharides present on the mucin precursors must occupy less than 3% of threonines in the core protein(s).

**Table I**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thr</th>
<th>α-ABA</th>
<th>Substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>993</td>
<td>22</td>
<td>2.2</td>
</tr>
<tr>
<td>170,000</td>
<td>1,654</td>
<td>44</td>
<td>2.6</td>
</tr>
<tr>
<td>260,000</td>
<td>3,163</td>
<td>46</td>
<td>1.4</td>
</tr>
<tr>
<td>Mature</td>
<td>157</td>
<td>489</td>
<td>75.7</td>
</tr>
</tbody>
</table>

**Discussion**

We have identified novel intermediates in the biosynthesis of mucin glycoproteins by the breast carcinoma cell line, MCF7. Four proteins of M, 170,000, 185,000, 260,000, and 275,000 were immunoprecipitated by antibody W1 from MCF7 cells which had been pulse-labeled with [3H]threonine and [3H]proline. Kinetic evidence suggested that the M, 185,000 and 275,000 proteins were precursors of the M, 170,000 and 260,000 proteins, respectively. That the M,
170,000 and 260,000 proteins were direct precursors of high molecular weight mucin(s) was supported by kinetic evidence, the results of inhibitor and cell surface immunoprecipitation studies, and immunoprecipitation analysis with different antibodies and cell lines. In most cell lines examined (Fig. 4), the numbers and sizes of the precursor proteins observed after a 1-h labeling period correlated closely with the numbers and sizes of high molecular weight mucin(s). In four of seven lines, two precursor proteins and two high molecular weight mucin(s) were observed. In one line, W5-6, only a single precursor protein and high molecular weight mucin(s) were detected. Electrophoretic mobilities of precursor proteins from these five cell lines correlated roughly with the mobilities of mature mucin(s) in their respective lines, suggesting that different sized precursors gave rise to different sized mature mucin(s). This was further supported by results of a pulse-chase experiment performed with the ZR75.1 cell line, which gave two precursor proteins and two high molecular weight mucin(s) (see discussion for Fig. 4).

It would seem likely that the $M_1$, 170,000 and 260,000 precursors represent products of different alleles at the polymorphic PUM locus (21). Swallow et al. (21) suggested that different alleles of PUM locus encode different sized core proteins. Thus, the $M_1$, 170,000 and 260,000 mucin precursors may be derived from different sized core protein genes. This explanation is consistent with observations of Gendler et al. (20) who showed that the cell lines MCF7 and T47-D, which we have shown to express two different sized mucin precursors (Fig. 4), express two different sized mRNAs for the mucin core protein(s).

The intermediates in mucin biosynthesis which we have identified are significantly larger than estimates (20) of the size of the mucin core protein from human milk ($M_1$, ~68,000). Since the mucin precursors are glycosylated, some of this size difference is due to N-linked oligosaccharide side chains added cotranslationally. It is also possible that a small number of O-linked oligosaccharides, undetectable by the methods we used in Table I, are present on the mucin precursors. It is well known that the addition of a relatively low mass of O-linked oligosaccharide can result in disproportionate effects on glycoprotein mobility during SDS-PAGE (24–25).

Our data indicate that factors other than size of the core protein(s) can affect the mobility of high molecular weight mucin(s) during SDS-PAGE. The cell lines MCF7, Hep2, and HTB81 all expressed mucin precursors of the same sizes, but expressed high molecular weight mucin(s) which differed in mobility. Since the degree of threonine-linked glycosylation in MCF7 cells increased greatly as mucin precursors matured (Table I), the different electrophoretic mobilities of mature mucin(s) from these three cell lines may be due to differences in numbers or compositions of O-linked oligosaccharide chains. It is also possible that other posttranslational modifications such as sulfation (see Fig. 1) may affect electrophoretic mobility.

The mucin precursors contained high mannose N-linked oligosaccharide side chains, as suggested by their susceptibility to endoglycosidase H (Fig. 6). Initiation of N-linked oligosaccharide synthesis thus preceded initiation of most O-linked oligosaccharides. Evidence for N-linked glycosylation of breast carcinoma associated mucin(s) has not been previously published, although a preliminary report on carcinoma associated mucin biosynthesis also claimed similar results (31).

Current evidence is conflicting on the timing of initiation of O-linked glycosylation. Some investigators have reported evidence that initiation of O-linked glycosylation is essentially cotranslational. Strous (23) showed that nascent peptides (between 40 and 60 amino acids in length) from rat gastric membrane-bound polysomes contain GalNAc. Cummings et al. (24) showed that addition of GalNAc to the low density lipoprotein receptor preceded the processing of N-linked oligosaccharides in the Golgi. Likewise, Johnson et al. (25) observed the addition of O-linked oligosaccharides as an early event in the biosynthesis of glycophrin. In contrast, other investigators have suggested that initiation of O-linked oligosaccharides is a later biosynthetic event, occurring in smooth membrane organelles (presumably the Golgi) after N-linked glycosylation (26–29). Finally, Spielman et al. (30) have proposed that initiation of O-linked glycosylation of rat asialotransferrin is a continuous event, occurring throughout the period of transit between the rough endoplasmic reticulum and the cell surface.

Our data indicate that most of the O-linked oligosaccharide side chains on breast carcinoma associated mucin(s) are initiated relatively late in their biosynthetic pathway, well after initiation of N-linked chains, which occurs within 5 min of synthesis. Thus, our results on the timing of initiation of O-linked oligosaccharide chains differ from those reported by others (23–25, 30). It is possible that different sites of O-linked glycosylation are characteristics of different systems. Our evidence for late initiation of O-linked oligosaccharide chains on breast carcinoma associated mucin(s) may reflect the unique properties of these proteins, which are unusual in their extremely high degree of glycosylation.

Acknowledgments—We wish to thank Steve Hartman for performing amino acid analyses, Vince Ochs for technical assistance, and Drs. Jaques Garriques and Wen Chang for critical reviews of the manuscript.

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