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Biosynthesis of the subunits of factor VIIIR by bovine aortic endothelial cells

(immunoprecipitation/peptide mapping/tunicamycin/von Willebrand factor)

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ABSTRACT **The biosynthesis of the subunit of factor VIIIR was studied in bovine aortic endothelial cells by the techniques of immunoprecipitation and NaDodSO$_4$/polyacrylamide gel electrophoresis. It was determined that the subunit is first produced as a M_r 240,000 glycoprotein precursor, which appears to undergo proteolytic cleavage at or about the time of secretion into the medium with a resultant change in apparent M_r to 225,000, the size of the mature subunit found in plasma. The M_r 240,000 species was detected within 10 min of the start of labeling of cells, but factor VIIIR was not detected in the culture medium until ≈50 min. Treatment of the cells with tunicamycin resulted in a decrease in the apparent M_r of both species but did not grossly inhibit processing of precursor to product or the secretion of the latter. Thus, much or all *N*-linked glycosylation of factor VIIIR is not essential for these steps. Accumulation of factor VIIIR in the medium continued over a 24-hr period of cell labeling with [^{35}S]-methionine, without significant net intracellular accumulation of the precursor, suggesting that a large storage pool of factor VIIIR is not present in endothelial cells under these conditions.**

Mammalian clotting factor VIII is a complex of two distinct molecules, factor VIIIR (von Willebrand factor) and factor VIIIC (antihemophilic factor). Disorders of factor VIIIR function are manifest in the diverse clinical presentations of von Willebrand disease, an autosomal disorder (1).

Factor VIIIR circulates in plasma as a series of aggregated structures composed of an apparently single glycosylated subunit of M_r 225,000 (2, 3). These structures appear to consist of at least two subunits, and the reported M_rs of these complexes fall in the range of 450,000 to 20,000,000 (4–10). The aggregates appear to be held together by disulfide bonds, as they are stable in high ionic strength medium and in 2% NaDodSO$_4$, but dissociate into monomers upon mild reduction (2, 3). Different forms of von Willebrand disease have been characterized as having (*i*) decreased amounts of total plasma factor VIIIR but a normal distribution of aggregate sizes; (*ii*) abnormal aggregate distribution but normal total amount of factor VIIIR; and (*iii*) near normal amount and size distribution but altered functional state of these molecules (4–10). In addition, abnormalities in the carbohydrate portion of the molecule have been reported (11). Thus, a number of distinct genotypes appear to underlie the appearance of this disease.

Factor VIIIR is synthesized in megakaryocytes, but the primary site of synthesis in all species studied to date appears to be the endothelial cell (12, 13). Although it is a secreted glycoprotein, little is known of the mechanism of biosynthesis of the mature, secreted molecule. Experiments to be described here address this subject.

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MATERIALS AND METHODS

Cells. Aortae were obtained from just killed 7- to 10-day-old calves, and endothelial cells were isolated by the collagenase method of Gimbrone (14). Cells were grown at 37°C in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (GIBCO) in a 7% CO_2-containing atmosphere. Cells were studied between their second and ninth passages.

Reagents. Purified factor VIIIR, bovine plasma fibronectin, and monospecific rabbit antibodies to these proteins were isolated as described (2, 15). Tunicamycin was obtained from Calbiochem–Behring, and [^{35}S]methionine (≈1,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} Bq) was purchased from New England Nuclear. Other chemicals were generally of reagent grade or highest purity available.

Labeling of Cultures. Confluent dishes of cells were labeled with [^{35}S]methionine (50–125 μCi/ml) in methionine-free, Dulbecco's modified Eagle's medium lacking serum but containing 5 mg of bovine serum albumin (Sigma) per ml (labeling medium). Albumin was added as a carrier to help prevent degradation of proteins secreted into the medium and their adherence to surfaces during immunoprecipitation. After the growth medium was drained, 0.8 ml of labeling medium was added to 60-mm dishes, and 1.5 ml was added to 100-mm dishes. For the chase segments of pulse–chase experiments, pulse-labeled dishes were rinsed once with Hanks' buffered saline and incubated in serum-free Dulbecco's medium containing 0.2 mM methionine and 5 mg of albumin per ml. When tunicamycin was added to various cultures, it was present at a concentration of 3 μg/ml for 3 hr in growth medium prior to the start of labeling.

Immunoprecipitation. At the indicated times, medium was removed from a labeled culture and centrifuged at room temperature in an Eppendorf Microfuge for 1–2 min to remove any cellular debris. Nonidet P-40 (Sigma), NaDodSO$_4$, Trasylol (Mobay Chemicals, New York), and phenylmethylsulfonyl fluoride were added to final concentrations of 1%, 0.1%, 100 units/ml, and 100 μg/ml, respectively. To prepare cell lysates from such cultures, dishes were rinsed twice with Hanks' buffered saline and then rocked at 4°C for 15 min with lysing buffer (40 μl/cm^2) containing 0.02 M Tris·HCl (pH 8), 1% Nonidet P-40, 0.1% NaDodSO$_4$, 0.15 M NaCl, phenylmethylsulfonyl chloride (100 μg/ml), and Trasylol (100 units/ml). Each plate was then scraped with a rubber policeman, and the lysate was centrifuged for 1–2 min in an Eppendorf Microfuge to remove debris. Subsequent anti-factor VIIIR immunoprecipitation of this pelleted material revealed a small amount (<5%) of the same spe-

cific band present in the soluble portion; no additional, specific bands were noted. Samples (0.8 ml) were then preabsorbed for 30–60 min at 4°C with 50 μl of formalin-treated *Staphylococcus aureus* (IgGsorb, Enzyme Center, Boston) and 5 μl of normal rabbit serum. Rabbit antiserum to bovine factor VIIIR or, where indicated preimmune rabbit serum (rabbit serum prior to immunization; 0.6 μl), and a 5-μl packed volume of protein A-Sepharose beads (Sigma) were then mixed with 1 ml of lysing buffer, and the suspension was incubated on a rocking platform for 1 hr at room temperature. The beads were pelleted, and the preadsorbed sample, noted above, was added to them. Immunoprecipitation was then carried out for ≈16 hr at 4°C. The beads were washed four times in lysing buffer made 1% in 2-mercaptoethanol, and the bound antigen(s) were eluted by boiling for 5 min in 0.02 M Tris·HCl, pH 9/10% 2-mercaptoethanol/5% $NaDodSO_4$/20% glycerol.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis and Partial Protease Digestion. $NaDodSO_4$ gel electrophoresis was performed by the method of Laemmli (16). Gels were stained with Coomassie brilliant blue to visualize marker proteins, fluorographed with EN^{3}HANCE (New England Nuclear), and dried. For quantitation, dried gel bands were located from the fluorograph and excised from the gel, and their radioactive content was measured in a scintillation counter.

Partial protease digestion experiments were performed by the method of Cleveland *et al.* (17) with minor modifications. Radioactive bands were localized in stained gels by reference to markers, excised from the wet gel, crushed, and eluted overnight at 37°C in 50 mM ammonium bicarbonate (pH 7) containing 50 μg of ovalbumin per ml and 0.1% $NaDodSO_4$. The eluate was lyophilized and dissolved in ≈0.1 vol of buffer A (0.125 M Tris·HCl, pH 6.5/0.01 M EDTA/20% glycerol). The samples were then loaded atop a 16 cm × 25 cm × 1 mm 7.5–20% acrylamide gradient gel overlaid with various concentrations of *Staphylococcus aureus* V8 protease (Miles–Yeda, Rehovot, Israel) in buffer A containing 10% glycerol. Gels were electrophoresed at room temperature until the marker dye (Coomassie brilliant blue) reached the bottom. Gels were then stained and fluorographed as noted above.

RESULTS

Factor VIIIR Antigen Species Detected by Specific Immunoprecipitation of *in Vivo* Labeled Protein. As noted in Fig. 1, anti-factor VIIIR immunoprecipitation of the culture medium after a 4-hr labeling period with [^{35}S]methionine revealed a single band of M_r 225,000 comigrating with authentic, purified bovine factor VIIIR. No such band was identified with the preimmune serum. Because of the similar migration positions of the M_r 225,000 band and fibronectin, the fact that under some conditions both polypeptides can bind nonspecifically to solid surfaces, and the fact that fibronectin is also a glycoprotein produced and secreted by bovine endothelial cells in considerable excess relative to factor VIIIR (15), special care was taken to ensure that the specific bands observed after anti-factor VIIIR immunoprecipitation lacked fibronectin. First, prolonged electrophoresis of the M_r 225,000 anti-factor VIIIR reactive band from the culture medium revealed it to migrate faster than authentic fibronectin (Fig. 1 *Right*). Second, addition of sufficient purified fibronectin to inhibit >95% of [^{35}S]-labeled fibronectin immunoprecipitation by rabbit antibody to bovine fibronectin did not interfere with the anti-factor VIIIR immunoprecipitation of the M_r 225,000 band (Table 1). Thus, this polypeptide is immunologically and physically distinct from fibronectin.

The endothelial cell lysate also was immunoprecipitated, and

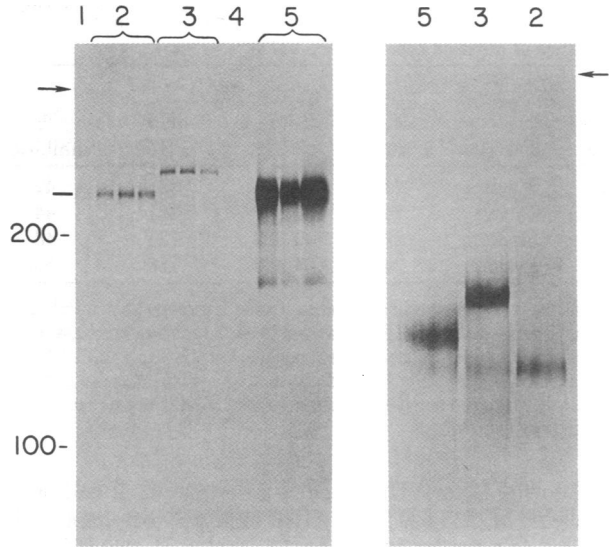

FIG. 1. Anti-factor VIIIR immunoprecipitation of cell medium and cell lysate from a culture of bovine endothelial cells. The arrows indicate the interface of stacking and running gels. M_rs are shown × 10^{-3}. (*Left*) The acrylamide concentration of the running gel was 6%. The bar indicates the migration position of purified M_r 225,000 bovine plasma factor VIIIR. Cells were labeled for 4 hr with [^{35}S]methionine. At that time, the cell medium was harvested, and a cell lysate was prepared. Lanes: 1, immunoprecipitation of cell medium with normal rabbit serum; 2, same fraction immunoprecipitated with rabbit anti-bovine factor VIIIR; 3, immunoprecipitation of cell lysate with anti-factor VIIIR; 4, same as lane 3 but with normal rabbit serum; 5, immunoprecipitation of half the volume of medium used for lanes 1 and 2 by rabbit anti-bovine fibronectin. (*Right*) To demonstrate clear resolution of fibronectin from the M_r 225,000 anti-factor VIIIR band, the same proteins were electrophoresed for 24 hr through a 5% acrylamide gel. Lanes in *Right* correspond to those in *Left* except that the anti-fibronectin immunoprecipitation was performed with 1/20th the volume of medium used in the anti-factor VIIIR immunoprecipitations (lanes 2, both gels). The slowly migrating minor band seen in lane 2 *Left* was infrequently noted. It is not seen in any other immunoprecipitations presented in this paper.

there was virtually no M_r 225,000 band present. Instead, an immunoreactive species migrating with an apparent M_r of ≈240,000 was detected. A band comigrating with the intracellular protein was not found in the medium in repeated experiments, and only trace amounts of the 225,000 protein were ever detected in the cellular fraction. To demonstrate that the M_r 240,000 intracellular species was specifically immunoprecipitated by antibody to factor VIIIR, competition experiments with factor VIIIR purified from bovine plasma were performed. Limiting amounts of purified factor VIIIR were used here with the goal of assessing the relative affinities of the two proteins for the anti-factor VIIIR antibody. The amount of pu-

Table 1. Immunoprecipitation of medium in the presence and absence of purified bovine fibronectin (FN)

Gel band	Antibody	cpm Without FN	cpm With FN	% inhibition
M_r 225,000	Factor VIIIR	905	1,068	None
FN	FN	20,050	755	96

Immunoprecipitation of extracts of [^{35}S]methionine-labeled bovine endothelial cells was performed as described in *Materials and Methods* except that, in duplicate reactions, excess purified bovine fibronectin (4 μg) was added along with the radioactive extract. Gel bands were located from the fluorogram, excised from the dried gel, and assayed in a scintillation counter. Results are expressed in counts per minute.

Table 2. Factor VIIIR immunoprecipitation in the presence and absence of purified bovine factor VIIIR

Exp.	Fraction	$M_r \times 10^{-3}$	cpm Without VIIIR	cpm With VIIIR	% inhibition
1	Medium	225	905	529	42
	Lysate	240	1,008	561	44
2	Medium	225	1,389	723	48
	Lysate	240	1,483	736	50

Samples were assayed as noted in Table 1, except that purified M_r 225,000 bovine factor VIIIR was added to each reaction mixture (at the time of addition of the crude, radioactive antigen) to inhibit the immunoprecipitation of the labeled product by ≈50%. Results with two separately prepared fractions of purified M_r 225,000 bovine plasma factor VIIIR are shown.

rified bovine factor VIIIR needed to inhibit about half of the ^{35}S-labeled M_r 225,000 factor VIIIR immunoprecipitation, also inhibited to a similar extent the ^{35}S-labeled M_r 240,000 factor VIIIR immunoprecipitation (Table 2). This demonstrates that the antibody to factor VIIIR has approximately the same affinity for the M_r 240,000 and M_r 225,000 factor VIIIR species, and that these species are immunologically indistinguishable.

Because of the relatively high carbohydrate content of bovine plasma factor VIIIR (2), it was possible that observed differences in NaDodSO$_4$ gel migration between the two species might relate to the degree of glycosylation. To test this possibility, cells were labeled in the presence of tunicamycin to prevent dolichol-pyrophosphate-mediated glycosylation. When extracted from cells exposed to this drug, both the intracellular band and the species identified in the medium migrated faster than the same polypeptides isolated from untreated cells (Fig. 2). Although some reduction in the apparent migration difference between the anti-factor VIIIR reactive intracellular and extracellular polypeptides was apparent after drug treatment, it remained significant and suggested an apparent M_r difference of more than 10,000.

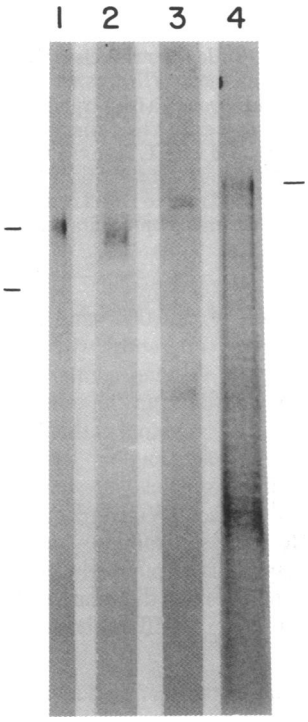

FIG. 2. Effect of tunicamycin on the gel migration of anti-factor VIIIR reactive proteins. Bovine endothelial cells were labeled with [^{35}S]-methionine for 4 hr in the presence and absence of tunicamycin. The upper bar on the left represents the migration position of purified M_r 225,000 bovine plasma factor VIIIR. The lower bar on the left marks migration of myosin (M_r, 200,000). The bar on the right represents the migration position of the factor VIIIR intracellular precursor (M_r, 240,000). Lanes: 1 and 4, anti-factor VIIIR immunoprecipitation of medium and lysate, respectively, from a culture labeled in the absence of tunicamycin (as in Fig. 1); 2 and 3, anti-factor VIIIR immunoprecipitation of medium and lysate from an identical culture, labeled in the presence of tunicamycin.

To further evaluate the relationship between the M_r 240,000 and the M_r 225,000 species, both bands were eluted from gels and subjected to partial degradation with increasing concentrations of *S. aureus* V8 protease. The two species yielded very similar partial digestion patterns, although two additional bands were noted in the digest of the intracellular species, perhaps due to additional sequence in the M_r 240,000 protein (Fig. 3). Thus, the primary structures of these two polypeptides are highly homologous. Further support for this contention came from the results of a pulse–chase experiment (Fig. 4). Specifically, during the 60-min pulse period, the M_r 240,000 band was the major labeled species. During the 10-hr chase period, most of the radioactivity in this band disappeared and became apparent in the anti-factor VIIIR-precipitated M_r 225,000 band in the medium. Thus, a M_r 240,000 precursor–225,000 product relationship is suggested by these data. Moreover, these results again demonstrate that significant amounts of mature M_r 225,000 factor VIIIR did not appear in the cell extract under the conditions used here.

Time Course of Biosynthesis and Distribution of the Various Factor VIIIR Polypeptides. The M_r 240,000 intracellular species was readily detected 10 min after the start of labeling, and accumulation of label in the M_r 240,000 band proceeded through an initial, slow phase (Fig. 5). This lag probably reflects (*i*) incorporation of radioactive amino acid into carboxyl-terminal ends of polypeptide chains whose synthesis was initiated prior to the start of labeling and (*ii*) gradual equilibration of radioactive methionine with the existing intracellular methionine pool. Labeling for between 0.5–33 hr resulted in a rapid accumulation of the M_r 240,000 intracellular form, culminating in the appearance of a steady-state plateau concentration of this

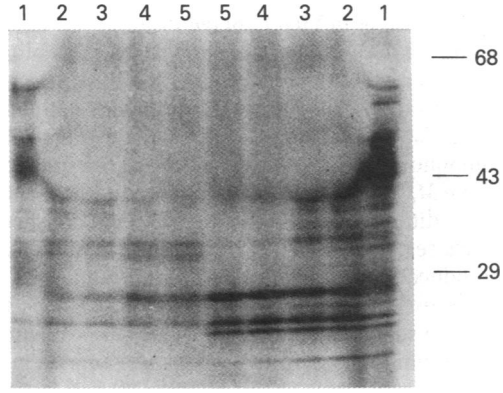

FIG. 3. Protease V8 digestions of M_r 240,000 and M_r 225,000 anti-factor VIIIR reactive proteins. Aliquots of each of these two bands excised and eluted from NaDodSO$_4$/polyacrylamide gels were exposed to various concentrations of V8 protease, and the resulting products were analyzed. The numbers at the top of the lanes refer to concentrations of V8: 5, 50 μg/ml; 4, 30 μg/ml; 3, 10 μg/ml; 2, 5 μg/ml; and 1, 1 μg/ml. Digests of the M_r 225,000 species are in five left lanes, and digests of the 240,000 species are in the five right lanes. M_rs are shown × 10^{-3}. Because no digestion products of higher M_r than those shown were identified, only the lower half of the gel is reproduced here.

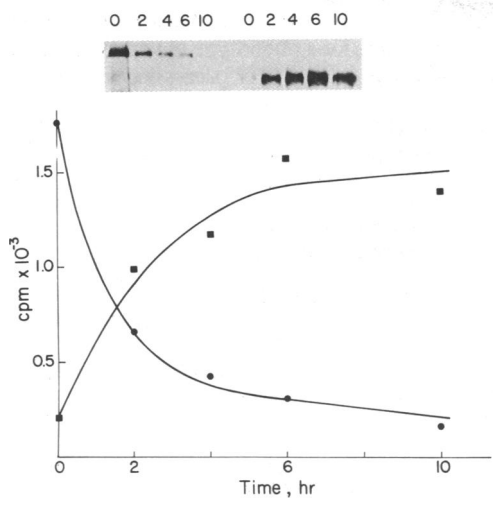

FIG. 4. Pulse–chase analysis of replicate endothelial cell cultures. Five cultures were labeled for 1 hr with [^{35}S]methionine and then chased in nonradioactive methionine-containing medium. (*Upper*) Immunoprecipitation results with cell lysate are shown in the left five lanes and with medium are shown in the right five lanes. Numbers at the tops of the lanes indicate hours of chase. (*Lower*) Radioactivity was measured in the excised M_r 240,000 (●) and M_r 225,000 (■) bands in a scintillation counter as described.

molecule (Fig. 6). In contrast, the results of pulse–chase experiments, where a short labeling period (15 min) was used, and results of labeling vs. time studies revealed that the first appearance of M_r 225,000 factor VIIIR in the medium did not occur until 50 min (data not shown). This is significantly longer than the 30-min delay found for bovine endothelial cell fibronectin in another cell type (18). From that point on, M_r 225,000 factor VIIIR accumulated continuously during a labeling period of 24 hr (Fig. 6). In experiments where labeling was performed in the presence of fetal bovine serum, incorporation of [^{35}S]methionine into the M_r 225,000 band continued for at least 48 hr (data not shown). This result is compatible with the finding of a continual increase in the concentration of immunoreactive factor VIIIR over a 6-day period (19) in prior studies of factor VIIIR accumulation in the medium of endothelial cells cultivated in the presence of serum. These observations clearly show that, under the conditions studied, bovine endothelial cells do not accumulate a major intracellular storage pool of M_r 240,000 protein relative to the amount of M_r 225,000 mature factor VIIIR which is secreted.

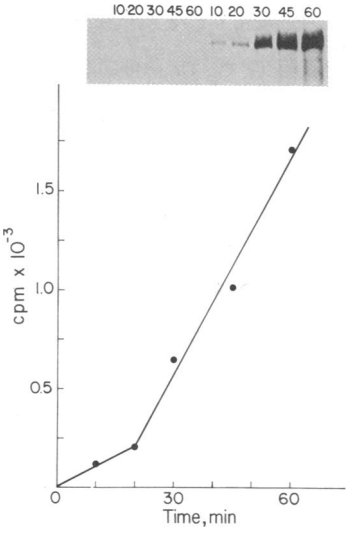

FIG. 5. Immunoprecipitation of endothelial cell extracts and culture medium during 1 hr of labeling. Five replicate cultures (in 60-mm plates) of endothelial cells were labeled for the indicated times with radioactive methionine. Cell extracts and medium from each was then collected and subjected to anti-factor VIIIR immunoprecipitation analysis. (*Upper*) Immunoprecipitation of medium (left five lanes) and cell lysates (right five lanes). Numbers at the tops of lanes indicate the time of labeling in minutes. (*Lower*) Radioactivity was assayed in the excised M_r 240,000 band. (In the original fluorograph a faint M_r 225,000 band was visible at 60 min.)

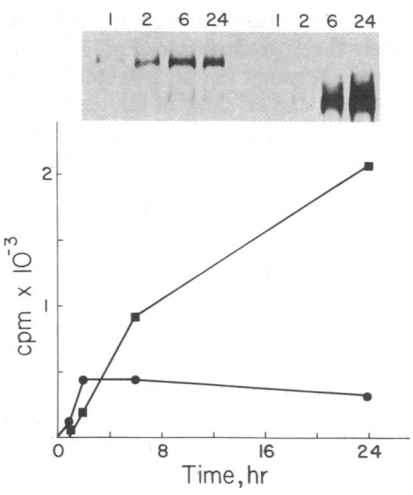

FIG. 6. Immunoprecipitation of endothelial cell extracts and culture medium during a 24-hr labeling period. The same methods outlined in the legend to Fig. 5 were used here. (*Upper*) Immunoprecipitation of cell lysates (left four lanes) and medium (right four lanes). Numbers at the tops of lanes indicate hours of labeling. (*Lower*) Radioactivity was assayed in the excised M_r 240,000 band (●) and the M_r 225,000 band (■).

DISCUSSION

The results presented here describe events in the biosynthesis of factor VIIIR subunits by bovine aortic endothelial cells. Our findings indicate that an intracellular precursor form of factor VIIIR is produced first, followed by a rather long time interval before the molecule is secreted in a form considerably smaller (M_r, 225,000) than its intracellular precursor (M_r, 240,000). Moreover, an intracellular pool of M_r 240,000 molecules did not continue to accumulate. Rather such molecules were continuously processed, and the M_r 225,000 product(s) was exported from the cell, resulting in only a limited intracellular content of the 240,000 species.

When the precursor was first detected at 10 min after labeling, it was found to be glycosylated, as defined by a significant alteration in mobility after tunicamycin treatment. The major portion of the apparent migrational difference between the intracellular and extracellular forms of factor VIIIR is probably due to extra amino acid sequence present in the former and absent in the latter. Support for this contention arises from the observation that, after tunicamycin treatment (Fig. 2), a substantial $NaDodSO_4$ gel migrational difference between the two forms persisted. In addition to *N*-linked oligosaccharide moieties, the synthesis of which is blocked by tunicamycin, bovine factor VIIIR likely contains significant amounts of *O*-linked oligosaccharide. [Human factor VIIIR, a structurally and functionally similar protein, is reported to have 30% of its carbohydrate in *O*-linkage (20, 21).] Serine- and threonine-linked sugars are thought to be added by individual enzyme systems in a less concerted fashion than is the case for the asparagine-linked system (22). If these piecemeal carbohydrate additions

were responsible for the observed migration differences, it would seem more likely that a gradual transition in gel migration position would occur between the M_r 240,000 precursor and the M_r 225,000 product, and an array of intermediate-size species might be seen during a pulse–chase experiment. In fact, the presence of a relatively sharp intracellular precursor band, under all conditions tested, suggests an insensitivity of migration rate to carbohydrate composition after the original dolichol/pyrophosphate reaction has occurred. In addition, if an apparent M_r difference of >10,000 were derived from small carbohydrate linkages scattered over the length of the polypeptide chain, numerous differences might have been expected in the protease maps of the M_r 240,000 and M_r 225,000 species, rather than the high degree of similarity noted. Moreover, it is unlikely that carbohydrate addition would result in more rapid gel migration. Such additions generally retard electrophoretic migration (23). It is likely, then, that the observed size difference reflects a difference in primary amino acid sequence between the two forms. In effect, then, it suggests that the M_r 240,000 species is a pro or prepro species of factor VIIIR.

The functional significance of pro-factor VIIIR is unclear. In some systems, a precursor form is felt to be instrumental in the proper assembly of tertiary structure, as in the case of proinsulin (24), or of quarternary structure, as in the case of procollagen (25). A molecule as large as factor VIIIR may display a high degree of each type of structure, and if assembly of factor VIIIR aggregates occurs intracellularly, this might explain the relatively long time between pro-factor VIIIR synthesis and the secretion of mature factor VIIIR. Because only insignificant amounts of mature factor VIIIR are found in cellular lysates, it seems unlikely that the putative cleavage causing the reduction in apparent M_r could occur until just prior to secretion. However, because no pro-factor VIIIR has been detected in the medium, cleavage likely occurs prior to or during release. Therefore, the conversion of pro-factor VIIIR to mature factor VIIIR may be part of the secretion mechanism. An explanation of this effect may lie in a bifunctional role for factor VIIIR (as both a plasma and a membrane protein). In this regard, some of the factor VIIIR present in megakaryocytes appears to be incorporated into the platelet membrane (26, 27). Moreover, the fact that the conversion of pro-factor VIIIR to mature factor VIIIR and the compartmentalization of each form continue in the presence of tunicamycin suggests that the putative cleavage process is largely insensitive to the *N*-linked carbohydrate content of the protein.

In conclusion, the biosynthesis of factor VIIIR subunits proceeds by a complex mechanism commencing with the synthesis of a discrete intracellular precursor molecule. If one or more specific cleavages are required for the secretion and normal biologic function of factor VIIIR in the circulation and the enzyme(s) involved is (are) relatively specific for this system, then at least one other gene product is involved in production of normal factor VIIIR. In that case, deficiencies in this gene product could be responsible for a phenotype of von Willebrand disease involving ineffective or improper processing of pro-factor VIIIR.

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