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Membrane Binding Properties of Blood Coagulation Factor V and Derived Peptides†

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ABSTRACT: The interactions of factor V and factor Va light chain with phospholipid vesicles were compared. The results showed that the factor Va light chain bound with the same parameters as factor V when the proteins were present at similar densities on the membrane. The protein–vesicle collisional efficiency was 30–50% for both factor V and factor Va light chain. The factor Va light chain bound at a higher density, and the additional binding interactions had lower affinity. The dissociation process showed negative cooperativity, possibly due to competition for acidic phospholipids in the membrane. The higher molar packing density produced more rapid protein–membrane dissociation rate constants. However, when factor V and Va light chains were present at similar molar densities on the vesicle, the dissociation rates, estimated by two methods, were similar. Analysis of dissociation rates also showed that factor Va interacted with factor Xa on the membrane surface while factor Va light chain did not. Factor Va generated by thrombin digestion of factor V did not result in a major loss of membrane-bound protein mass unless ethylenenediaminetetraacetic acid was present; in the latter case the mass changes indicated that all peptides were removed from the membrane except factor Va light chain. Equilibrium and dynamic measurements showed that ionic strength had a major effect on the dissociation rate but not on the association process. The salt effect indicated interaction between oppositely charged species with the product of the number of charges equal to at least −5.5. Factor Va light chain appeared to interact with phospholipids via a general charge interaction rather than via a specific charge stoichiometry. Lysine modifications readily prevented protein–membrane binding while tryptophan modification had little effect.

Blood coagulation factor Va interacts with factor Xa on membrane surfaces to form the prothrombinase complex [see Jackson & Nemerson (1980) for a review]. All three proteins involved in this reaction bind to membranes containing acidic phospholipids (Papahadjopoulos & Hanahan, 1964; Nelsestuen et al., 1976; Bloom et al., 1979). Thorough understanding of the mechanism of the prothrombinase reaction requires knowledge of the physical interactions between each of the components and how these participate in ternary and quaternary (factor Va, factor Xa, prothrombin, and phospholipid) macromolecular assemblies. The three proteins each exist as inactive precursors, as active, proteolytically cleaved proteins, and as isolated protein fragments with partial functions. One important question is whether the protein–membrane interactions are affected by these cleavages and whether the changes affect the prothrombinase reaction. Factors X and Xa appear to have identical membrane binding properties (Nelsestuen et al., 1976; Van de Waart et al., 1983). Prothrombin and its isolated 156-residue amino-terminal fragment display at most small differences in their membrane interactions (Nelsestuen, 1976).

Factor V has been studied recently and the conclusions are inconsistent. Bloom et al. (1979) reported a K_D for the factor V–membrane complex of about 10^{-7} M, and Higgins & Mann (1983) reported that factor Va light chain bound somewhat less tightly. Pusey et al. (1982) reported that assumptions made in these studies were not valid under similar circumstances and that the dynamics of factor V–membrane binding indicated a K_D of about 10^{-10} M. First-order rate plots for dissociation of the complex were not linear so this was an average value (Pusey et al., 1982). This tighter binding correlated closely with reported binding of factor Va to cell membranes (Tracy et al., 1981; Kane & Majerus, 1982; Tracy et al., 1983). The discrepancy in the K_D for factor V–phospholipid vesicle binding is therefore important for interpretation of factor V–cellular interactions; the tighter binding constant implies that factor V–cellular interactions may be mediated by phospholipids alone while the weaker binding constant strongly implies that other forces (e.g., a cell surface protein receptor) are involved in factor V–cellular interactions.

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van de Waart et al. (1983) reported a K_D for the factor V–phospholipid complex of 10^{-7}–10^{-8} M with the factor Va light chain binding somewhat tighter. These authors used synthetic phospholipids with homogeneous fatty acyl chains that differed from the previous studies.

We would also like to know the physical bases for changes in protein–membrane binding affinity that result from the proteolytic events. For example, there may be a protein conformational change that affected the membrane binding site, a portion of the membrane binding region may have been separated by proteolysis, or there may be other factors involved.

This study examined several aspects of this protein–membrane interaction. The factor Va light chain appeared to contain the intact factor V–membrane interaction site. A difference was that factor Va light chain could bind at higher density on the vesicle surface. Both dynamic and equilibrium measurements showed that averaged dissociation constants for the factor V–vesicle complex were subnanomolar. This interaction was strongly ionic, which agreed with changes in binding due to lysine modification. Factor Va but not factor Va light chain formed a complex with factor Xa on the membrane surface. New methods were developed for study of protein–membrane binding interactions.

Materials and Methods

Egg PC,[1] bovine brain PS, egg PG, and synthetic DPPC were purchased from the Sigma Chemical Co. *N*-Dansyl-PE was synthesized by the procedure of Waggoner & Stryer (1970). PI was prepared from bovine brain Folch fraction I by the method of Comfurius & Zwaal (1977). Single bilayer vesicles containing PS and PC were prepared by sonication and gel filtration (Nelsestuen & Lim, 1977; Huang, 1969), while PG/PC containing vesicles were prepared by ethanol injection (Batzri & Korn, 1973). Phospholipid concentrations were determined by phosphate assay (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 25. Weight average molecular weights of vesicle preparations were determined by light scattering methods described previously (Pusey et al., 1982).

Picryl sulfate (2,4,6-trinitrobenzenesulfonic acid), 2-hydroxy-5-nitrobenzyl bromide, Tris buffers, phenylmethanesulfonyl fluoride, and QAE, SP, and G-25 Sephadex were purchased from the Sigma Chemical Co. Citraconic anhydride was from Pierce Chemical Co. Bio-Gel A.5M was from Bio-Rad. All other chemicals were of reagent grade or better.

Proteins. Single-chain factor V was prepared from venipuncture bovine blood by the method of Pusey et al. (1982). Factor Va was generated by thrombin digestion of factor V (1:1000 w/w) at 37 °C for 10 min. The factor V_a light chain was isolated by the method of Lindhout et al. (1982). Factor Xa was prepared as outlined by Nelsestuen & Lim (1977). The protease from Russell's viper venom that cleaves factor V (RVV-V) was isolated as described (Kisiel, 1979) from lyophilized Russell's viper venom (Sigma Chemical Co.). Values of $E^{1\%}_{280\,nm}$ used were as follows: factor V, 9.6 (Nesheim et al., 1979a), factor Va derived light chain, 15.8 (Esmon, 1979). Alternatively, protein was determined by the method of Bradford (1976), using BSA as a standard. When modified PLBP was quantitated by this latter technique, a solution of unmodified PLBP was used for the standard.

Amino Groups Were Blocked with Citraconic Anhydride. Aliquots of citraconic anhydride in diethyl ether (1 mg/mL) were dried under argon in a tube, and an aliquot of protein solution in 0.05 M phosphate buffer, pH 8.0, was added. Alternatively, the citraconic anhydride was added directly to the protein solution with rapid stirring and the ether removed by a stream of argon blown over the solution at 37 °C for about 2 min. Appropriate aliquots of 4 M NaOH were slowly added to maintain pH. Amino groups were determined with 2,4,6-trinitrobenzenesulfonic acid by using the procedure of Fields (1972).

Tryptophan was modified with HNB-Br by using the procedure of Horton & Koshland (1972). The HNB-Br solution (0.2 M dioxane) was slowly added to the protein solution, which was stirred at room temperature. Immediately after addition of each aliquot, an equal volume of 0.2 M NaOH was added. Protein was separated from excess reagent by gel filtration on Sephadex G-25 at room temperature. The extent of tryptophan modification was determined by dilution of an aliquot of the protein material into 2.5 M NaOH and determination of the absorbance at 410 nm. An extinction coefficient of 18 450 M^{-1} cm^{-1} (Loudon & Koshland, 1970) was used to estimate the extent of tryptophan modification.

Membrane binding protein was determined by using 90° relative light scattering (Nelsestuen & Lim, 1977). These measurements were used to determine the mass of membrane binding protein present in the factor V preparation as described by Pusey et al. (1982). The factor V concentrations given in the text are taken from the mass of membrane binding protein. The PLBP was quantitated by an $E^{1\%}_{280\,nm} = 15.8$ (Lindhout et al., 1982). Protein vesicle binding was also followed by using fluorescence energy transfer measurements in an Hitachi Perkin-Elmer 44A fluorescence spectrophotometer. Typically, tryptophan excitation was at 285 nm and dansyl emission was monitored at 540 nm with slit widths of 5 and 17 nm, respectively. Vesicles used for energy transfer determinations typically contained 10% dansyl-PE. The latter derivative did not alter the protein–phospholipid binding interaction significantly (Pusey et al., 1982).

Protein–vesicle association rates were obtained from the increased fluorescence energy transfer when peptide and fluorescent-labeled vesicles were mixed in the stopped-flow apparatus described by Lampe et al. (1983). Excitation was achieved with a 450-W xenon lamp source (SLM Instruments) with a 277-nm band-pass filter (LKB instruments). Emission was monitored through a 410-nm cutoff filter. The data were analyzed as an irreversible bimolecular association between protein (P) and protein binding sites (V) on the membrane:

$$P + V \rightarrow PV \quad (1)$$

The assumption of irreversibility is valid because the very slow dissociation process (Pusey et al., 1982) does not contribute to the signal change on the stopped-flow time scale. The rate of product formation then becomes

$$dPV/dt = k_1(P_0 - P)(V_0 - V) \quad (2)$$

where P_0 and V_0 are the initial concentrations of P and V after mixing, respectively, and PV is the concentration of product at time t. For these experiments, the final concentration of PV was equal to the initial concentration of the limiting component (P or V) present, and the signal intensity was proportional to the concentration of PV at time t. Integration of eq 2 and rearrangement gives

$$\ln [(1 - PV/V_0)/(1 - PV/P_0)] = (V_0 - P_0)k_1t \quad (3)$$

[1] Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; dansyl-PE, *N*-dansyldipalmitoylphosphatidylethanolamine; PLBP, the phospholipid binding peptide of factor Va also referred to as peptide E by Higgins & Mann (1983) or the factor Va light chain by van de Waart et al. (1983); HNB-Br, 2-hydroxy-5-nitrobenzyl bromide; EDTA, ethylenediaminetetraacetic acid.

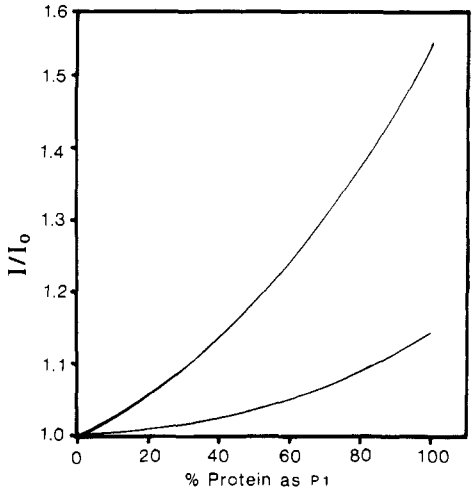

FIGURE 1: Light scattering intensity as a function of protein distribution over two vesicle populations. The protein, vesicle population 1, and vesicle population 2 are present at a ratio of 1.5:1.0:10 (w/w/w). The upper curve is for a vesicle population 1 of M_r 8 × 10^6 and vesicle population 2 of M_r 3.2 × 10^6. The lower curve is for both vesicle populations of M_r 3.2 × 10^6. I is the intensity of scattered light at the protein distribution shown, and I_0 is the intensity of scattered light where all protein is associated with vesicle population 2.

where P_0 is the limiting component. A plot of ln $[(1 - PV/V_0)/(1 - PV/P_0)]$ vs. t gives a straight line with slope $(V_0 - P_0)k_1$, from which k_1 was obtained. This rate constant is for the interaction of protein with binding sites on the vesicle. The number of binding sites per vesicle was obtained from maximum changes in light scattering intensity as described previously (Pusey et al., 1982).

Dissociation Rate Constants Were Determined by Light Scattering or Fluorescence Energy Transfer Techniques. The total light scattering (I_t) from a mixture of particles is equal to the sum of light scattering from the individual components (I_i). Given two populations of protein–vesicle complexes, a summary equation can be written: $I_t = k[(\partial n_1/\partial c_1)^2 M_{r1} c_1 + (\partial n_2/\partial c_2)^2 M_{r2} c_2]$ where M_r is the molecular weight of the individual protein–membrane complexes, c is their weight concentration (g/mL), $\partial n/\partial c$ is the refractive index increment estimated as described previously (Nelsestuen & Lim, 1977; Wei et al., 1982), and k is a machine constant. This equation assumes that the second virial coefficient is negligible, a condition that has been shown to hold for these systems (Nelsestuen & Lim, 1977). Given known concentrations of two vesicle populations (V_1 and V_2), each of known molecular weight, plus a known total protein concentration (P_t) that is bound to the vesicles, one can calculate a value of I_t for different protein distributions on the two vesicle populations. For example, if all protein is associated with vesicle population 1, $c_1 = c_{V1} + c_{CPt}$ and $M_{r1} = M_{rV1} + c_{Pt}$ (mol of V_1)$^{-1}$ mL. M_{r2} and c_2 are equal to the values for the second phospholipid population without bound protein. Figure 1 shows theoretical curves for I_t/I_0, where I_t is the light scattering intensity at the distribution of protein shown on the abscissa and I_0 is the intensity when all protein is associated with the second vesicle population.

From these plots it is apparent that upon addition of vesicles to a protein–vesicle complex, I_t will decrease if the protein distributes itself over the total vesicle population. The signal change is maximized by starting with vesicles densely populated with protein and by adding a large excess of the second vesicle population. The signal change is also greatly enhanced if the first vesicle population has a higher molecular weight (upper curve, Figure 1).

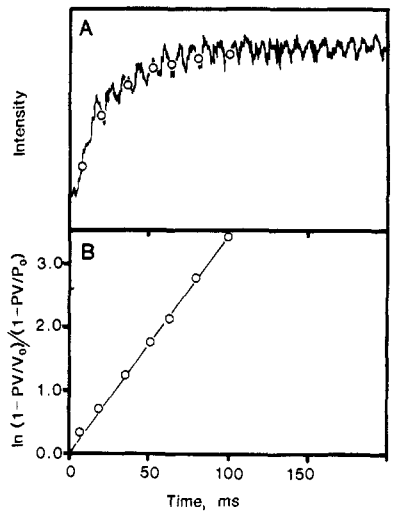

FIGURE 2: Stopped-flow analysis of PLBP–membrane binding. Phospholipid [PG–dansyl-PE–PC (40:10:50), 100 μg/mL after mixing] and PLBP (179 μg/mL after mixing) were mixed in the stopped flow at 1 °C with fluorescence excitation at 277 nm. The fluorescence emission was monitored above 410 nm by the apparatus outlined under Materials and Methods. Part A shows the result from a sum of four runs. Part B shows a second-order rate plot of the data. The experimental points are those shown in part A. From the slope of the curve, an association rate constant of 0.37 × 10^8 M^{-1} s^{-1} was obtained. This is expressed on the basis of protein binding interactions given 75 interactions per vesicle of M_r 3.2 × 10^6. The buffer was 0.05 M Tris (pH 7.5)–0.1 M NaCl–0.1 mM $CaCl_2$. Control experiments with vesicles containing no fluorescent label showed no signal change.

Protein–vesicle dissociation rates were also estimated by the fluorescence energy transfer method of Pusey et al. (1982). Briefly, this method involves binding protein to vesicles containing a fluorescent probe such as the dansyl moiety that can accept fluorescence energy from tryptophan residues in the protein. When distance and orientation requirements are met, fluorescence energy transfer occurs and is monitored by the emission intensity from the dansyl moiety. A large excess of nonfluorescent vesicles are added at zero time to effectively trap protein molecules dissociating from the fluorescent vesicles. A decrease in the fluorescence signal occurs, and the rate of decrease can be used to estimate the rate of protein–vesicle dissociation.

Iodide ion quenching of protein fluorescence was done at 20 °C using KI as the quenching titrant. Excitation (5-nm slit) and emission (12-nm slit) were at 285 and 335, respectively. The buffer was 0.02 M phosphate, pH 8.0. Phospholipid vesicles used in the experiment were PG–PC (40:60).

Results

Association Rates for PLBP–Vesicle Association. Figure 2A shows the fluorescence intensity due to energy transfer from protein tryptophan to membrane-bound dansyl groups after mixing PLBP with vesicles of PG–dansyl-PE–PC (40:10:50). The curve is the sum of four runs. The experiment was run at 1 °C in order to obtain a slower rate. Instrument dead time was 5–8 ms (Wei et al., 1982). The data points indicated in Figure 2A were plotted according to eq 3 (Figure 2B). The association rate constant derived from this line was 0.37 × 10^8 M^{-1} s^{-1}. At saturation there were about 75 peptides bound per vesicle of M_r = 3.2 × 10^6 as determined from maximum light scattering intensity changes (Pusey et al., 1982). The protein–vesicle association rate constant was therefore 2.8 × 10^9 M^{-1} s^{-1}. This value approached the theoretical collisional rate constant (6.6 × 10^9 M^{-1} s^{-1} at 1 °C). This latter value was estimated as described previously (Wei et al., 1982) by

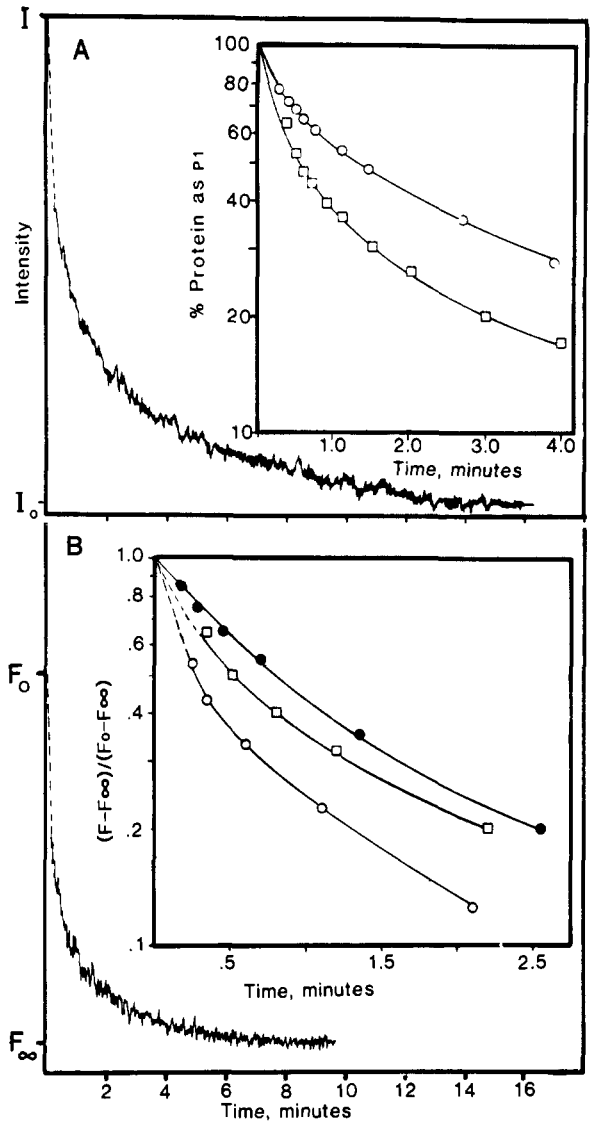

FIGURE 3: Protein–vesicle dissociation measured by light scattering (part A) and fluorescence energy transfer (part B). Phospholipid vesicles (3.92 μg of PS–PC, 20:80, M_r 8 × 10^6) and factor V (6.2 μg) were mixed in 1.5 mL of buffer (0.05 M Tris, pH 7.5, 0.1 M NaCl, 0.1 mM $CaCl_2$). At zero time, 42.7 μg of vesicle [PS–PC (20:80), M_r 3.6 × 10^6] was added and the decrease in light scattering monitored. The initial intensity is the sum of light scattering for protein bound to the large vesicle plus that of the small vesicles with no protein bound. I/I_0 at zero time was 1.45. Part A, inset: first-order rate plots of the dissociation data for (○) factor V and (□) PLBP. The percent protein as P_1 was calculated from the observed I/I_0 and the theoretical curve in Figure 1. Part B shows fluorescence due to energy transfer after mixing 32.4 μg of vesicles (PS–PC, 20:80) with a complex of fluorescent vesicles (3.14 μg of PS–dansyl-PE–PC, 20:10:70) and PLBP (7.3 μg). Fluorescence intensity at 540 nm (excitation at 285) was measured. F_0 is the fluorescence due to energy transfer before adding the unlabeled vesicles. F_∞ is the intensity at equilibrium measured separately. Part B inset shows first-order rate plots of data obtained for (□) factor V and (○) PLBP where each was present at about 90% protein saturation on the membrane. This gave about 4 times as many PLBP as factor V molecules per vesicle. Also shown is PLBP that was only 25% saturating on the membrane (●). This latter concentration was similar to the molar density of factor V on the membrane. All experiments were done at 25 °C.

using the reported hydrodynamic properties of PLBP (Laue et al., 1983) and phospholipid vesicles (Pusey et al., 1982).

Dissociation Rate Constants. An example of a light scattering intensity tracing used for determining the protein–vesicle dissociation rate is shown in Figure 3A. The distribution of factor V on vesicle population 1 was obtained from the appropriate theoretical curve (Figure 1), and the rate of protein dissociation from vesicle population 1 was estimated from a first-order rate plot of the data (Figure 3A, inset). When negligible reassociation of protein with vesicle population 1 is assumed, the process can be treated as an irreversible first-order reaction. This latter assumption will be correct when the second vesicle population is present at infinitely greater concentration than the first vesicle population. A 10-fold excess of the second vesicle population appeared to be satisfactory since addition of larger amounts did not change the kinetics perceptably. The first-order rate plots for this protein–membrane dissociation were not linear, which was similar to the results obtained by fluorescence energy transfer [Figure 3B and Pusey et al. (1982)]. The first half-life of this dissociation was a value that compared closely to that obtained by the fluorescence energy techniques [Pusey et al. (1982) and see below]. Figure 3A (inset) also shows a first-order rate plot for dissociation of PLBP using this technique.

Figure 3B shows the dissociation curve for PLBP–phospholipid as determined by fluorescence energy transfer. The inset shows a first-order rate plot of the data plus data for factor V. Two rate plots are given for PLBP. One shows dissociation from densely packed lipid (about 70 peptides/vesicle) and the second for vesicles containing the same protein density as factor V (about 20 peptides/vesicle). The maximum energy transfer for the former experiment was proportionally greater than for the latter experiment. It was clear that dissociation rates were comparable for factor V and PLBP when they were present at similar densities on the membrane surface [Figure 3B, (□) and (●)]. In contrast, densely packed PLBP dissociated more rapidly than factor V. The dissociation rate plots obtained by the latter method were only slightly faster than those obtained by the light scattering method (Figure 3A).

Estimation of Binding by Equilibrium Techniques. The studies presented above and previously (Pusey et al., 1982) provided estimates of rate constants for protein–membrane interaction. It would be desirable to estimate apparent binding constants by using equilibrium measurements as well. At physiological ionic strength there is not an adequate concentration of free factor Va for measurement by the techniques employed (Pusey et al., 1982). Previously published data indicated a pronounced salt effect on factor V-phospholipid binding and that high salt will dissociate the complexes (Pusey et al., 1982; van de Waart et al., 1983). We made use of this effect to determine the concentration of free protein at half membrane saturation. Fluorescence energy transfer was used to monitor the interaction. At 285 nm there is a small direct excitation of the dansyl moiety (F_{PL}) that served as a useful internal standard. Fluorescence energy transfer was measured relative to this internal standard (F/F_{PL}, where F is total fluorescence intensity). As salt was added, the value of F/F_{PL} dropped to 1.0, indicating that the factor V was completely dissociated. The value of F_{PL} was found to decrease only slightly due to the added salt, and no correction for this effect was necessary. A series of titrations were performed at varying protein concentrations, keeping the protein:phospholipid ratio constant at about 90% saturation of the vesicles (Figure 4A). The initial value of F/F_{PL} was constant over the concentration range used (1.4 × 10^{-9} to 1.8 × 10^{-7} M factor V), indicating that similar proportions of the protein were bound at all concentrations. The midpoint for dissociation should be related to an averaged dissociation constant (the concentration of free protein at half saturation of the membrane). These values were plotted by the linear relationship shown in Figure 4B ($\log K_D = \log (K_D^0 - 2AZ_AZ_B)(I/\text{mol kg}^{-1})^{1/2}$, where $2A$ equals 1.02

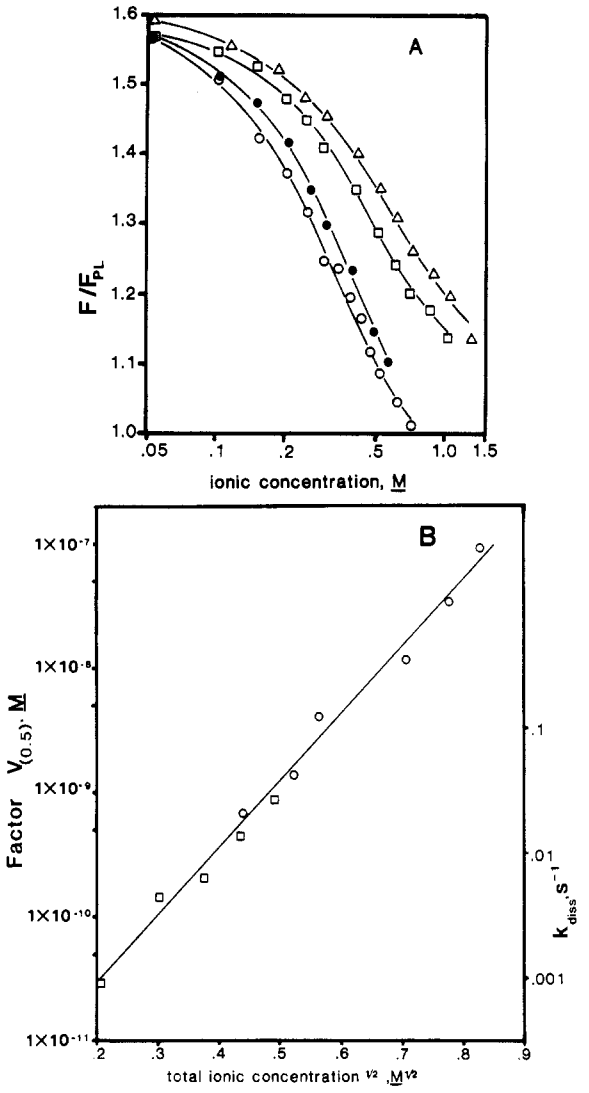

FIGURE 4: Dissociation of factor V–membrane complexes by salt. The ratio of factor V to phospholipid (PS–dansyl-PE–PC, 20:10:70) was constant in all experiments, and the energy transfer was about 90% of maximum saturation. The factor V concentrations shown in part A were (○) 2.8×10^{-9}, (●) 8.4×10^{-9}, (□) 2.5×10^{-8}, and (△) 7.5×10^{-8} M. Fluorescence intensity at 540 (excitation at 285 nm) was measured at 25 °C in 0.05 M Tris (pH 7.5). Aliquots of a concentrated NaCl solution were added, and the ratio of fluorescence intensity due to energy transfer (F) to fluorescence due to direct excitation of the dansyl moiety (F_{PL}) was plotted as a function of total ionic concentration. Part B shows the log of the free factor V concentration at half-titration (e.g., in part A) plotted vs. the square root of the ionic concentration (○). At the lowest protein concentration (1.4×10^{-9} M), the signal to noise ratio was 7.5. Also shown are dissociation rate constants (□) obtained from the first half-life for factor V–membrane dissociation at the ionic concentrations shown [data from Figure 6 of Pusey et al. (1982)].

at 25 °C in water and K_D^0 is the constant at zero ionic strength). Extrapolation to physiological ionic strength gave half-saturation at (2–3) $\times 10^{-10}$ M for factor V binding to membranes of PS/PC (20/80). This is very similar to an average dissociation constant estimated from the association rate constant and median dissociation rate constant under these conditions (about 10^{-10} M; Pusey et al., 1982). Also shown on Figure 4B are dissociation rate parameters estimated from the data of Pusey et al. (1982, Figure 6). These are median first-order rate constants calculated from the first half-time for factor V–membrane dissociation. The slopes (about 5.5) of the curves in Figure 4B were similar and should be equal to $-1.02Z_AZ_B$ where Z is the charge on the interacting species. While interpretation of this number as the absolute number

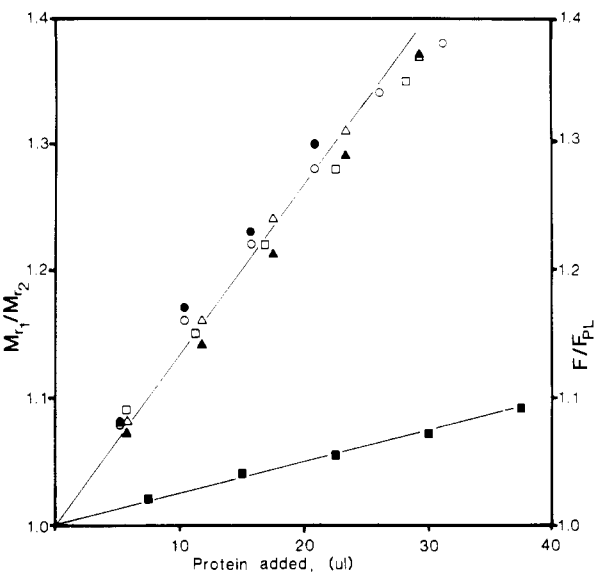

FIGURE 5: Comparison of fluorescence energy transfer (open symbols) and mass of membrane bound protein (closed symbols). Solutions of factor V (○, ●), thrombin-digested factor V (△, ▲), and thrombin-digested plus EDTA (5 mM)-treated factor V (□, ■) were obtained from the same protein preparation. The mass of membrane binding peptide was determined as the ratio of M_{r2} (relative molecular weight of the protein–vesicle complex) to M_{r1} (relative molecular weight of the vesicles alone). Fluorescence energy transfer is expressed by the terms described in Figure 4. The buffer was 0.05 Tris (pH 7.5)–0.1 M NaCl containing 0.1 mM calcium (25 °C).

of charges involved in this macromolecular system is probably unwarranted, the positive slope indicated that the charges were of different sign. Similar slopes for these two plots indicated that binding affinity and dissociation rate constants varied with ionic strength in the same manner. The association rate constant must therefore be essentially independent of ionic strength over the range tested. This agreed with the previous experimental measurement of association rates (Pusey et al., 1982).

A similar series of experiments was conducted with the PLBP at a protein/vesicle ratio of 70 (data not shown). A plot similar to Figure 4B gave a slope of +5, which extrapolated to 8×10^{-10} M protein at 0.15 M ionic strength.

Comparison of Molar Concentration and Mass of Membrane-Bound Proteins. Figure 5 shows comparison of the degree of fluorescence energy transfer and the mass of membrane-bound protein determined from light scattering measurements for factor V, factor Va, and EDTA-treated factor Va. The same factor V preparation was used so the proteins were present in equal molar concentrations. Factor V was treated with thrombin to produce Va and then with EDTA to dissociate the Va peptides (Esmon, 1979; Nesheim & Mann, 1979; Hibbard & Mann, 1980; Guinto & Esmon, 1982). Fluorescence energy transfer from these solutions gave the same fluorescence yield per aliquot of protein. The 90° light scattering measurements showed little loss of membrane binding protein after thrombin treatment. This was similar to a previous report (Pusey et al., 1982) where changes in the mass of membrane-bound protein were 10% or less. In contrast, after EDTA treatment only 20–25% of the original protein mass was still bound to the membrane. This agreed with the molecular weight of 74000–80000 for the membrane-binding peptide (Higgins & Mann, 1983; van de Waart et al., 1983) vs. 330000 for factor V (Nesheim et al., 1979a). Apparently, only the membrane binding peptide was bound after EDTA treatment and all fluorescence energy transfer occurred from the PLBP portion of factor Va.

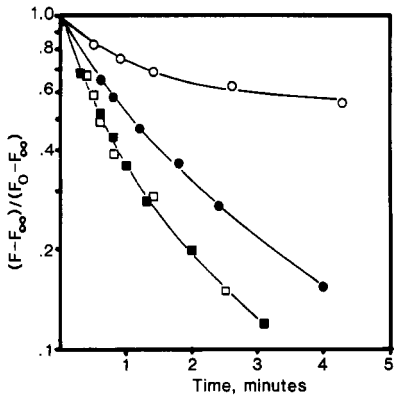

FIGURE 6: Effect of factor Xa on factor Va- and PLBP-membrane dissociation rates. Factor Va (○) or EDTA (5 mM)-treated factor Va (□) were mixed with fluorescent vesicles (5 μg of PG-dansyl-PE-PC, 40:10:50) and Xa (0.12 μM) in buffer [0.05 M Tris (pH 7.5)-0.1 M NaCl] containing 2 mM calcium. The EDTA in the treated sample did not significantly alter the free calcium concentration in this final solution. At zero time 80 μg of unlabeled vesicles (PG-PC, 40:60) was added. The method and analysis were that described in Figure 2B. The closed symbols show experiments corresponding to the open symbols but run in the absence of factor Xa.

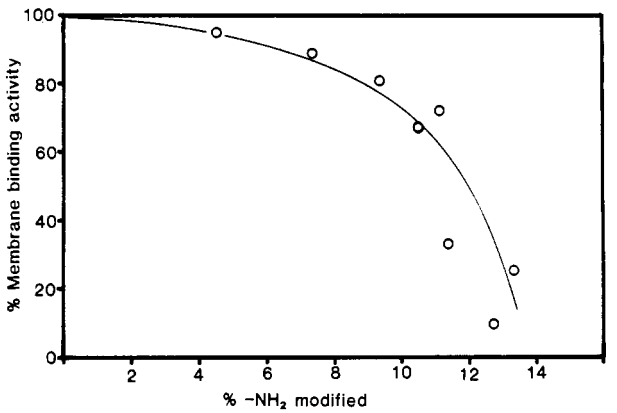

FIGURE 7: Effects of amino group modification on binding of PLBP to membranes. A solution of PLBP was treated with citraconic anhydride and the free amino groups were quantitated. Membrane binding was determined by the amount of fluorescence energy transfer to fluorescent labeled phospholipid (PG-dansyl-PE-PC, 40:10:50). The unmodified protein was assigned a value of 100%. The energy transfer was measured at 25 °C in 0.05 M Tris (pH 7.5)-0.1 M NaCl.

Effect of Factor Xa on the Dissociation of Va and PLBP from the Membrane. Previous results have shown that factor Xa had a pronounced effect on the factor Va-phospholipid dissociation rate (Pusey et al., 1982). Figure 6 shows an extension of those experiments to EDTA-treated factor Va. The results show that the dissociation rates for factor Va and PLBP were about the same, while those for the factor Va-Xa-phospholipid dissociation were greatly prolonged. The latter suggested a factor Va-Xa interaction on the membrane surface. Failure of factor Xa to affect the dissociation rate of EDTA-treated factor Va (Figure 5) indicated that there was no interaction between PLBP and factor Xa that stabilized their binding to the phospholipid surface. The same result was obtained for purified PLBP and factor Xa (data not shown).

Modification of Lysine Residues. The effects of blocking primary amine groups on membrane binding peptide-phospholipid interactions were investigated by using citraconic anhydride. Membrane binding was assayed by fluorescence energy transfer. Figure 7 shows that partial blocking of the amine groups with accompanied charge reversal led to loss of the phospholipid binding function. Both light scattering and fluorescence energy transfer experiments conducted over a 10-fold concentration range gave similar results, indicating that the effects were due to virtual loss of membrane binding activity by a population of the protein molecules and not to changes in the dissociation constant of the total protein population. Amino groups appear to be essential for phospholipid binding.

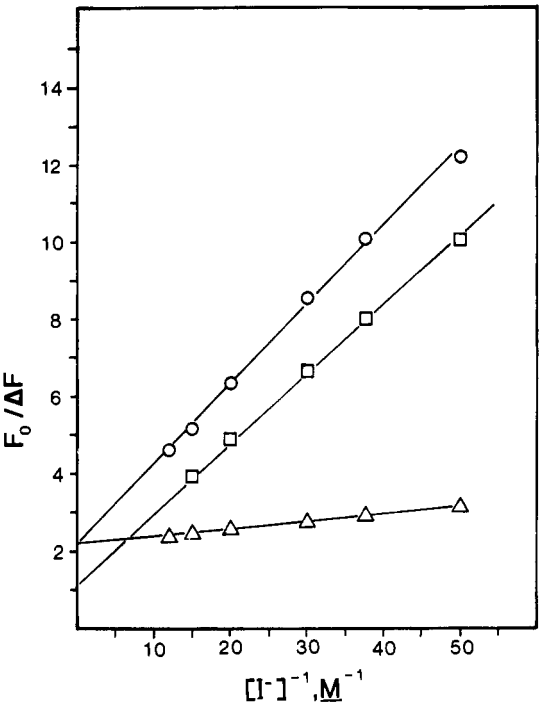

FIGURE 8: Modified Stern-Volmer plot of PLBP fluorescence quenching by iodide ion. The fluorescence intensity of PLBP (19.2 μg/mL) in the presence (○) and absence (△) of phospholipid (21 μg/mL of PS-PC, 20:80) was determined at 25 °C as a function of iodide ion concentration. The buffer was 0.02 M sodium phosphate (pH 8.0) and the total ionic concentration was 0.15 M. Also shown is a titration of protein alone in 6 M guanidine hydrochloride (□).

Iodide Quenching of Membrane Binding Peptide Fluorescence. The tryptophans involved in energy transfer are located on PLBP, and experiments were carried out to determine if these residues were more protected from solvent ion access when bound to the membrane. Figure 8 shows the results of an experiment on quenching of solvent accessible tryptophans with iodide using intact peptide, denatured peptide, and a peptide-phospholipid (PS-PC, 20:80) complex. A modified Stern-Volmer plot (Lehrer, 1971) of the quenching data gives a direct indication of the extent to which the fluorescent residues are accessible to the solvent when I^- is used as quencher. For peptide denatured in 6 M quanidine hydrochloride, all residues should be equally accessible and the plot should have an intercept of 1. The actual intercept was 1.1, which appeared to be within experimental error of the theoretical value. Quenching experiments on native protein indicated that about 45% of the fluorescence intensity was accessible to solvent (Figure 8). This was still the case after peptide-phospholipid complex formation, indicating that no additional tryptophan residues were totally shielded in the immediate binding region. The slopes of the curves were different, indicating a decreased accessibility to I^-. For example, steric factors caused by the membrane and other nearby proteins could change the slope of this curve for membrane-bound protein.

Tryptophan Modification. Figure 9 shows the effects of tryptophan modification on protein-membrane binding. Progressive tryptophan modification had a pronounced effect on fluorescence energy transfer to the membrane but had little

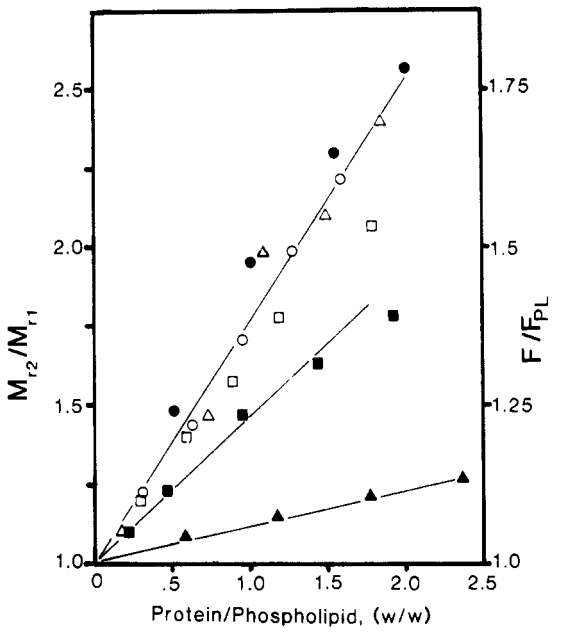

FIGURE 9: Effects of HNB-Br on PLBP–phospholipid interaction measured by light scattering (open symbols) and fluorescence energy transfer (closed symbols). Light scattering was determined by using 6.67 μg of PG–PC (40:60)/mL, while fluorescence energy transfer experiments were done by using 8.36 μg/mL PS–dansyl-PE–PC (20:10:70). Symbols are (○, ●) no treatment, (□, ■) 37% of the tryptophans reacted with HNB-Br, and (△, ▲) 68% reacted with HNB-Br. Full tryptophan modification was estimated by the absorbance change produced by treatment of PLBP in 8 M urea.

effect on peptide binding measured by light scattering. It appeared that the tryptophans responsible for energy transfer were not critical to membrane binding. The light scattering studies showed nearly quantitative membrane binding by the unmodified peptide (Figure 9).

Discussion

For stepwise association of multiple protein molecules to a phospholipid vesicle surface, a sequential binding model can be written:

$$P + V \underset{k_{2\cdot1}}{\overset{k_{1\cdot1}}{\rightleftharpoons}} P_1V + P \underset{k_{2\cdot2}}{\overset{k_{1\cdot2}}{\rightleftharpoons}} P_2V \ldots + P \underset{k_{2\cdot n}}{\overset{k_{1\cdot n}}{\rightleftharpoons}} P_nV \quad (4)$$

where V is the vesicle, P is protein, n is the maximum number of proteins that can bind to a vesicle, and $k_{1\cdot i}$ and $k_{2\cdot i}$ are the association and dissociation rate constants for the individual steps, respectively. V and n are average values due to the small polydispersity of these unilamellar vesicles.

Each second-order association rate constant obtained from application of eq 3 has units of (molar protein interaction sites)$^{-1}$ s^{-1}. The data in Figure 2 reasonably fit a single bimolecular interaction process (eq 1–3) so that in eq 4 $k_{1\cdot1} = k_{1\cdot2} = \ldots = k_{1\cdot n}$. In this case, a protein–vesicle association rate constant (k_1') can be obtained from: $k_1' = nk_{1\cdot i}$. This latter rate constant is related to the association of any protein molecule with the vesicle and is the appropriate value to use for comparison with the protein–vesicle collisional rate constant to obtain estimates of collisional efficiency. The association rate constants for several proteins are shown in Table I. All of these peripheral membrane binding proteins showed a high collisional efficiency, indicating little molecular fitting during the association process. Factor V and the PLBP show similar protein–vesicle association rate constants (k_1'; Table I), indicating that similar structures and processes are involved in these associations.

Table I: Protein–Vesicle Association Rate Constants at 10 °C

protein	k_1[a]	n	k_1'[b]	reference
prothrombin	1×10^7	70	7×10^8	Wei et al. (1982)
factor X	1×10^7	100	1×10^9	G. L. Nelsestuen et al., unpublished results
factor IX	1.3×10^7	100	1.3×10^9	G. L. Nelsestuen et al., unpublished results
myelin basic protein	2×10^8	50	1×10^{10}	Lampe et al. (1983)
factor V	1×10^8	70	2×10^9	Pusey et al. (1982)
factor V_a–LC	5.5×10^7	75	4.2×10^9	Figure 2 above (adjusted to 10°)

[a] k_1 has units of (molar protein binding sites)$^{-1}$ s^{-1}. [b] k_1' has units of (molar vesicles)$^{-1}$ s^{-1}.

First-order rate plots for protein–vesicle dissociation showed curvature by two different methods (insets, Figure 3; Pusey et al., 1982). The results showed that dissociation was faster when protein density on the vesicle was high. This behavior corresponds to anticooperative binding of proteins to the vesicle and is expected for a system where multiple proteins each bind to multiple mobile receptors on a surface (Dwyer & Bloomfield, 1981). The receptors in this case are acidic phospholipids; at higher protein densities there are fewer acidic phospholipids per protein and lower average protein–membrane binding affinity. Such anticooperative binding could also result from steric crowding of the proteins as saturation is reached. In any event, comparison of the membrane-binding parameters of two similar proteins must be carried out at similar protein–membrane binding ratios so the same constants are compared. Under these conditions it was clear that the dissociations of factor V and PLBP from the membrane were very nearly identical [Figure 3B, (□) and (●)]. We therefore conclude that the structures and processes involved in factor V–membrane dissociation are essentially identical with those involved in PLBP–membrane dissociation. Thus, the PLBP region of factor V appears to contain the entire membrane binding region of factor V.

More PLBP than factor V molecules were able to associate with a given vesicle. This was similar to previous reports (Higgins & Mann, 1983; Van de Waart et al., 1983). As anticipated from this study, the additional molecules of PLBP associate with lower affinity due to higher dissociation rate constants. As a result, methods or conditions that detect apparent dissociation constants, averaged over all sites (e.g., Figure 4A), will indicate that PLBP has a lower average membrane binding affinity than factor V. This was found to be the case for the experiments shown in Figure 4 (see above). Such results should nevertheless be useful for the comparisons shown in Figure 4B. They give dissociation constants that are similar to those obtained from averaged kinetic parameters (Pusey et al., 1982).

Apparent dissociation constants for factor V association with various cell types have been reported to be approximately 10^{-10} M (Tracy et al., 1981, 1983; Kane & Majerus, 1982). These values were obtained by Scatchard analysis of protein–cell binding measurements. The validity of such analyses has been questioned (Klotz, 1982), and this apparent dissociation constant may also represent an average or maximum value. An important observation is that this apparent dissociation constant corresponds to averaged values for factor V–phospholipid complexes (Pusey et al., 1982; Figure 4B). It is therefore possible but not necessary that factor V–cellular

interactions are mediated by phospholipids.

Since factor Xa did not participate in the fluorescence energy transfer process when dansyl PE was used as the acceptor of tryptophan energy transfer, this technique was used to investigate the interactions of factor Xa with the membrane binding peptide and with factor Va on the membrane. No interaction between factor Xa and PLBP on the vesicle surface was detected. In contrast, intact factor Va showed a strong interaction with factor Xa. We concluded from this that PLBP alone was insufficient for interaction with factor Xa on the vesicle surface. In independent studies van de Waart (1984) arrived at the same conclusion. Other workers have presented data suggesting that the PLBP (component E) was involved in binding factor Xa to the platelet surface (Tracy & Mann, 1983). The effect of PLBP on factor Xa binding to the platelet (Tracy & Mann, 1983) therefore may not be analogous to a protein–protein binding on the phospholipid surface. The results to date do not rule out the possibility that PLBP contains a factor Xa binding region that becomes functional upon association with the factor Va heavy chain or is formed jointly with the heavy chain.

Determination that the PLBP–membrane binding site was virtually identical with that of factor Va made it an excellent subject for further investigations. Our initial efforts concerned the role of tryptophan and lysine residues, the latter due to the requirement for acidic phospholipids and the former because of the fluorescence energy transfer process and its potential utility in determining protein–membrane interaction geometry. After reaction with citraconic anhydride, charge reversal of the modified lysine residues occurs and the membrane binding peptide was found to have reduced membrane binding ability as measured by fluorescence energy transfer. By altering the concentration over a 10-fold range, it was determined that the effects were not due to small changes in binding affinity and could be treated as the loss of functional protein. As expected, progressive tryptophan modifications had dramatic effects on the fluorescence energy transfer process. However, little effect was seen on the mass of membrane-bound protein determined by light scattering. Experiments involving iodide quenching of protein tryptophan indicated that no additional residues were shielded from access to the bulk solvent upon binding to vesicles. Higher quenching constants were observed and could be due to reduced access to the peptide on the vesicle surface due to steric factors. It appears that most or all of the protein is external to the membrane.

Factor Va activity can be generated from only two peptides of total M_r 180000 (Esmon, 1979). The results obtained here (Figure 5) indicated that factor Va, as generated by thrombin cleavage of factor V, contained approximately the same mass as factor V. This latter observation was consistent with the observation that thrombin digestion had little effect on electrophoretic mobility of factor V under nondenaturing conditions (Nesheim & Mann, 1979). The function of approximately half of the factor Va molecule is therefore unknown. It is interesting that thrombin cleavage greatly altered the degree to which the protein extended outward from the vesicle surface (Pusey et al., 1982). The relationship of this observation to factor Va function is still undefined.

Registry No. Blood coagulation factor V, 9001-24-5; blood coagulation factor Va, 65522-14-7; blood coagulation factor Xa, 9002-05-5; lysine, 56-87-1.

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Construction of DNA Sequences Complementary to Rat α_1 and α_2 Collagen mRNA and Their Use in Studying the Regulation of Type I Collagen Synthesis by 1,25-Dihydroxyvitamin D†

C. Genovese, D. Rowe,* and B. Kream

ABSTRACT: Type I collagen mRNA from fetal rat calvaria was used as a template for the synthesis of a cDNA that was subsequently inserted in the *Pst*I site of the plasmic vector pBR322 and cloned. Three recombinant plasmids containing type I collagen specific sequences were characterized: pα_1R1 is 1600 bp and spans approximately 500 amino acid residues within the triple helical region of α_1(I) and pα_1R2 is 900 bp in size and covers the entire 3′ noncoding and about half of the C-terminal propeptide region of α_1(I) collagen mRNA. The third recombinant pα_2R2 is 1500 bp and contains α_2(I) sequences specific for the entire 3′ noncoding and C-terminal propeptide region. Partial nucleic acid sequence data revealed that the decreasing order of amino acid and nucleotide homology to similar regions of the rat cDNA was mouse > human > chick. Northern hybridization of mRNA after electrophoresis in 0.8% agarose revealed two distinctly different molecular weight patterns characteristic of α_1(I) (4.7 and 5.7 kb) and α_2(I) (4.2 and 4.5 kb) collagen mRNA when hybridized with the corresponding cDNA probe. Despite the high degree of sequence homology, DNA probes from rat or human produced a significantly reduced hybridization signal when used as an interspecies hybridization probe than to its corresponding mRNA. The rat cDNA probes were used in a dot hybridization assay to measure the type I collagen mRNA content in the fetal rat calvaria. The 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] reduced collagen synthesis and type I collagen mRNA levels in osteoblasts located in the central bone segment of calvaria but had no effect on cells in the periosteum. Furthermore, 1,25-$(OH)_2D_3$ appeared to regulate the levels of the α_1(I) and α_2(I) collagen mRNA in a coordinated manner.

Collagen is the most abundant structural protein in vertebrates. It provides support for body organs and plays an important role in development and cell–cell interactions. Thus, it is not surprising that its production is highly regulated. For example, transcriptional regulation has been observed in the Rous sarcoma transformed chick embryo fibroblasts (Sandmeyer & Bornstein, 1981; Sobel et al., 1981) and during development of chick calvaria (Moen et al., 1979). Translational control is mediated by the cleaved N-terminal propeptide of type I procollagen (Horlein et al., 1981). Posttranslational regulation of the collagen synthesis is well documented at the level of intracellular degradation (Berg et al., 1980). Our laboratory has studied the mechanism(s) by which parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$][1] regulate bone collagen synthesis. Using cell-free translation we have shown that the collagen synthesis and the steady-state mRNA levels are diminished to the same degree in fetal rat calvaria incubated in the presence of these hormones (Kream et al., 1980; Rowe & Kream, 1982). To define further the mechanism of the regulation, cDNA probes to chick and human type I collagen were hybridized to rat mRNA extracted from cultured rat calvaria. Because this heterologous hybridization signal was weak, we developed rat-specific type I collagen cDNA probes. This report describes the synthesis and characterization of these probes. These cDNA probes have sufficient sensitivity to measure changes in type I collagen mRNA in the central bone and periosteal components of fetal rat calvaria. Using a dot hybridization assay, we determined that 1,25-$(OH)_2D_3$ reduced the relative type I collagen mRNA content and collagen synthesis in the central bone but not the periosteum.

Experimental Procedures

Construction and Cloning of cDNA. Calvaria from 40–50, 19-day-old fetal rats were removed, cleaned free of contaminating tissues and surrounding epithelium, and immersed in liquid N_2. The RNA was extracted in SET buffer (1% SDS, 5 mM EDTA, 10 mM Tris, pH 7.5) containing 50 μg/mL proteinase K (Rowe et al., 1978). The 28S RNA was isolated by SDS–sucrose gradient centrifugation, and the mRNA was poly(A) selected by passage through an oligo(dT)-cellulose

† From the Departments of Pediatrics (C.G. and D.R.) and Medicine (B.K.), the University of Connecticut Health Center, Farmington, Connecticut 06032. *Received January 10, 1984; revised manuscript received June 28, 1984.* Supported by NIH Grants AM29983 and AM29850. D.R. is a recipient of RCDA HD00330, and B.K. is a recipient of RCDA AM01017.

[1] Abbreviations: PTH, parathyroid hormone; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethylcellulose; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; SSC, 0.15 M NaCl–0.015 M sodium citrate; dGTP, deoxyguanosine triphosphate; dATP, deoxyadenosine triphosphate; dTTP, thymidine triphosphate; dCTP, deoxycytidine triphosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TCA, trichloroacetic acid; CDP, collagenase-digestible protein.

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