We examined the biosynthetic processing and assembly of the platelet glycoprotein (GP) IIb-IIIa complex in [35S]methionine-labeled HEL cells, a human cell line with features of megakaryocytes. Both GP IIb and GPIIIa were synthesized as single-chain precursors to which high mannose N-linked oligosaccharides were added in the endoplasmic reticulum (ER). A 5-fold excess of the major IIb precursor, preIIb, was synthesized relative to GPIIIa. Two smaller proteins immunologically related to GP IIb were synthesized in smaller amounts. Assembly of the preIIb and GPIIIa precursors required 4–6 h for completion. All GPIIIa molecules were eventually assembled; the excess preIIb precursors were degraded without reaching the cell surface. Following assembly, preIIb-IIIa complexes were rapidly transported to the Golgi apparatus where preIIb underwent modification of high mannose chains into complex oligosaccharides and proteolytic cleavage to yield disulfide-linked heavy and light chains. Pre-treating cells with the ionophore monensin blocked cleavage of preIIb but not its carbohydrate modification or its assembly with GPIIIa. These studies suggest that 1) assembly of the precursors of GP IIb and GPIIIa in the ER is a slow process requiring conformational maturation of one or both subunits, and 2) only heterodimers assembled in the ER are transported to the Golgi apparatus for additional processing and, ultimately, expression on the cell surface.

The platelet membrane glycoprotein (GP) IIb-IIIa complex plays a central role in the hemostatic response to vessel injury. Following platelet activation, GP IIb-IIIa becomes competent to bind the extracellular adhesive proteins fibrinogen, von Willebrand factor, fibronectin, and vitronectin. In addition, it interacts with intracellular cytoskeletal proteins. These interactions support platelet aggregation, spreading of platelets on subendothelial surfaces, and clot retraction (reviewed in Phillips et al., 1988). The importance of GP IIb-IIIa in platelet function is underscored by the bleeding complications observed in patients with Glanzmann’s thrombasthenia, a congenital disorder in which platelets manifest qualitative or quantitative deficiencies in this receptor complex (reviewed in McEver and Majerus, 1989).

The structural features of GP IIb-IIIa have been analyzed in detail. GP IIb (M, 140,000) and GPIIa (M, 105,000) are large cysteine-rich glycoproteins that form noncovalently associated heterodimers in the platelet membrane (McEver et al., 1985, 1986; Jennings and Phillips, 1982; Carrell et al., 1985). GPIIb consists of a heavy chain (GPIIba, M, 123,000) linked by disulfide bonds to a light chain (GPIIb\α, M, 23,000) (Phillips and Agin, 1977). Each protein contains 15% carbohydrate (McEver et al., 1982). The CDNA-derived primary structures of GPIIb and GPIIIa indicate that each protein has a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail at the carboxyl terminus (Fitzgerald et al., 1987; Poncz et al., 1987; Bray et al., 1987; Zimrin et al., 1988; Rosa et al., 1988; Uzan et al., 1988). GPIIb contains four internal repeats homologous to Ca$^{2+}$-binding sites in calmodulin and troponin C (Poncz et al., 1987). Binding of divalent cations to this region may stabilize the association of GPIIb and GPIIIa as heterodimers (Runicki et al., 1981; Fitzgerald and Phillips, 1985). GPIIb-IIIa is a member of the integrin superfamly of cell-surface molecules that mediate attachment of cells to other cells or to extracellular matrix (Hynes, 1987). Most integrins recognize the sequence of Arg-Gly-Asp (RGD) found in many extracellular matrix components (Ruoslathi and Pierschbacher, 1986). Each receptor contains an \( \alpha \) subunit homologous to GPIIb and a \( \beta \) subunit homologous to GPIIIa (not to be confused with the \( \alpha \)-heavy and the \( \beta \)-light chains of GPIIb). The integrins were originally divided into three subfamilies, each characterized by a common \( \beta \) subunit bound to distinct \( \alpha \) subunits (reviewed in Hynes, 1987; Ruoslathi and Pierschbacher, 1987). Recently, a fourth subfamily has been identified in epithelial cells (Tamura et al., 1989). One of the subfamilies uses GPIIIa (also known as \( \beta 3 \)) as its \( \beta \) subunit. The receptors in this group include 1) GPIIb-IIIa, which uses GPIIb as the \( \alpha \) subunit and is presumably restricted to megakaryocytes and platelets (Bray et al., 1987), and 2) the vitronectin receptor, which has an \( \alpha \) subunit distinct from GPIIb and has been best characterized in human osteosarcoma cells (Pytela et al., 1985) and M21 melanoma cells and endothelium (Cheresh, 1987; Cheresh and Harper, 1987). Recent evidence suggests that this receptor is also present in platelets and can bind thrombospondin (Lam et al., 1988; Lawler and Hynes, 1988).
Comparatively little information is available concerning the biosynthesis of GPIIb-IIIa. Such information might improve our understanding of how the receptor becomes stably associated in cell membranes and would be of particular relevance to understanding the molecular defects in Glanzmann's thrombasthenia, in which both the GPIIb and GPIIIa subunits are deficient. Studies employing cell-free translation of RNA from HEL cells, a human leukemia cell line with some features of megakaryocytes (Tabilio et al., 1984), have demonstrated that GPIIb and GPIIIa are derived from separate precursors (Bray et al., 1986; Silver et al., 1987). The translation studies also indicated that the GPIIb precursor is a single-chain molecule containing both the α-heavy and the β-light chains. The assembly of GPIIb and GPIIIa could not be examined in the cell-free system, since only the GPIIb precursor could be incorporated into microsomes (Bray et al., 1986). Duperray et al. (1987) confirmed the synthesis of distinct precursors for GPIIb and GPIIIa in metabolically labeled bone marrow stem cells isolated from patients with chronic myelocytic leukemia. These investigators also demonstrated that the GPIIb precursor undergoes proteolytic cleavage to produce a two-chain molecule and that the high mannose N-linked oligosaccharides in GPIIb, but not GPIIIa, are converted to complex forms. However, the relationship between post-translational modification and assembly of the two subunits was not addressed.

In the present study we have analyzed both the processing and assembly of GPIIb and GPIIIa in HEL cells. We demonstrate that the two subunits assemble relatively late after synthesis, just before transport to the Golgi apparatus where GPIIb undergoes further modification.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal rabbit antisierum to nonreduced GPIIb, nonreduced GPIIIa, and reduced/alkylated GPIIbα and GPIIIaβ were prepared as described previously (Bray et al., 1986).

The monoclonal antibodies TIB 1 and T10, which are specific for GPIIb and a divalent cation-dependent epitope on the GPIIIa molecule, respectively, have been described (McEvie et al., 1983). AP-3, a monoclonal antibody specific for GPIIIa (Newman et al., 1986), was a generous gift from Dr. Peter Newman (Blood Center of Southwestern Wisconsin). The monoclonal antibodies AP-2 and 10E5, which recognize epitopes on the GPIIb-IIIa complex (Pardal et al., 1985; Coler et al., 1983), were kindly provided by Dr. Thomas Kunicki (Blood Center of Southwestern Wisconsin) and Dr. Barry Coller (State University of New York at Stony Brook), respectively.

**Cell Culture**—HEL cells (Martin and Papayannopoulou, 1982) were kindly provided by Dr. Thalia Papayannopoulou, University of Washington, Seattle. The cells were cultured at 37 °C in 5% CO2 in RPMI 1640 medium containing 4 mM glutamine and 10% fetal bovine serum. Under these conditions the cells attached to the bottom of flasks. Cells were grown to confluence and passed every 3-4 days. Delay of passage resulted in detachment and clustering of the cells in suspension. Preliminary experiments showed that attached cells incorporated approximately 40% more methionine into trichloroacetic acid-precipitable material than when grown in suspension. Therefore all experiments were conducted with attached cells.

**Metabolic Labeling of HEL Cells**—Cells were detached with a cell scraper, counted, centrifuged at 120 × g for 5 min, and incubated at 2 × 106 cells/ml for 1 h at 37 °C in methionine-free RPMI 1640 medium (GIBCO) containing 10% dialyzed bovine serum. They were then pulsed at 37 °C with [35S]methionine (1000-1400 Ci/mmol; Amersham Corp.) at a final concentration of 400 μCi/ml. When chased after pulse, cells were diluted quickly with 1 volume of warm complete RPMI 1640 medium (containing 10% nondialyzed bovine serum supernate) containing 1 mM methionine and then incubated further at 37 °C. At the end of pulse or chase, cells were diluted 10-fold with ice-cold RPMI 1640 medium, centrifuged, and immediately solubilized in lysis buffer (500 μl/106 cells). After vortexing for 30 s, lysates were incubated on ice for 30 min and centrifuged at 10,000 × g for 10 min at 4 °C. Supernatants were collected and stored at -70 °C. Lysis buffer consisted of 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, and 10 μl/ml of a 1:10 dilution of protease inhibitor stock solutions I and II (Ronnett et al., 1984). Stock solution I contained 1 mg/ml leupeptin (Peninsula Laboratories); 2 mg/ml antipain (Peninsula Laboratories); 10 mg/ml benzamidine (Sigma); 10,000 kallikrein-inhibiting units/ml apronin (Sigma). Stock solution II, dissolved in water, contained 300 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin (Peninsula Laboratories) and 1 mg/ml chymostatin (Peninsula Laboratories), dissolved in dimethyl sulfoxide. Both stock solutions were kept at -70 °C in aliquots.

**Immunoprecipitations**—The quantity of labeled protein used for each immunoprecipitation was standardized by determining the incorporation of [35S]methionine into proteins was monitored by precipitation and was referred to as total extract. Cells from the other aliquot were resuspended in 10 mM PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), 1 mM PMSF, 1% Triton X-100, 0.5 M Tris-HCl, pH 8.0, 100 mM NaCl. A second immunoprecipitation was performed by incubation with 2 μl of a 1:1 mixture of anti-GPIIb and anti-GPIIIa polyclonal antisera at 37 °C for 1 h. The antigen-antibody complexes were then precipitated and washed as described above. Bound proteins were eluted by boiling the 5 μl of 60 mM Tris-HCl, pH 6.8, 5 mM iodoacetamide, 5 mM EDTA, 2% SDS, 10% glycerol, 0.05% bromphenol blue.

**Detection of GPIIb-IIIa Expressed on the Surface of HEL Cells**—This procedure is a modification of the method of Kranzel et al. (1979). Metabolically labeled HEL cells were divided into two aliquots. One aliquot was lysed and subjected to the standard immunoprecipitation and was referred to as total extract. Cells from the other aliquot were resuspended in 10 mM PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl), containing 0.02% sodium azide to stop further cellular metabolism. The cells were incubated with antibodies (2 μg/ml) at 37 °C for 1 h, collected by centrifugation, washed twice in ice-cold PBS to remove unbound antibodies, and then incubated with lysis buffer which also contained an extract of 5 × 106 unlabeled HEL cells/ml. The excess unlabelled cell lysate was used to prevent binding of dissociated antibody molecules to labeled intracellular antigens released during the lysis procedure. Immunoprecipitations were then conducted as described above.

**Digestion with Endo-β-N-Acetylgalcosaminidase H**—The stock so...
olution of endo H (Miles) consisted of 1 unit of enzyme dissolved in 1 ml of sterile-filtered 50 mM sodium acetate, pH 5.5, 0.1% SDS (endo H buffer), to which were added 20 μl of protease inhibitor stock solutions I and II. The endo H stock solution was stored frozen in aliquots at −20 °C. Immunoprecipitates prepared as above were re-
ret al., 200, 92.5, and 68 kDa are indicated at the
left. 200,92.5, and 68 kDa are listed at the
right lane.

RESULTS

Assembly of GPIIb with GPIIIa—To determine the kinetics of assembly of GPIIb with GPIIIa, we pulsed HEL cells for 30 min with [35S]methionine and then chased the cells with excess unlabeled methionine as indicated under “Experimental Procedures.” The immunoprecipitations shown in Fig. 1A were conducted with the monoclonal antibody Tab, which is specific for GPIIb. After pulse Tab precipitated a 135-kDa protein designated as preIIb, since it appeared identical to the single-chain precursor of GPIIb incorporated into microsomes during cell-free synthesis from HEL cell mRNA (Bray et al., 1986). After 2 h of chase, Tab also precipitated mature two-chain GPIIb. Only the 123-kDa heavy chain GPIIbα was detected in the fluorogram. The 23-kDa light chain GPIIbβ was not seen since it migrated at the tracking dye front; however, on nonreducing gels, the mature GPIIb molecule has an apparent M̄, of 140,000 and comigrated with platelet GPIIb (not shown). The sequential detection of preIIb and GPIIbα during chase confirmed post-translational proteolytic cleavage of preIIb into the disulfide-linked α and β chains of mature GPIIb. Fig. 1B illustrates parallel immunoprecipitations with AP-3, a monoclonal antibody specific for GPIIIa. The GPIIIa precipitated by AP-3 comigrated with mature GPIIIa even at the earliest time point measured. As with AP-3, half-maximal precipitation of GPIIIa by polyclonal anti-GPIIIa occurred at 45 min of chase (see also Figs. 5 and 8). This suggested that the precursor of GPIIIa required this period to fold sufficiently to allow recognition by the polyclonal antibody. The polyclonal anti-GPIIIa antibody, like Tab, precipitated preIIb but in significantly greater quantities than that precipitated by the monoclonal antibody (discussed below). In addition, the polyclonal antibody precipitated 126- and 116-kDa proteins. These latter two proteins were also precipitated by polyclonal antibodies raised against purified GPIIbα and GPIIbβ, suggesting that they were immunologically related to GPIIb and not merely contaminating proteins in the GPIIb used for immunization (not shown). PreIIb, the 126-kDa, and the 116-kDa proteins all had half-lives of 3–5 h. Some GPIIIa was precipitated by anti-GPIIb after the 30-min pulse, due to a minor population of contaminating anti-GPIIIa antibodies (identified by Western blotting, not shown); therefore, coprecipitation of GPIIIa could not be assessed with this antibody.

With polyclonal anti-GPIIIa, assembly with GPIIb was detected beginning at 3–5 h of chase. The kinetics of assembly paralleled the appearance of two-chain GPIIIa, although a small amount of preIIb was coprecipitated by anti-GPIIIa after the initial pulse, similar to the preIIb coprecipitated by AP-3 in Fig. 1. After prolonged chase only GPIIIa and mature GPIIb were immunoprecipitated. This indicates that preIIb and the 126- and 116-kDa proteins were either converted to mature two-chain GPIIb or proteolytically degraded.

HEL Cells Synthesize preIIb in Excess Compared with GPIIIa—As estimated by scanning densitometry, polyclonal anti-GPIIb precipitated five times more preIIb than the max-

Fig. 1. Kinetics of biosynthesis of GPIIb and GPIIIa in HEL cells as detected by immunoprecipitation with the monoclonal antibodies Tab and AP-3. HEL cells were pulsed for 0.5 h with [35S]methionine (P) and then chased for the indicated times. Cells were then lysed and immunoprecipitated with the monoclonal antibody Tab, specific for GPIIb (A), or the monoclonal antibody AP-3, specific for GPIIIa (B). Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions, followed by fluorography. GPIIbβ was not detectable in this gel system. Molecular mass standards of 200, 92.5, and 88 kDa are indicated at the left. In panel B, the GPIIIa band at the 0.5-h chase interval was inappropriately intense; in other similar experiments AP-3 always immunoprecipitated less GPIIIa at 0.5 h than at 1 h.

Fig. 2. Kinetics of biosynthesis of GPIIb and GPIIIa in HEL cells as detected by immunoprecipitation with polyclonal antibodies. HEL cells were pulsed for 0.5 h (P) and chased for the indicated times. Cells were then lysed and immunoprecipitated with rabbit polyclonal antibodies specific for GPIIb (Anti-GPIIb) or GPIIIa (Anti-GPIIIa). Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions, followed by fluorography. Nonimmune serum (Control) was used after pulse (left lane) and after the 18-h chase (second lane from right). Molecular mass standards of 200, 92, and 68 kDa are listed at the left. 125I-Surface-labeled platelets (right lane) were also used as standards for GPIIbα and GPIIIa.
imal amount of two-chain GPIIb (Fig. 2), indicating that most of the IIb precursor was degraded without further processing. The polyclonal antibody also precipitated substantially more preIIb than did the monoclonal antibody Tab (compare Figs. 1 and 2), suggesting that only a fraction of preIIb underwent sufficient maturation to express the Tab epitope. Analysis of the fluorogram in Fig. 2 further suggested that HEL cells synthesized preIIb in excess relative to GPIIIa. To quantitate this difference, we assumed, first, that all methionines in both preIIb and GPIIIa were labeled equally and, second, that the ratio of methionines in preIIb compared with those in GPIIIa was 0.8, based on 12 methionines per preIIb (Poncz et al., 1987) and 15 methionines per GPIIIa (Rosa et al., 1988). The densitometric value of preIIb after pulse was four times that of the maximal value for GPIIIa seen during chase, suggesting that five preIIb molecules were synthesized for every GPIIIa molecule. An alternative explanation is that additional GPIIIa precursors were synthesized but underwent degradation prior to reaching a conformation recognized by the anti-GPIIIa antibodies. To determine the proportion of GPIIIa forming complexes with two-chain preIIb, we measured the relative densitometric values of GPIIIa and GPIIIa immunoimprecipitated by antibodies to GPIIIa. After 20 h of chase, AP-3 precipitated GPIIIa with a densitometric value of 0.65 ± 0.05 (mean ± S.D. of three experiments) relative to GPIIIa. Similar ratios were measured when polyclonal anti-GPIIIa was used for immunoimprecipitation (Fig. 2). Since the GPIIIa heavy chain contains nine methionines (Poncz et al., 1987), the methionine ratio of GPIIIa relative to GPIIIa is 0.6. This suggests that after extended chase periods all GPIIIa assembled with two-chain preIIb in a 1:1 molar ratio, consistent with the heterodimeric structure of mature GPIIb-IIIa complexes (Jennings and Phillips, 1982; Carrell et al., 1985). Likewise, all preIIb molecules undergoing conversion to the two-chain form assembled with GPIIIa, since antibodies to both GPIIb and GPIIIa precipitated similar quantities of GPIIIa after prolonged chase (Fig. 2).

**Structural Features of preIIb Probed with the Monoclonal Antibodies Tab and T10**—To examine further the structure of preIIb, we used the GPIIb-specific monoclonal antibody Tab as well as T10, a monoclonal antibody that identifies a divalent cation-dependent epitope on the GPIIb-IIIa complex (McEver et al., 1983). HEL cells were pulsed for 30 min and then lysed with a chase period, which allowed immunoprecipitation of preIIb prior to proteolytic cleavage and assembly with GPIIIa (Fig. 3). Like Tab, T10 precipitated preIIb (lanes 1 and 2), thereby localizing the T10 epitope to GPIIIa. Since the T10 epitope is lost when GPIIIa-IIIa is exposed to EDTA (McEver et al., 1983), this experiment suggests that at least some binding sites for divalent cations are present on preIIb. Like polyclonal anti-GPIIIa (Fig. 2), in contrast to Tab, T10 also precipitated the 126-kDa protein (lane 2). Two other proteins of slightly lower apparent molecular weight precipitated in lane 2 were not seen in other experiments. The 126-kDa protein was consistently precipitated from lysates prepared in the presence of multiple protease inhibitors, suggesting that it was not a proteolytic product of preIIb. The 9-kDa difference between preIIb and the 126-kDa protein was retained after enzymatic removal of carbohydrate (not shown), implying that the difference between the two molecules occurred at the protein level. When electrophoresed without prior disulfide reduction both preIIb and the 126-kDa molecule migrated faster with apparent $M_s$ of 123,000 and 122,000, respectively (lanes 3 and 4). This supports the presence of internal disulfide bonds in these molecules. Since preIIb underwent a sharper mobility shift after disulfide reduction than did the 126-kDa protein, it may contain a disulfide-bonded domain not present in the 126-kDa protein. This domain could include the Tab epitope, since Tab did not precipitate the 126-kDa protein.

**Sensitivity of preIIb-IIIa and GPIIIa Heterodimers to Dissociation with EDTA**—The platelet GPIIb-IIIa complex can be dissociated into its component subunits by exposure to chelators of divalent cations such as EGTA or EDTA (Kunicki et al., 1981; Fitzgerald and Phillips, 1985; Shattil et al., 1985). This structural feature is thought to be due to the binding of Ca$^{2+}$ ions to GPIIb (Poncz et al., 1987; Phillips et al., 1988). To examine whether the mature HEL cell heterodimer shared similar features, we pulsed HEL cells for 2 h and then chased the cells in unlabeled medium for 4 h to allow processing and assembly of the GPIIb and GPIIIa subunits. Cell lysates were then incubated in the presence or absence of EDTA prior to immunoprecipitation with antibodies to GPIIb or GPIIIa (Fig. 3). EDTA completely dissociated
be dissociated by exposure to EDTA. Mature GPIIb from GPIIIa. In contrast, the small amount of single-chain preIIb assembled with GPIIIa has been modified into complex structures resistant to endo H. Exposure to endo H, suggesting that the oligosaccharides had not complex N-linked oligosaccharides from glycoproteins indicating the removal of high mannose oligosaccharides. In addition, the minor fraction of preIIb coprecipitated by AP-3 remained sensitive to endo H.

PreIIb Molecules Are Not Expressed on the Surface of HEL Cells—Expression of preIIb and GPIIIa-IIIa at the cell surface was analyzed in Fig. 6. HEL cells were pulsed for 2 h, chased for 6 h, and then divided into two aliquots. One aliquot was immediately lysed and subjected to immunoprecipitation with a panel of antibodies. Intact cells from the other aliquots were incubated with antibodies and these washed and lysed in the presence of a 100-fold excess of unlabeled HEL cell lysate before immunoprecipitation (see “Experimental Procedures”). GPIIb-IIIa was detected at the surface of HEL cells by both polyclonal anti-GPIIb and by 10E5, a monoclonal antibody specific for the GPIIb-IIIa complex (lanes 7 and 8). In contrast, polyclonal antibodies to the GPIIb heavy chain, GPIIbα, detected preIIb in cell lysates (lane 2) but not at the cell surface (lane 6).

Treatment of HEL Cells with the Ionophore Monensin Prevents Cleavage of preIIb but Not Carbohydrate Modification or Assembly with GPIIbα—To determine further the sequence of events leading to maturation of GPIIb-IIIa, we treated HEL cells with monensin, an ionophore that alters the pH of many organelles, including the mid- and trans-Golgi, and hence interferes with certain Golgi-mediated processing steps (Tar- takov, 1983). Cells preincubated with or without monensin were pulsed for 2 h and then chased for 4 h. In preliminary experiments, it was determined that monensin did not affect the synthesis or mobility of preIIb or GPIIIa during the initial pulse. After chase (Fig. 7), monensin blocked cleavage of preIIb into two-chain GPIIb, since GPIIbα was neither precipitated by Tab (compare lanes 1 and 3) nor coprecipitated by anti-GPIIbα (compare lanes 5 and 7). Instead a larger proportion of uncleaved preIIb was noted to assemble with GPIIIa (lanes 5 and 7). This was not due to increased synthesis of preIIb because identical quantities of preIIb were precipitated by Tab in the presence or absence of the ionophore (lanes 1 and 3).

To determine the effect of monensin on modification of high mannose N-linked oligosaccharides into complex forms (another Golgi-mediated event), we digested immunoprecipitates from monensin-treated or control HEL cells with endo H. In the presence of monensin, about 10% of preIIb molecules precipitated by Tab developed resistance to digestion with endo H, as estimated by scanning densitometry (lanes 3 and 5).
4). This indicated that some preIIb molecules could develop complex carbohydrate side chains without prior proteolytic processing. The entire population of preIIb molecules containing complex carbohydrate chains appeared to have assembled with GPIIIa, based on densitometric analysis of the endo H-resistant band coprecipitated by anti-GPIIIa (lane 8). As seen previously (Fig. 5), a small proportion of preIIb molecules sensitive to endo H digestion could be coprecipitated by anti-GPIIIa (lanes 7 and 8). This endo H-sensitive population was also detected in the presence of monensin (lane 8, bottom “preIIb” arrow). This again suggested that at least some preIIb molecules could assemble with GPIIIa in the ER, prior to modification of carbohydrate in the Golgi apparatus.

Inhibition of Cleavage of preIIb by Monensin Does Not Affect the Kinetics of Assembly with GPIIIa—We next examined whether inhibition of cleavage of preIIb by monensin affected the rate of assembly of preIIb with GPIIIa. HEL cells treated with or without monensin were pulsed for 30 min and then chased at short intervals up to 3 h prior to lysis and immunoprecipitation with anti-GPIIIa. Prior to SDS-PAGE, the immunoprecipitates were treated with endo H in order to maximize mobility differences between preIIb molecules containing high mannose (endo H-sensitive) or complex (endo H-resistant) oligosaccharides (see also Fig. 7, lane 8). As illustrated in Fig. 8, GPIIIa first assembled with containing high mannose oligosaccharides (“preIIb” arrow in left panel, lower “preIIb” arrow in right panel). Without monensin, small amounts of two-chain GPIIb were coprecipitated by anti-GPIIIa as early as 70 min of chase, but assembly still appeared to be increasing at the 3-h time point. In the monensin-treated cells, the modification of preIIb oligosaccharides into complex forms could be detected by slightly slower mobility of the molecule (upper “preIIb” arrow, right panel). Assembly of this form of preIIb with GPIIIa occurred with kinetics indistinguishable from those of two-chain GPIIb.

**DISCUSSION**

Our studies confirm and extend previous studies of the biosynthesis of GPIIb-IIIa, a member of the integrin family of cell-adhesion receptors.

We earlier identified a precursor for GPIIIa in cell-free translation products from HEL cell mRNA (Bray et al., 1986). This precursor of M, 95,000 contained no carbohydrate and could not be incorporated into microsomal vesicles in the cell-free system. In the current study, intact HEL cells synthesized a precursor of GPIIIa of M, 105,000, which comigrated with mature platelet GPIIIa after a 30-min pulse and even a brief 5-min pulse (not shown). This precursor contained high mannose oligosaccharides added in the ER, which could be removed by digestion with endo H even after prolonged chase. This indicates that HEL cells do not convert the high mannose oligosaccharides on GPIIIa into complex forms in the Golgi apparatus. The same observation was previously noted in transformed megakaryocytes from patients with chronic myelocytic leukemia (Duperray et al., 1987). High mannose oligosaccharides are also characteristic of GPIIIa in nonmalignant human platelets, since 1) the carbohydrate composition of platelet GPIIIa contains a large proportion of mannose residues (McEver et al., 1982) and 2) platelet GPIIIa migrates more rapidly on SDS-polyacrylamide gels after digestion with endo H (not shown).

The processing of GPIIb is more complex than that of GPIIIa. The major precursor, termed preIIb, is a single-chain molecule containing both the α-heavy and β-light chains of the mature two-chain molecule. During the initial pulse, core high mannose oligosaccharides are added to the protein in the ER. Therefore, the preIIb identified in the current study is identical to the molecule translated from cell-free HEL cell RNA after incorporation into microsomes (Bray et al., 1986). Subsequently, preIIb is cleaved to yield the two-chain molecule and undergoes modification of high mannose oligosaccharides into complex forms. These two processing events, which occur in the Golgi apparatus, proceed with indistinguishable kinetics in HEL cells. Similar concurrent cleavage and carbohydrate modification have been noted for the GPIIb precursor in transformed megakaryocytes from patients with chronic myelocytic leukemia (Duperray et al., 1987) and for the α subunit of the vitronectin receptor in M21 melanoma cells (Cheresh and Harper, 1987). In the current study, pre-treatment of HEL cells with the ionophore monensin dissociated these two events, resulting in detection of single-chain GPIIb containing complex carbohydrate. This indicates that modification of N-linked oligosaccharides do not require prior cleavage of the single-chain precursor.

After the initial pulse, two minor proteins immunologically related to preIIb were also identified. These two proteins, M, 126,000 and 116,000, were detected with polyclonal antibodies to GPIIb, and the 126-kDa form was also precipitated by the monoclonal antibody T10. These two species do not appear to be related to the α subunits of the vitronectin receptor because, in a previous immunocytochemical survey of human tissue sections, neither the polyclonal nor monoclonal antibodies to GPIIb reacted with tissues such as endothelium or osteoclasts, where the other receptor is expressed (Beckstead et al., 1986). Our studies do not resolve whether these minor proteins represent proteolytic products of preIIb or precursors derived from alternatively spliced forms of GPIIb mRNA. The two molecules may not be processed further. Alternatively, they could reflect different forms of GPIIbβ, the light chain of GPIIb. In the gel system used, GPIIbβ migrated with the tracking dye under reducing conditions; therefore, variations in GPIIbβ would not have been detected following proteolytic cleavage.
Several lines of evidence suggest that preIIb assembles with GPIIIa in the ER, prior to delivery to the Golgi apparatus where further processing occurs. First, we detected the assembly of a small population of endo H-sensitive preIIb with GPIIIa immediately following pulse-labeling of HEL cells. Since resistance to cleavage with endo H develops only after modification of high mannose oligosaccharides in the Golgi apparatus, these complexes must have assembled in the ER. Second, treatment of cells with monensin, an inhibitor of some Golgi-mediated processes, prevented proteolytic cleavage of preIIb but did not affect the kinetics of assembly with GPIIIa. Third, in the monensin-treated cells, all single-chain IIb precursors which developed resistance to endo H also had already assembled with GPIIIa, suggesting that assembly was a prerequisite for further Golgi-mediated carbohydrate modification.

Although the IIb and IIIa subunits appeared to assemble in the ER, most of the GPIIb molecules has already undergone proteolytic cleavage and carbohydrate modification at the time assembly was first detected. This suggests that, following assembly of preIIb with GPIIIa in the ER, transport to the Golgi apparatus and subsequent processing of preIIb occurred extremely rapidly. However, assembly itself required several hours. This may reflect the requirement for an unusually long period for folding of either or both subunits in a conformation suitable for assembly. A delay for proper folding to proceed is consistent with three observations: 1) half-maximal precipitation of GPIIIa by monoclonal or polyclonal antibodies did not occur until nearly 1 h following pulse; 2) only a minority of preIIb molecules precipitable by polyclonal antibodies to GPIIIa were recognized by the monoclonal antibodies T9 or T10; 3) unlike mature heterodimers, the small number of complexes in which endo H-sensitive preIIb assembled with GPIIIa could not be dissociated with EDTA. The proper folding and assembly of preIIb and GPIIIa in the ER may depend in part on interactions with the ER-resident protein, BiP (Bole et al., 1986). This protein, first identified in B-cells, has subsequently been determined to be present in many tissues, where it may have a general role in binding subunits of oligomeric proteins until they have reached a conformation suitable for assembly (Haas and Wabl, 1983; Haas and Meo, 1988). The importance of suitable folding for assembly of oligomeric proteins has been particularly well demonstrated for the influenza virus hemagglutinin (Gething et al., 1986; Copeland et al., 1988) and is consistent with a model in which transport from the ER to the Golgi apparatus is the limiting step in movement of proteins to the cell surface (Rothman, 1987; Lodish, 1988). In HEL cells, virtually all GPIIIs eventually assemble with preIIb and is transported to the Golgi apparatus. In contrast, approximately five times more preIIb is synthesized than GPIIIs. The excess preIIb, which neither assembles with GPIIIa nor reaches the cell surface, is slowly degraded over many hours. The site of degradation may be a protease-rich pre-Golgi compartment, analogous to the site where excess free subunits of the pentameric T-cell receptor are digested (Lippincott-Schwartz et al., 1988).

Other investigators have studied the processing and assembly of two additional members of the integrin family: the Mac-1 adhesion receptor of macrophages (Ho and Springer, 1983) and the vitronectin receptor in M21 melanoma cells (Cheresh and Harper, 1987; Cheresh and Spiro, 1987). In both of these receptors, assembly of \( \alpha \) and \( \beta \) subunits preceded additional processing, suggesting that assembly also occurred in the ER. In contrast to the current study, where the \( \alpha \) subunit preIIb was synthesized in excess, cells expressing Mac-1 or the M21 molecule synthesized excess \( \beta \) chains. In M21 cells, assembly of \( \alpha \) and \( \beta \) chains occurred much more rapidly than in HEL cells; however, subsequent transport to the Golgi apparatus (assessed by proteolytic cleavage of the \( \alpha \) subunit) was relatively slow (Cheresh and Spiro, 1987). This suggests that additional conformational changes in the complex were required prior to transport. In addition, the \( \beta \) subunit of the M21-cell receptor, which appears to be identical to GPIIIa, underwent modification of its core high mannose oligosaccharides into complex forms in the Golgi apparatus (Cheresh and Harper, 1987). This is clearly different than GPIIIa in platelets and HEL cells (our studies) or transformed megakaryocytes (Duperray et al., 1987), which retains high mannose carbohydrate chains. The enzymatic machinery for Golgi-mediated carbohydrate modification is present in HEL cells and megakaryocytes, since GPIIb undergoes modification of its high mannose side chains. Therefore the differential carbohydrate processing of GPIIIs in HEL and M21 cells may reflect conformational differences resulting from association with distinct \( \alpha \) subunits.

Our studies are relevant to the potential molecular defects in Glanzmann's thrombasthenia, where both GPIIb and GPIIIa are markedly deficient (McEver and Majerus, 1989). Theoretically, mutations could occur in the genes encoding either subunit. If so, the abnormal subunit might not fold properly and would have difficulty assembling with the normal subunit. The unassembled subunits, whether mutant or normal, would not be transported to the Golgi apparatus for further processing. Instead, they would be degraded gradually over several hours. As a result, circulating thrombasthenic platelets, which, like normal platelets, survive for about 10 days in the circulation after release from megakaryocytes, would have little detectable GPIIb or GPIIIa. The residual proteins detected in such patients may represent a minority of heterodimers that were able to assemble in the ER and be transported to the cell surface. This is consistent with a previous study in which platelet lysates from patients with thrombasthenia were studied by SDS-PAGE and immunoblotting using polyclonal antibodies to GPIIb and GPIIIa (Nurden et al., 1985). Most of the patients studied had small amounts of immunoreactive GPIIb and GPIIIa. Of note, the detectable GPIIb conigrated with mature two-chain GPIIb under both reducing and nonreducing conditions. No immunoreactive band corresponding to single-chain preIIb was identified. This suggests that the residual GPIIb and GPIIIa in these patients had undergone assembly in the ER, followed by further processing in the Golgi apparatus.

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