gardless of the means of inducing unfolding provides strong support for representing the N ⇄ U equilibrium by the two-state model. The connection between thermal- and denaturant-induced unfolding is rarely ever made because the thermally unfolded state is not generally considered to be as extensively unfolded as it would be in GdnHCl or urea. In the presence of very high and constant chloride ion concentration, the connection between GdnHCl and thermal unfolding of thioredoxin appears to be remarkably simple in this particular system. Only further studies with other proteins will tell whether the linear relationship is of a general nature.

Registry No. GdnHCl, 50-01-1; urea, 57-13-6.

REFERENCES

Alonso, D. O. V., & Dill, K. A. (1991) *Biochemistry 30*, 5974–5985.
Bolen, D. W., & Santoro, M. M. (1988) *Biochemistry 27*, 8069–8074.
Dill, K. A. (1985) *Biochemistry 24*, 1501–1509.
Dill, K. A., & Shortle, D. (1991) *Annual Review of Biochemistry* (Richardson, C. C., Abelson, J. N., Meister, A., & Walsh, C. T., Eds.) pp 795–825, Annual Reviews Inc., Palo Alto, CA.
Greene, R. F., & Pace, C. N. (1974) *J. Biol. Chem. 249*, 5388–5393.
Langsetmo, K., Fuchs, J., & Woodward, C. (1989) *Biochemistry 28*, 3211–3220.
Laurent, T. C., Moore, E. C., & Reichard, P. (1964) *J. Biol. Chem. 239*, 3436–3444.
Lim, C.-J., Geraghty, D., & Fuchs, J. A. (1985) *J. Bacteriol. 163*, 311–316.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1–43.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266–280.
Pace, C. N., & Vanderburg, K. E. (1979) *Biochemistry 18*, 288–292.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063–8068.
Schellman, J. A. (1987) *Biopolymers 26*, 549–559.

Pentraxin Family of Proteins Interact Specifically with Phosphorylcholine and/or Phosphorylethanolamine†

Ruth A. Schwalbe,‡ Björn Dahlbäck,§ John E. Coe,∥ and Gary L. Nelsestuen*,‡

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108, Department of Clinical Chemistry, Lund University, Malmö General Hospital, S-214 01 Malmö, Sweden, and Laboratory of Persistent Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana 59840

Received November 1, 1991; Revised Manuscript Received March 13, 1992

ABSTRACT: Pentraxins are a family of serum proteins characterized by five identical subunits that are noncovalently linked. The two major types of pentraxins are C-reactive protein (CRP) and serum amyloid P component (SAP). CRP proteins are identified by their calcium-dependent interaction with phosphorylcholine. This study showed that SAP also bound to phosphorylated compounds but had a high specificity for phosphorylethanolamine. Thus, human CRP and SAP show high specificity that is complementary for the related compounds, phosphorylcholine and phosphorylethanolamine, respectively. This relationship suggests a complementary and/or related function for the pentraxins. Pentraxins from other species were also examined. Mouse SAP showed binding interactions and specificity similar to human SAP. Female protein (FP) from hamster and rat CRP showed a hybrid specificity and bound to both phosphorylethanolamine and phosphorylcholine. All of the proteins that bound phosphorylethanolamine also associated with human C4b-binding protein (C4BP). With the exception of human and rat CRP, all the proteins also bound to vesicles containing acidic phospholipids. All of these binding interactions were calcium-dependent and mutually exclusive, suggesting that they involved the same site on the protein. These findings suggest possible ways to examine the function of the pentraxins.

The pentraxin family of proteins have been highly conserved throughout evolution in vertebrate species (Pepys et al., 1978; Baltz et al., 1982). A common structural feature is five identical subunits noncovalently bound in a cyclic manner (Gotschlich & Edelman, 1965; Kushner & Sommerville, 1970; Bach et al., 1977; Oliveria et al., 1980; Pepys et al., 1978, 1982; Pontet et al., 1981; de Beer et al., 1982; Baltz et al., 1982; Pepys & Baltz, 1983). In fact, molecular structure is a vital criterion for classification as a pentraxin (Osmand et al., 1977). The members of this family include C-reactive protein (CRP),[1] serum amyloid P component (SAP), and female protein (FP) from hamster (Skinner & Cohen, 1988). CRPs and SAPs are distinguished by specific binding properties and whether or not the pentraxin is associated with amyloid deposits (Pepys

† Supported in part by Grant HL15728 (to G.L.N.) from the National Institutes of Health and Grant B91-13X-07143-07B (to B.D.) from the Swedish Medical Research Council.
‡ University of Minnesota.
§ Lund University.
∥ National Institutes of Health.

[1] Abbreviations: PE, phosphorylethanolamine; PC, phosphatidylcholine; CRP, C-reactive protein; SAP, serum amyloid P component; FP, female protein from Syrian hamster; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; dansyl-PtdEtn, dansyl phosphatidylethanolamine; CPS, pneumococcal C-polysaccharide; EDTA, ethylenediaminetetraacetate.

0006-2960/92/0431-4907$03.00/0

& Baltz, 1983; Coe & Ross, 1985). Pentraxins that bind phosphorylcholine and pneumococcal C-polysaccharide (CPS) are classified as CRPs, and those that bind agarose with high affinity are SAPs (Pepys et al., 1978). Also, if the pentraxin is associated with amyloid deposits it is categorized as a SAP (Pepys et al., 1982; Coe & Ross, 1985; Skinner & Cohen, 1988). FP isolated from Syrian hamsters binds phosphorylcholine (Coe, 1977; Coe et al., 1981) and is found in amyloid deposits (Coe & Ross, 1985). It is therefore classified as a CRP/SAP pentraxin (Coe et al., 1981).

Human CRP was identified by Abernathy and Avery (1941) as the material that precipitated CPS in acute-phase serum (Tillet & Francis, 1930). This precipitation reaction was calcium-dependent (Abernathy & Avery, 1941; Gotschlich et al., 1982). Subsequently, a number of phosphomonoesters were reported to inhibit the interaction between CPS and human CRP, by binding to CRP (Gotschlich & Edelman, 1967; Anderson et al., 1978; Gotschlich et al., 1982). Indirect and direct measurements showed that phosphorylcholine was much more effective than the other phosphomonoesters (Volanakis & Kaplan, 1971; Anderson et al., 1978). In contrast, SAP did not associate with phosphorylcholine.

A distinguishing feature of human CRP and the majority of other CRPs that have been studied is the change in serum concentration in relation to various physiological conditions. For example, in humans, CRP is present in very low concentration under normal circumstances (Baltz et al., 1982; Skinner & Cohen, 1988) but will rise as much as 1000-fold in response to tissue damage or inflammation that cause the acute-phase response (Morley & Kushner, 1982; Skinner & Cohen, 1988). In rat, as well as other species, the phosphorylcholine binding protein, CRP, is normally found in substantial amounts and increases about 2- or 3-fold due to the acute phase (Pontet et al., 1981; Nagpurkar & Mookerjea, 1981; de Beer et al., 1982). Because CRP is associated with this type of physiological response, it is described as an acute-phase reactant. Thus, human CRP is a major acute-phase reactant, and rat CRP is a minor acute-phase reactant. However, designation of the acute-phase reactants as major and minor on the basis of the change in protein concentration does not deal with the total amount of protein. For instance, the serum levels of rat CRP are about 3-fold greater than those in human serum. It is therefore possible that denoting the acute-phase reactants as major and minor can be somewhat misleading by emphasizing the change in protein serum levels and minimizing the significance of the pentraxin that is already available and/or functioning.

Human SAP is a normal serum and tissue protein (Dyck et al., 1980; Breathnach et al., 1981) and a minor acute-phase reactant. Recently, human SAP was reported to bind to C4b-binding protein (C4BP) in human serum (Schwalbe et al., 1990b). This interaction might have a number of functions including delivery of SAP to other sites in the circulation or in regulating complement activities. C4BP is an inhibitory regulator of complement (Scharfstein et al., 1978; Nagasaw & Stroud, 1980). C4BP is also complexed with protein S, an inhibitory regulator of blood clot formation (Dahlbäck & Stenflo, 1981). Furthermore, the protein S–C4BP complex will associate with negatively charged membranes through the protein S component (Schwalbe et al., 1990a). Heparin disrupts the high-affinity SAP–C4BP interaction (Schwalbe et al., 1991), and this may also play a role in modulating complement activity.

SAP also interacts with various forms of DNA (Pepys & Butler, 1987; Breathnach et al., 1989; Butler et al., 1990), whereas CRP binds to the protein moiety of chromatin (Robey et al., 1984; Du Clos et al., 1991). FP was shown to share binding to chromatin (like CRP) and DNA (like SAP) (L. Saunero-Nova, J. E. Coe, and T. W. Du Clos, manuscript in preparation).

An unusual relationship has been found in mice. While they have both SAP and CRP, the major acute-phase reactant is SAP, not CRP (Pepys et al., 1979; Baltz et al., 1980). Nevertheless, mouse SAP is similar to other SAPs in that it does not bind phosphorylcholine and it is a constituent of amyloid deposits (Skinner et al., 1974; Baltz et al., 1980).

The biosynthesis of the SAP/CRP homologue in hamster serum, FP, has been reported to be under hormonal (Coe, 1977) and acute-phase control (Coe & Ross, 1983). FP is actually a negative acute-phase reactant in female hamsters but a major positive acute-phase reactant in males (Coe & Ross, 1983). FP shares a phosphorylcholine binding specificity with CRP and also a agarose binding specificity with SAP (Coe et al., 1981). The extraordinary high serum level of FP in female hamsters (1–3 mg/mL) may be associated with early amyloidosis and premature death (Coe & Ross, 1990).

This study demonstrated that the pentraxins can be separated into three types, based on their binding specificity. As reported previously, CRP binds specifically to phosphorylcholine. A new finding was that SAP homologues show specific interaction with phosphorylethanolamine. The SAP/CRP homologue, FP, as well as CRP from rat, showed intermediate specificity and interacted with both phosphorylcholine and phosphorylethanolamine. Proteins that bound phosphorylethanolamine all shared an interaction with C4BP. These general patterns of specificity may be relevant to the function of the pentraxins.

Materials and Methods

Human SAP was either purchased from the Sigma Chemical Co. or was prepared by standard procedures (Thompson & Enfield, 1978). No difference in the various interactions of protein from these preparations was detected (Schwalbe et al., 1990b). Mouse SAP was isolated according to Pepys et al. (1978), and rat CRP was purified by phosphorylcholine–Sepharose absorption (Coe et al., 1981). FP was purified from female hamster serum by standard procedure (Coe et al., 1981). Human CRP was purchased from Sigma.

The calcium salt of phosphorylcholine was purchased from Sigma; the calcium ions were removed by passage through Chelex 100 (Bio-Rad Laboratories) resin. The cadmium chloride complex of L-α-glycerophosphorylcholine was purchased from Sigma. To produce free L-α-glycerophosphorylcholine, an ion-exchange column was used as described in the pamphlet that accompanied this product. Both products were concentrated under vacuum, and the concentration was determined by organic phosphate (Chen et al., 1956) content. *O*-Phosphorylethanolamine and glycerophosphate were also purchased from Sigma. Protein molar ratios were calculated from protein molecular weights of 151 000 for FP (Coe et al., 1981), mouse SAP, and rat CRP, 235 000 for human SAP (Painter et al., 1982), and 570 000 for C4BP (Dahlbäck, 1983). Protein concentrations were determined by the methods described in the preparation procedures.

Proteins were radiolabeled by the reductive methylation procedure outlined by Jentoft and Dearborn (1983). Radiolabeled formaldehyde (specific activities of 56.4 mCi/mmol for [^{14}C]formaldehyde and 100 mCi/mmol for [^{3}H]formaldehyde) was obtained from Du Pont–New England Nuclear. $NaCNBH_3$ (20 mM, Sigma) was added to proteins (0.5–1.8

mg/mL) in 50 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. This was followed by addition of 50 μCi of radiolabeled formaldehyde/mg of protein. The reaction mixtures were allowed to stand at room temperature for 4 h and were then dialyzed against 50 mM Tris buffer (pH 7.5) containing 0.1 M NaCl until all the free radiolabeled formaldehyde was removed. The purity of the final protein preparations were analyzed by their sedimentation patterns in a sucrose density gradient centrifugation (see below). Samples that did not give a single sedimentation peak were purified by chromatography on a *O*-phosphorylethanolamine affinity column (Sigma). The proteins were applied in buffer (50 mM Tris, pH 7.5, 100 mM NaCl) containing 1 mM calcium. The columns were washed with the same buffer, and the purified proteins were eluted with buffer containing EDTA. The proteins were dialyzed against 50 mM Tris buffer (pH 7.5) containing 0.1 M NaCl. With the exception of mouse SAP, this procedure always produced radiolabeled protein that gave a single radioactive component when evaluated by sucrose density gradient centrifugation. The final protein preparations and specific activities were ^{14}C-human SAP, 5490 cpm/μg; ^{14}C-FP, 5534 cpm/μg; ^{14}C-mouse SAP, 4364 cpm/μg; ^{14}C-rat CRP, 3875 cpm/μg; ^{3}H-human CRP, 16929 cpm/μg.

Sucrose density gradient ultracentrifugation was carried out in isokinetic gradients as described by McCarty et al. (1974). Gradients were formed by continuous gradient production using a constant mixing volume (10 mL per tube). The mixing chamber contained 10% sucrose solution, and the reservoir contained 31% sucrose solution. Solution was pumped out of the mixing chamber into centrifuge tubes (14- × 95-mm polyallomer, Beckman). The tubes were allowed to stand at 4 °C for 2 h, and samples (0.3 mL) were applied to the top of the gradient. Buffers used for centrifugation contained 50 mM Tris (pH 7.5) containing 0.1 M NaCl and 0.1% bovine serum albumin (Sigma). Calcium (1 mM) and EDTA (2 mM) were added as needed. Some samples contained 0.05 mL of serum in a total sample volume of 0.3 mL. The samples were centrifuged for 26 h at 36000 rpm in a Beckman Model SW 40 rotor and Beckman Model L5-50 preparative ultracentrifuge. Temperature for all runs was 4 °C. Samples were eluted by continuous injection of 31% sucrose solution into the bottom of the tube so that fraction number 1 is from the top of the tube. The fractions were collected by drop counting, and fraction volume was determined to be 0.465 mL. Reproducibility of a sedimentation position was ±0.2 mL (Schwalbe et al., 1990b). Other details regarding the reproducibility of replicate runs and the standards used are as reported by Schwalbe et al. (1990b).

Egg phosphatidylcholine (PtdCho), phosphatidylserine (PtdSer) from bovine brain, and dansyl-phosphatidylethanolamine (dansyl-PtdEtn) were purchased from Sigma Chemical Co. and were reported to be greater than 95% pure. The procedure outlined by Nelsestuen and Lim (1977) was followed for preparation of phospholipid vesicles. In short, the phospholipids were mixed in organic solvents and dried under a stream of nitrogen. The phospholipids were then suspended in a 50 mM Tris buffer containing 0.1 M NaCl at pH 7.5 and subjected to sonication for 2-s bursts followed by a 3-s rest period for a total sonication time of 5 min. The sonicator was a Heat Systems Model W385 sonifier-cell disruptor. The phospholipid solution was kept in a water–ice bath. After sonication, the phospholipid mixture was chromatographed on a column of Sepharose 4B to isolate the small unilamellar vesicles (Huang, 1969). The phospholipid concentrations were determined from phosphate measurements (Chen et al., 1956) using a phosphorus to phospholipid weight ratio of 25.

Protein–membrane interaction was detected by a fluorescence energy transfer technique. The excitation wavelength was 280 nm and emission was 520 nm. The composition of the phospholipid vesicles used in all the reported studies was 20% PtdSer, 70% PtdCho, and 10% dansyl-PtdEtn. Fluorescence intensity due to direct excitation of the phospholipid vesicle at 280 nm provided the reference emission (I_o). Fluorescence energy transfer due to protein–membrane interaction was calculated as a fractional change in emission intensity $(I - I_o)/I_o = \Delta Fl$, where I is the total emission intensity of the protein–membrane complex. The protein–membrane interactions are expressed as percentages of the maximum fluorescence intensity $(\Delta Fl \times 100)/\Delta Fl(max)$. All binding interactions were reversed by addition of excess EDTA, demonstrating that the interactions were calcium-dependent.

The interaction of pentraxins with C4BP was measured by light-scattering intensity at 90° using light of 320 nm (Schwalbe et al., 1990b). The results were expressed as a percentage of maximum light-scattering intensity change $(\Delta I \times 100)/\Delta I(max)$. Excess light scattering (ΔI) is the total light scattered minus that from the individual components. Since intensity is proportional to molecular weight, interaction between the particles will result in excess light-scattering intensity. Light scattering was also used to detect calcium-induced aggregation of SAP. In every case, signal changes were reversed by addition of excess EDTA.

Fluorescence energy transfer and excess light-scattering measurements were performed with a Hitachi Perkins–Elmer Model MPF 44A fluorescence spectrophotometer. Unless indicated, the buffer used to study protein–protein and protein–membrane interactions was 50 mM Tris, pH 7.5, containing 100 mM NaCl and calcium or EDTA as indicated. The temperature for all interactions was 25 °C. Experimental uncertainty is provided by signal to noise values. Repetitive titrations gave results that were indistinguishable.

Results

Interactions of FP with C4BP and Phospholipid Vesicles. FP and SAP have similar characteristics in that both have been found in amyloid deposits (Coe & Ross, 1985), they have homologous amino acid sequences, and both are members of the pentraxin family of proteins (Coe et al., 1981). Recent reports have demonstrated that human SAP binds to phospholipid vesicles and to C4BP (Schwalbe et al., 1990b, 1991). Other pentraxins were included in this study, and Figure 1 illustrates the type of results obtained. The interaction with C4BP was readily detected from excess light-scattering intensity. The results showed a saturable signal change, indicating formation of a discrete complex. The complex appeared to be a 1:1 complex since the signal change was similar to that produced by SAP–C4BP interaction (Schwalbe et al., 1990b). The stoichiometry of the FP–C4BP complex also appeared to be about 1:1 on the basis of the mass ratio at the saturation point (Figure 1A).

FP interaction with membranes was detected by fluorescence energy transfer (Figure 1B). The tryptophan residues in FP are able to transfer fluorescence energy to dansyl-PtdEtn incorporated in the phospholipid vesicle when the two fluorophores are in close proximity. The increase in fluorescence intensity indicated that FP bound to negatively charged membranes in a calcium-dependent fashion. The calcium concentration at the midpoint of the titration curve was 2 mM which was about 2-fold higher than the calcium required for SAP–membrane interaction (Schwalbe et al., 1990b).

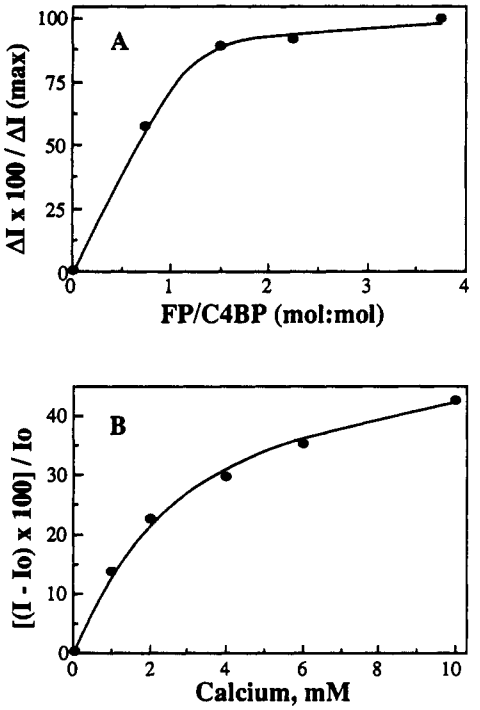


FIGURE 1: Interaction of FP with C4BP and phospholipids. (Panel A) Relative excess light-scattering intensity resulting from addition of FP to a solution of C4BP (26 μg/mL; 0.046 μM) in buffer containing 1 mM calcium is shown. Signal to noise was greater than 15:1. (Panel B) The results detect protein–membrane binding by fluorescence energy transfer. Fluorescence intensity of the vesicles alone (I_0) and of the protein–vesicle mixture (I) was detected as calcium was added. The solution contained FP (15 μg/mL; 0.097 μM) and phospholipid vesicles (16 μg/mL) in 50 mM Tris, pH 7.5, containing 0.1 M NaCl. Signal to noise was approximately 30:1.

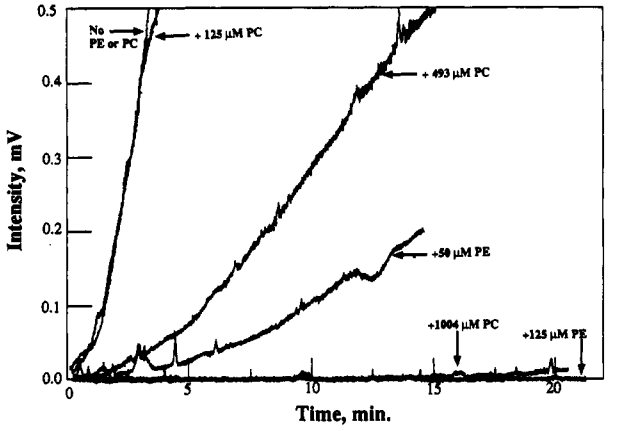


FIGURE 2: Inhibition of SAP aggregation by phosphorylethanolamine or phosphorylcholine. The light-scattering intensity of a solution of human SAP (31 μg/mL) to which calcium (4 mM) was added at zero time is shown. The tracings result from solutions containing various amounts of phosphorylethanolamine or phosphorylcholine. The intensity of the instrument output is expressed in millivolts. The six light-scattering tracings presented, beginning with the uppermost curve are no phosphomonoester; 125 μM phosphorylcholine; 493 μM phosphorylcholine; 50 μM phosphorylethanolamine; 1004 μM phosphorylcholine; and 125 μM phosphorylethanolamine. EDTA, added to these mixtures, returned the light-scattering signals to their original values.

Phosphorylethanolamine and Phosphorylcholine Affect SAP Aggregation. SAP aggregation is calcium-dependent and a highly cooperative process (Schwalbe et al., 1990b). Figure 2 shows time-dependent changes in the light-scattering intensity of human SAP in the presence of various amounts of phosphorylethanolamine or phosphorylcholine. Both phosphate monoesters affected SAP aggregation, but phosphorylethanolamine was much more effective. For example, during the time shown in Figure 2, 125 μM phosphorylethanolamine inhibited SAP aggregation completely, while the same amount of phosphorylcholine did not have a detectable effect. However, if phosphorylcholine concentrations were high enough, SAP aggregation could be prevented for the time period indicated (Figure 2). If the reactions were followed for longer periods of time, some aggregation always occurred. For example, the experiment with 125 μM phosphorylethanolamine began to show SAP aggregates after 2.5 h. A sample that contained 250 μM phosphorylethanolamine had virtually no detectable aggregation after 16 h (data not shown). All the aggregation reactions were reversed by EDTA addition. This data indicated that SAP interacted with both phosphomonoesters but with considerable specificity for phosphorylethanolamine. Pontet et al. (1981) did not detect human SAP interaction with this ligand. The procedure consisted of detecting an interaction by the ability to dissociate preformed SAP aggregates. It apparently had lower sensitivity than the procedure of preventing aggregation (Figure 2).

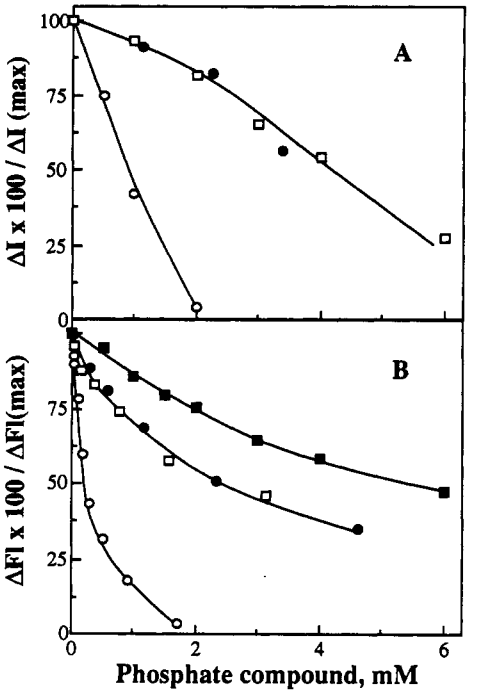


FIGURE 3: Dissociation of human SAP–C4BP and human SAP–membrane complexes by various phosphate compounds. (Panel A) The dissociation of preformed SAP–C4BP complexes was monitored as a function of phosphorylethanolamine (○), phosphorylcholine (●), or glycerophosphate (□) added to the solution. The concentration of SAP was 5.3 μg/mL, and C4BP was 26 μg/mL. ΔI is the excess light scattering from the solution due to SAP–C4BP complex formation while $\Delta I_{(max)}$ is the maximum value. Signal to noise was at least 12 to 1. (Panel B) The dissociation of SAP (16 μg/mL) from phospholipid vesicles (16 μg/mL) was monitored as a function of the concentration of phosphorylethanolamine (○), phosphorylcholine (●), glycerophosphate (□), or inorganic phosphate (■) in the solution. The standard Tris buffer was used with a calcium content of 1 mM. The signal is fluorescence intensity of the sample that is due to energy transfer (ΔFl) expressed relative to its maximum value [$\Delta Fl_{(max)}$]. Signal to noise was at least 17:1.

Dissociation of Complexes of Human SAP or FP with C4BP and Phospholipids. As previously reported, human SAP and C4BP form a 1:1 calcium-dependent complex (Schwalbe et al., 1990b). The human SAP–C4BP interaction was detected by excess light-scattering intensity by the same procedure illustrated in Figure 1A for the FP–C4BP interaction. Ligand binding specificities were determined by competition experiments. Human SAP and C4BP were added to calci-

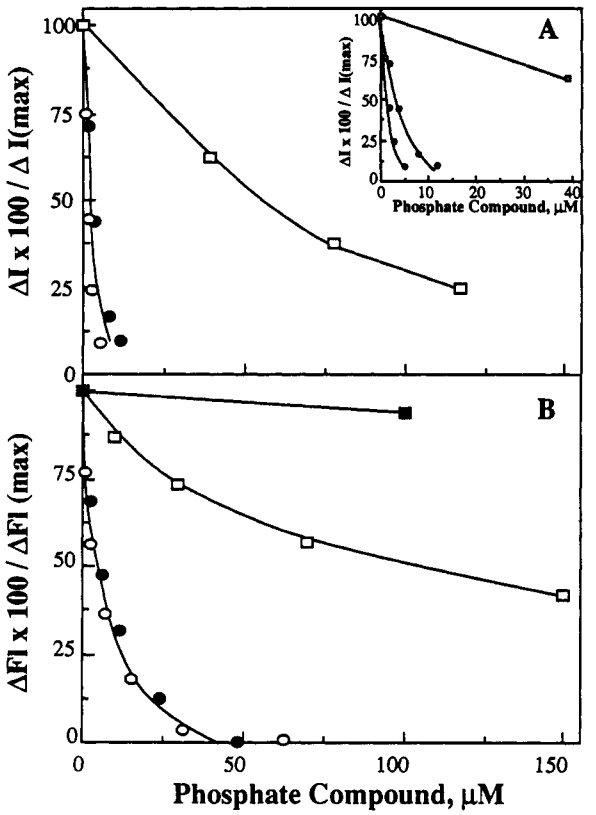


FIGURE 4: Dissociation of FP–C4BP and FP–phospholipid complexes with phosphate compounds. (Panel A) Phosphorylethanolamine (○), phosphorylcholine (●), or glycerophosphate (□) were added to a solution of FP (5.8 μg/mL) and C4BP (25.8 μg/mL). The buffer contained 1 mM calcium. (Panel B) The dissociation of the FP–phospholipid complex was monitored as a function of phosphorylethanolamine (○), phosphorylcholine (●), glycerophosphate (□), or inorganic phosphate (■) that was added to the solution. The solution contained FP (14.2 μg/mL), phospholipid (16.1 μg/mL), and calcium (2 mM). The signal intensities for light scattering (panel A) and fluorescence energy transfer (panel B) are as described in the legend to Figure 3. The inset shows an expanded scale.

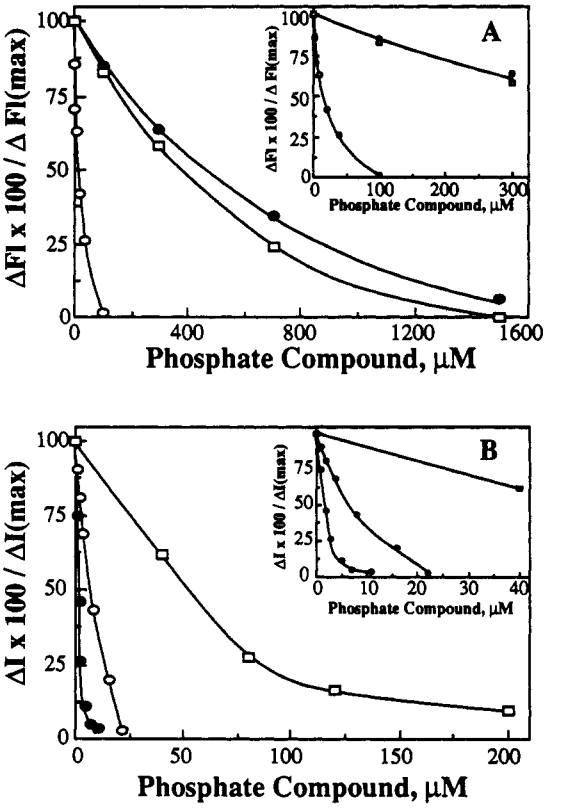


FIGURE 5: Dissociation of mouse SAP–membrane and rat CRP–C4BP complexes by phosphomonoesters. (Panel A) Phosphorylethanolamine (○), phosphorylcholine (●), or glycerophosphate (□) were added to a solution of mouse SAP (6.5 μg/mL) and phospholipid (6.5 μg/mL). (Panel B) Phosphorylethanolamine (○), phosphorylcholine (●), or glycerophosphate (□) were added to solution of rat CRP (6.5 μg/mL) and C4BP (25.8 μg/mL) in buffer containing 1 mM calcium. The signals for fluorescence energy transfer (panel B) and light scattering (panel A) are as described in the legend of Figure 3. The insets show an expanded scale.

um-containing buffer in a 1:2 ratio (Figure 3A). As phosphomonoesters were added to this solution, there was a decrease in excess light-scattering intensity. This resulted from competition between the phosphomonoesters and C4BP for binding to SAP. Once again, the results showed that phosphorylethanolamine was more effective than the other organic phosphates in dissociating the SAP–C4BP complex. It should be noted that the other phosphomonoesters were also capable of disrupting the SAP–C4BP complex.

Experiments similar to those in Figure 3 were conducted at a higher calcium concentration (2 mM) and at a higher pH value (8.4). When plotted (data not shown), the results were virtually superimposable on those generated at 1 mM calcium and at pH 7.5. The experiments at high calcium were important to show that the effect of the phosphomonoester was not due to chelation of calcium. Thus, human SAP binds organic phosphates in a calcium-dependent manner, with preference to phosphorylethanolamine, and this disrupts other interactions of SAP.

The data in Figure 3B also show that phosphomonoesters blocked the interaction of SAP with phospholipids. Initially, human SAP was bound to phospholipid vesicles in the presence of calcium. As the concentration of the phosphate compound was increased, the fluorescence signal decreased, suggesting dissociation of SAP from the membrane. Again, competition indicated that the site for interaction of human SAP with phosphomonoesters was either similar to or sterically overlapped the sites for SAP–membrane interaction. This experiment also showed a higher affinity (approximately 10-fold) for phosphorylethanolamine than for the other organic phosphate compounds. Inorganic phosphate was also capable of disrupting the SAP–membrane interaction and was almost as effective as phosphorylcholine.

The specificity for phosphomonoesters was also determined with FP (Figure 4). Organic phosphates dissociated the FP–C4BP interaction (Figure 4A). Surprisingly, both phosphorylcholine and phosphorylethanolamine were highly effective. Glycerol phosphate also dissociated the complex, but with about 50-fold lower affinity.

FP bound to negatively charged phospholipid vesicles, and this complex was also dissociated by phosphate compounds (Figure 4B). The results showed the same specificity so that FP bound phosphorylcholine and phosphorylethanolamine with similar affinity and interacted with glycerol phosphate as well but with lower affinity. Figure 4B shows that inorganic phosphate dissociated the FP–membrane complex, but required more than 7 mM to totally disrupt the complex (data not shown). This was similar to the concentrations of phosphate needed to dissociate the SAP–membrane complex (Figure 3B).

Interactions of Organic Phosphates with Mouse SAP and Rat CRP. When added to a solution of fluorescence-labeled phospholipid vesicles in a calcium buffer, mouse SAP produced an increase in fluorescence energy transfer, indicating interaction of SAP with membranes (Figure 5). Figure 5A shows dissociation of this complex by phosphate monoesters. The results suggested that mouse SAP was similar to human SAP

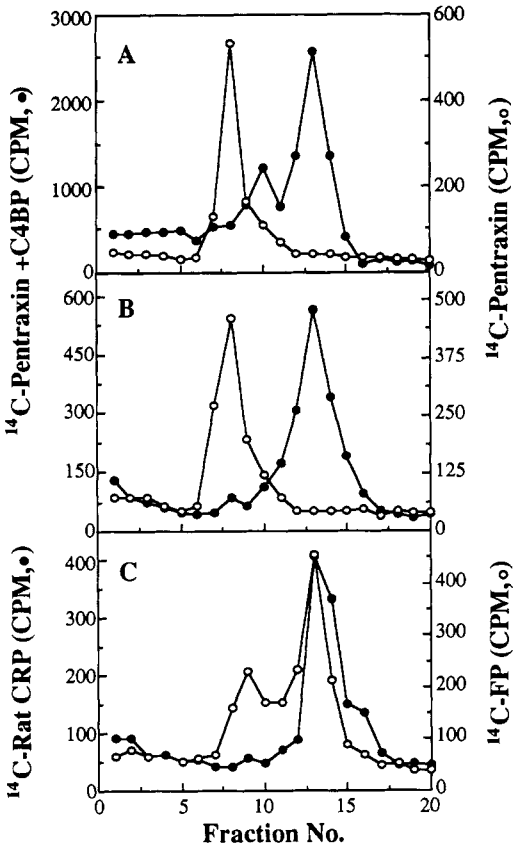


FIGURE 6: Association of C4BP with various pentraxins in a purified system and in human serum. (Panel A) Sedimentation of radiolabeled FP in sucrose density gradients was determined by procedures described under Materials and Methods. The samples include: [^{14}C]FP (●, 7 μg, 5,534 cpm/μg) plus unlabeled C4BP (17 μg) in buffer containing 1 mM calcium and [^{14}C]FP (○, 0.8 μg) in buffer containing 2 mM EDTA. (Panel B) Sedimentation patterns of ^{14}C-rat CRP (0.8 μg, 3,875 cpm/μg) and unlabeled C4BP (19 μg) in a buffer containing 1 mM calcium (●) and the same protein in buffer containing 2 mM EDTA (○) are shown. (Panel C) Sedimentation patterns of ^{14}C-rat CRP (●, 0.8 μg, 3,875 cpm/μg) or ^{14}C-FP (○, 0.8 μg) are shown. The samples contained 1 mM calcium and 0.05 mL of human serum. The total volume of all samples was 0.3 mL.

and interacted with phosphorylethanolamine with greater affinity than phosphorylcholine. It interacted with glycerophosphate in a fashion similar to phosphorylcholine.

Light-scattering intensity showed that rat CRP formed a calcium-dependent complex with C4BP (Figure 5B). This complex was dissociated by a number of organic phosphates (Figure 5B), confirming that rat CRP interacts with phosphorylcholine and phosphorylethanolamine (Pontet et al., 1981). Therefore, rat CRP was similar to FP in that both of these phosphomonoesters disrupted the complex to similar degrees, while glycerol phosphate was less effective. Rat CRP differed from FP in that it had a very weak interaction with membranes (data not shown). This latter interaction was not pursued further.

Association of the Pentraxins with C4BP in a Purified System and in Human Serum. Sucrose density gradient centrifugation is also a valuable method for analysis of SAP in purified and crude samples (Schwalbe et al., 1990b). This method was applied to the various interactions shown above to verify the interactions and to examine the interactions in serum.

FP (Figure 6A), rat CRP (Figure 6B), and mouse SAP (data not shown) sedimented with S values of about 7.5. These were similar to values previously determined for human SAP (Schwalbe et al., 1990b) and FP (S = 7.3; Coe et al., 1981).

Table I: Interactions of Various Pentraxins[a]

proteins	phosphoryl-ethanolamine	phosphoryl-choline	C4BP	phospho-lipid	amyloid deposit
true SAP					
human	+	−	+	+	+
mouse	+	−	+	+	+
hybrid pentraxin					
hamster FP	+	+	+	+	+
rat CRP	+	+	+	−	−
true CRP					
human	−	+	−	−	−

[a] Values of '+' and '−' are relative and are qualified by the actual quantitative data presented under Results.

Results with mouse SAP are not shown. The preparation of radiolabeled mouse SAP showed some radioactivity that remained at the top of the tube. Although considered preliminary, the results with mouse SAP were similar to those for FP and rat CRP. Mouse SAP showed a peak at an S value of approximately 7.5.

In the presence of C4BP and calcium, FP and rat CRP increased their sedimentation coefficients to about 11 (Figure 6A,B), a value similar to that reported for the SAP–C4BP complex (Schwalbe et al., 1990b, 1991). In the presence of C4BP, the major component of radiolabeled mouse SAP also showed a sedimentation coefficient of 11 (data not shown).

Figure 6C shows that in human serum, both FP and rat CRP sedimented at the position of a pentraxin–C4BP complex. Preliminary results were similar for mouse SAP (data not shown). Thus, despite some problems in preparing and purifying mouse SAP, sucrose density gradients support the light-scattering results. All three of these pentraxins appeared to form a 1:1 complex with C4BP.

Human CRP binds phosphorylcholine with much higher affinity than phosphorylethanolamine (Volanakis & Kaplan, 1971). Although human CRP has been reported to bind to lysophosphatidylcholine (Volanakis & Wirtz, 1979), it did not associate with C4BP or with intact phospholipids in a manner that was detected by the methods used in this study (data not shown). In order to demonstrate that the CRP used in this study was functional, its interaction with the various phosphorylated compounds was examined by radiolabeled human CRP (2.2 μg) bound to phsphorylethanolamine affinity column (0.3 mL) and eluted by a gradient of phosphorylcholine. Peak elution occurred at 40 μM phosphorylcholine. In contrast, nearly 2 mM phosphorylethanolamine or glycerophosphate was required for elution. In agreement with earlier reports the phosphodiester, glycerophosphorylcholine, was less effective than the corresponding phosphomonoester and about 200 μM was needed to elute CRP from this column. These results, which are not shown, served the primary purpose of establishing that the CRP used in these studies was fully functional and displayed the reported specificity for phosphomonoesters.

Discussion

The results of this study of the pentraxin family of proteins suggest a pattern of behavior that can be summarized by the grouping of proteins shown in Table I. Phosphorylethanolamine and/or phosphorylcholine binding specificities may be very helpful in differentiating the CRPs from the SAPs as well as the phylogenetics of the pentraxins in vertebrate species. The results demonstrate that, contrary to previous description, proteins that have been classified as SAP, both human and mouse, do interact with organic phosphate compounds. They show high specificity for phosphorylethanolamine. This study

also confirmed that human CRP had a high preference for interaction with phosphorylcholine. Thus, mouse or human SAP and human CRP provide two distinct groups based on binding specificity for phosphorylethanolamine versus phosphorylcholine. An interesting exception to this bimodel grouping was FP, the SAP/CRP homologue from hamster, which bound to phosphorylethanolamine and phosphorylcholine with similar affinities. This protein might be grouped as an intermediate hybrid specificity (Table I). An attractive speculation might be that this broad specificity may underlie the explanation for why another pentraxin has not been found in Syrian hamsters. That is, FP could be the sole pentraxin in Syrian hamsters that fulfills roles that require two pentraxins in other vertebrate species. Rat CRP was simialr to FP in that it bound both phosphomonoesters with similar affinities. However, rat has been reported to have two pentraxins (de Beer et al., 1982). Thus, the requirement for two pentraxins, with complementary specificities, versus one pentraxin with dual specificity, cannot entirely explain the distribution of these proteins in all species. Even three groups does not provide adequate description of the pentraxins since, of those with hybrid specificity for phosphorylcholine and phosphorylethanolamine, one (FP) is found in amyloid deposits and binds tightly to phospholipids while the other (rat CRP) displayed neither of these properties. Thus, the pentraxins display a continuum of different properties, and incorporation into amyloid is most closely correlated with binding to phospholipids.

An important point regarding the data in this study is that they provide relative values that allow sequencing of the affinities of pentraxins for various phosphorylated compounds. For example, about 7 μM phosphorylethanolamine was required to dissociate virtually all the FP from C4BP while approximately 2 mM was needed to dissociate SAP from C4BP. These relationships do not prove that FP binds phosphorylethanolamine with higher affinity than SAP. An alternative explanation is that the SAP–C4BP interaction is of much higher affinity than the FP–C4BP interaction so that more phosphorylethanolamine is needed to dissociate SAP–C4BP. The results are valuable for comparison of the relative affinities of one pentraxin.

It has been known for some time that CRP proteins will bind to certain phosphorylated compounds in a calcium-dependent manner. This study suggested that calcium-dependent binding to phosphorylated compounds may be a property shared by all pentraxins. The results verified that the human CRP binding site for phosphorylcholine recognizes two structural features, a phosphate group and a quaternary amine (Gotschlich et al., 1982). The new evidence demonstrated that human and mouse SAP were specific for binding to phosphorylethanolamine with recognition for two structures, the phosphate group and a positively charged primary amine. Therefore, in at least the human species, SAP and CRP showed complementary specificity for the primary and the quaternary amine, respectively. In both cases, the opposite structure was approximately as effective as a phosphomonoester that contained no cationic group. Thus, for both proteins, the site that recognizes the cationic unit has very high specificity.

In contrast, rat CRP and FP bound with similar affinities to phosphorylcholine and phosphorylethanolamine. These proteins also bound other organic phosphate compounds better than either SAP or human CRP, although not as well as they bound compounds with cationic groups. This indicated that the binding site of rat CRP and FP consisted of two regions, one for a phosphate group, which apparently dominates the interaction, and one for a positively charged group. The latter has relatively low specificity. These results may impact an older report that rabbit CRP would precipitate bovine serum albumin that was conjugated with phosphorylethanolamine and/or phosphorylcholine, whereas human CRP precipitated only albumin that was conjugated with phosphorylcholine (Oliveria et al., 1980; Gotschlich et al., 1982). The authors concluded that the difference in the precipitation reactions was due to the different binding sites in rabbit CRP and human CRP with rabbit CRPs having less specificity than human CRPs. The results of this current study suggest that rabbit CRP is like FP and rat CRP. Pontet et al. (1981) previously reported that rat CRP interacted with both phosphorylcholine and phosphorylethanolamine, but they did not detect an interaction of human SAP with either phosphorylcholine or phosphorylethanolamine. Therefore, they did not observe the complementary specificity for phosphorylcholine and phosphorylethanolamine that is displayed by the various pentraxins.

Some previous speculations for the function of CRP have concentrated on its interaction with CPS and subsequent initiation of complement. Thus, CRP may function at a level comparable to the immunoglobulins. Observation of related and complementary binding specificities for other pentraxins may suggest that this family of proteins functions in related and complementary roles. For example, the normal level of human SAP in serum is high (about 40–50 μg/mL; Pepys & Baltz, 1983; Skinner & Cohen, 1988) and increases by only about 3-fold during the acute-phase response.[2] In contrast, human CRP is found in trace levels in serum and increases up to 3000-fold as a result of cell death (Baltz et al., 1982). A possible speculation might be that, under normal physiological conditions, certain systems express receptors containing a binding site that correlates but is not necessarily identical to phosphorylethanolamine. During the acute-phase response, these binding sites may be methylated to quaternary amines. Alternatively, it is possible that acute phase results in expression of a new receptor corresponding to the site recognized as phosphorylcholine. The binding sites probed in this study may not be identical to phosphorylethanolamine or phosphorylcholine, but their specificity may be detected by these synthetic compounds.

A related possibility is that the pentraxins bind to phosphorylcholine or phosphorylethanolamine groups that are attached to various proteins. This would be analogous to interaction of human CRP with bovine serum albumin that has been conjugated with phosphorylcholine (Oliveria et al., 1980). Since four of the five pentraxins in this study bound to both phosphorylethanolamine and human C4BP, it is apparent that the binding site on C4BP mimics the specificity detected by phosphorylethanolamine but may be some structurally related material. There is no evidence for phosphorylation of C4BP. However, some glycosyl phosphatidylinositol membrane–protein anchors contain phosphorylethanolamine (Doering et al., 1990). Cell surface proteins containing glycolipid anchors include C8 binding protein and decay-accelerating factor, which are complement-related proteins. Further work will be needed to determine a direct link between these structures and a function of the pentraxins.

Under normal circumstances in the hamster, FP, which interacts with either phosphorylcholine or phosphorylethanolamine (Table I), may function in a role similar to human SAP. However, during infection or tissue damage, FP

[2] Dahlbäck et al., unpublished observation.

could also function like human CRP. Another significant modification is that in mice the major acute-phase protein is SAP (Pepys et al., 1979; Baltz et al., 1980). In this case, the acute-phase receptor may simply contain the site analogous to phosphorylethanolamine. Further studies are needed to determine if phosphorylcholine-containing and/or phosphorylethanolamine-containing receptors are used in these interactions. It is possible that the actual pentraxin binding sites are only related to these structures.

Another speculation might be that phosphorylethanolamine and phoshorylcholine (or related structures) are excreted by or released from cells under certain stimuli. Such compounds might dissociate SAP and/or CRP from other proteins and/or receptors. One class of binding sites might dissociate SAP from C4BP or from certain tissues, resulting in alteration of complement or the surface of these tissues. In addition to C4BP, a SAP-like molecule is associated with glomerular basement membranes (Dyck et al., 1980) and elastic fiber microfibrils (Breathnach et al., 1981). Overall, demonstration of a pattern of complementary specificities for interaction of the pentraxin family of proteins provides approaches that may aid in ultimate determination of their function. Much further work is needed to examine these and other possibilities.

Registry No. Calcium, 7440-70-2; phosphorylcholine, 107-73-3; phosphorylethanolamine, 1071-23-4.

REFERENCES

Abernathy, T. J., & Avery, O. T. (1941) *J. Exp. Med. 52*, 173–182.

Anderson, J. K., Stroud, R. M., & Volanakis, J. E. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1495.

Bach, B. A., Gerwurz, H., & Osmand, A. P. (1977) *Immunochemistry 14*, 215–219.

Baltz, M. L., Dyck, R. F., & Pepys, M. B. (1980) *Immunology 41*, 59–66.

Baltz, M. L., de Beer, F. C., Feinstein, A., Munn, E. A., Milstein, C. P., Fletcher, T. C., March, J. F., Taylor, J., Bruton, C., Clamp, J. R., Davies, A. J. S., & Pepys, M. B. (1982) *Ann. N.Y. Acad. Sci. 389*, 49–73.

Breathnach, S. M., Melrose, S. M., Bhogal, B., de Beer, F. C., Dyck, R. F., Tennent, G., Black, M. M., & Pepys, M. B. (1981) *Nature 293*, 652–654.

Breathnach, S. M., Kofler, H., Sepp, N., Ashworth, J., Woodrow, D., Pepys, M. B., & Hinter, H. (1989) *J. Exp. Med. 170*, 1433–1438.

Butler, P. J. G., Tennent, G. A., & Pepys, M. B. (1990) *J. Exp. Med. 172*, 13–18.

Chen, P. S., Toribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

Coe, J. E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 730–733.

Coe, J. E., & Ross, M. J. (1983) *J. Exp. Med. 157*, 1421–1433.

Coe, J. E., & Ross, M. J. (1985) *J. Clin. Invest. 76*, 66–74.

Coe, J. E., & Ross, M. J. (1990) *J. Exp. Med. 171*, 1257–1267.

Coe, J. E., Margossian, S. S., Slayter, H. S., & Sogn, J. A. (1981) *J. Exp. Med. 153*, 977–991.

Dahlbäck, B. (1983) *Biochem. J. 209*, 857–863.

Dahlbäck, B., & Stenflo, J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2512–2516.

de Beer, F. C., Baltz, M. L., Munn, E. A., Feinstein, A., Taylor, J., Bruton, C., Clamp, J. R., & Pepys, M. B. (1982) *Immulology 45*, 55–70.

Doering, T. L., Masterson, W. J., Hart, G. W., & Englund, P. T. (1990) *J. Biol. Chem. 265*, 611–614.

Du Clos, T. W., Zlock, L. T., & Marnell, L. (1991) *J. Biol. Chem. 266*, 2167–2171.

Dyck, R. F., Lockwood, C. M., Kershaw, M., McHugh, N., Duance, V. C., Baltz, M. L., & Pepys, M. B. (1980) *J. Exp. Med. 152*, 1162–1174.

Gotschlich, E. C., & Edelman, G. M. (1965) *Proc. Natl. Acad. Sci. U.S.A. 54*, 558–566.

Gotschlich, E. C., & Edelman, G. M. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 706–712.

Gotschlich, E. M., Liu, T.-Y., & Oliveria, E. (1982) *Ann. N.Y. Acad. Sci. 389*, 163–169.

Huang, C. (1969) *Biochemistry 8*, 344–352.

Jentoft, N., & Dearborn, D. G. (1979) *J. Biol. Chem. 254*, 4366–4370.

Kushner, I., & Somerville, J. A. (1970) *Biochim. Biophys. Acta. 207*, 105–114.

McCarty, K. S., Jr., Vollmer, R. T., & McCarty, K. S. (1974) *Anal. Biochem. 61*, 165–183.

Morley, J. J., & Kushner, I. (1982) *Ann. N.Y. Acad. Sci. 389*, 406–417.

Nagasawa, S., & Stroud, R. M. (1980) *Mol. Immunol. 17*, 1365–1372.

Nagpurkar, A., & Mookerjea, S. (1981) *J. Biol. Chem. 256*, 7440–7448.

Nelsestuen, G. L., & Lim, T. K. (1977) *Biochemistry 16*, 4164–4171.

Oliveria, E. B., Gotschlich, E. C., & Liu, T.-Y. (1980) *J. Immunol. 124*, 1396–1402.

Osmand, A. P., Friedenson, B., Gewurz, H., Painter, R. H., Hofmann, T., & Shelton, E. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 739–743.

Painter, R. H., De Escallon, I., Massey, A., Pinteric, L., & Stern, S. B. (1982) *Ann. N.Y. Acad. Sci. 389*, 199–213.

Pepys, M. B., & Baltz, M. L. (1983) *Adv. Immunol. 34*, 141–211.

Pepys, M. B., & Butler, P. J. G. (1987) *Biochem. Biophys. Res. Commun. 148*, 308–313.

Pepys, M. B., Dash, A. C., Fletcher, T. C., Richardson, N., Munn, E. A., & Feinstein, A. (1978) *Nature (London) 273*, 168–170.

Pepys, M. B., Baltz, M., Gomer, K., Davies, A. J. S., & Doenhoff, M. (1979) *Nature 278*, 259–261.

Pepys, M. B., de Beer, F. C., Milstein, C. P., March, J. F., Feinstein, A., Buttress, N., Clamp, J. R., Taylor, J., Bruton, C., & Fletcher, T. C. (1982) *Biochim. Biophys. Acta 704*, 123–133.

Pontet, M., D'Asnieres, M. D., Gache, D., Escaig, J., & Engler, R. (1981) *Biochim. Biophys. Acta. 671*, 202–210.

Robey, F. A., Jones, K. D., Tanaka, T., & Liu, T.-Y. (1984) *J. Biol. Chem. 259*, 7311–7316.

Scharfstein, J., Ferreira, A., Gigli, I., & Nussenzweig, V. (1978) *J. Exp. Med. 148*, 207–222.

Schwalbe, R. A., Dahlbäck, B., Hillarp, A., & Nelsestuen, G. L. (1990a) *J. Biol. Chem. 265*, 16074–16081.

Schwalbe, R. A., Dahlbäck, B., & Nelsestuen, G. L. (1990b) *J. Biol. Chem. 265*, 21749–21757.

Schwalbe, R. A., Dahlbäck, B., & Nelsestuen, G. L. (1991) *J. Biol. Chem. 266*, 12896–12901.

Skinner, M., & Cohen, A. S. (1988) *Methods Enzymol. 163*, 523–536.

Skinner, M., Cathcart, E. S., Cohen, A. S., & Benson, M. D. (1974) *J. Exp. Med. 140*, 871–876.

Thompson, A. R., & Enfield, D. L. *Biochemistry 17*, 4304–4311.
Tillet, W. S., & Francis, T., Jr. (1930) *J. Exp. Med. 52*, 561–571.
Volanakis, J. E., & Kaplan, M. H. (1971) *Proc. Soc. Exp. Biol. Med. 136*, 612–614.
Volanakis, J. E., & Wirtz, K. W. A. (1979) *Nature 281*, 155–157.

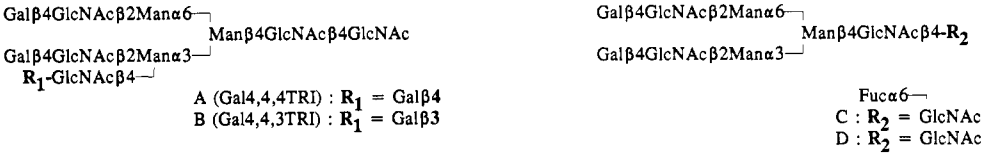
Comparison of N-Glycosides of Fetuins from Different Species and Human α_2-HS-Glycoprotein

Tetsuo Hayase,[†,§] Kevin G. Rice,[†,∥] Katarzyna M. Dziegielewska,[⊥] Mark Kuhlenschmidt,[#] Thomas Reilly,[#] and Yuan Chuan Lee*[,‡]

Department of Biology, Johns Hopkins University, Baltimore, Maryland, Research Center, Mitsubishi Kasei Corporation, Yokohama, Japan, Clinical Neurological Sciences, University of Southampton, Southampton, United Kingdom, and Department of Veterinary Pathobiology, University of Illinois, Urbana, Illinois

Received January 2, 1992; Revised Manuscript Received February 28, 1992

ABSTRACT: Complex type N-glycosides of commercial bovine fetuin preparations from pooled fetal calf serum have been shown to contain comparable amounts of Gal4,4,4TRI (see structure A below) and Gal4,4,3TRI (structure B) as major asialo-structures. To investigate whether there is a clear genetic specificity for synthesis of these oligosaccharides, N-glycosides from two preparations of bovine fetuin, each from a single calf, were examined. Both of these structures were present in each calf, and there was only a subtle quantitative difference in the ratio of these two structures between the calves. Thus, a specific galactosyltransferase, presumably required for the biosynthesis of the Gal4,4,3TRI structure, may exist in both of these individual calves. Comparison of fetuin N-glycosides was also extended to sheep, pig, and human α_2-HS-glycoprotein, the human counterpart of bovine fetuin, using high-pH anion-exchange chromatography of the reducing oligosaccharides as well as HPLC of their pyridinylamino derivatives. The N-glycosides of ovine fetuin also have both Gal4,4,4TRI and Gal4,4,3TRI structures in a ratio similar to that of bovine fetuin. However, the major N-glycoside of porcine fetuin is of a fucosyl biantennary complex type structure (structure C below) and human α_2-HS-glycoprotein has an N-glycoside which is almost exclusively a nonfucosylated biantennary structure (structure D). This species-specific presence of N-glycosides of fetuins and comparison with N-glycosides of other glycoproteins suggest that the polypeptide sequence of a glycoprotein may affect its N-glycan structure by regulating the activity of specific glycosyltransferases.



Fetuin is a fetal glycoprotein, expression of which is apparently regulated developmentally (Spiro, 1960; Dziegielewska et al., 1980a,b). The carbohydrate chains of bovine fetuin have been intensively studied (Nilsson et al., 1979; Townsend et al., 1986, 1989; Takasaki & Kobata, 1986; Green et al., 1988). Bovine fetuin was found to contain two different triantennary complex type oligosaccharides, Gal4,4,3TRI[1] and Gal4,4,4TRI (Townsend et al., 1986, Takasaki & Kobata, 1986; see structures in the abstract).

It has been shown previously that several commercial preparations of bovine fetuin contain different ratios of these two structures (Townsend et al., 1989). Since the commercial preparations are usually from pooled sources, each of these two different structures may be derived from individual calves. In this study, we obtained two preparations of bovine fetuin, each from a single calf, and examined the ratio of Gal4,4,4TRI and Gal4,4,3TRI structures in these preparations as compared to the ratio in a commercial preparation which was made by the same method.

Recently, the amino acid sequences of fetuins from sheep and pig have been elucidated (Brown et al., 1992). These fetuins as well as α_2-HS-glycoprotein, human fetuin, have close homology in amino acid sequence, especially around the N-glycosylation sites, with bovine fetuin. Therefore, we also compared the carbohydrate structures of fetuins from different species, by high-pH anion-exchange chromatography (HPAEC) of the released N-linked oligosaccharides and by HPLC of pyridinyl-2-amino derivatives of the oligosaccharides (PA-oligosaccharides; Hase et al., 1979).

* Author to whom correspondence should be addressed.
† Johns Hopkins University.
§ Mitsubishi Kasei Corp.
∥ Present address: Department of Pharmaceutics and Pharmaceutical Chemistry, The Ohio State University, Columbus, Ohio.
⊥ University of Southampton.
University of Illinois.

[1] Abbreviations: TRI, triantennary complex type oligosaccharide; HPAEC, high-pH anion-exchange chromatography; PA, pyridinylamino; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; CHO, Chinese hamster ovary; BHK, baby hamster kidney; Gn-T, *N*-acetylglucosaminyltransferase.

0006-2960/92/0431-4915$03.00/0