Regulated Export of a Secretory Protein from the ER of the Hepatocyte: A Specific Binding Site Retaining C-reactive Protein within the ER Is Downregulated during the Acute Phase Response

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Abstract. The half-time for secretion of the plasma protein C-reactive protein (CRP) by the hepatocyte decreases markedly in association with its increased synthesis during the acute phase response to tissue injury (Macintyre, S., D. Samols, and I. Kushner. 1985. *J. Biol. Chem.* 260:4169–4173). In studies in which subcellular fractions were prepared from cells incubated under pulse–chase conditions, CRP was found to be preferentially retained within the ER of normal hepatocytes, but secreted relatively efficiently in cells prepared from rabbits undergoing the acute phase response. On the basis of the detergent-dependency of specific binding of radiolabeled CRP, as well as EM visualization of biotinylated CRP identified with peroxidase-conjugated streptavidin, CRP was found to bind to the lumenal surface of permeabilized rough microsomes, while no binding was detected in Golgi fractions. As judged by both kinetic and equilibrium binding studies, rough microsomes from control rabbits were found to have two classes of specific binding sites for CRP; a high affinity site (K_d = 1 nM, B_{max} = 1 pmol CRP/mg microsomal protein) as well as a much lower affinity (K_d = 140 nM) site. In contrast, only the lower affinity class was detected in microsomes isolated from rabbits undergoing the acute phase response. On nitrocellulose blots probed with radiolabeled CRP a 60-kD protein, distinct from BiP, was detected in extracts of rough microsomes isolated from control rabbits, but not in Golgi fractions or rough microsomes from stimulated animals. These findings correlate with previous observations of changes in secretion kinetics of CRP and are consistent with the hypothesis that the intracellular sorting of CRP could be rerouted by downregulation of a specific ER binding site during the acute phase response.

PROTEINS that are cotranslationally inserted into the lumen of the ER during their synthesis have varied destinations, including the plasma membrane, the extracellular space, lysosomes, elements of the Golgi apparatus, and the ER itself. Thus, the need for specific delivery of a diversity of proteins to multiple locations represents a formidable task in protein trafficking. The mechanism by which newly synthesized lysosomal enzymes are specifically targeted to lysosomes has been well-characterized (reviewed in 37). Considerable progress has also been made in elucidating the role of the carboxy-terminal KDEL (reviewed in 57), or homologous (2, 3, 26), sequence in the continuous retrieval of soluble ER resident proteins from downstream compartments to their proper location within the lumen of the ER (42, 57, 74). More recently, several reports have begun to identify sequence and/or structural motifs of certain transmembrane proteins which allow for their specific localization to the membranes of the ER (32, 54) and Golgi elements (52, 55, 70).

In the case of plasma membrane and secretory proteins, the currently prevailing hypothesis suggests that in the absence of specific targeting signals, these proteins are transported to the cell surface by a default pathway of rapid bulk flow of vesicular contents (34). In a process referred to as quality control (reviewed in 31, 35, 60), proteins destined for exit from the ER appear to require a degree of proper folding and/or assembly, possibly facilitated by molecular chaperones such as BiP (grp78) (reviewed in 23), in order to be released from the ER. Proteins which do not meet these criteria are subject to degradation within the ER (14, 16, 77 and reviewed in 36). In the case of the T-cell antigen receptor, the processes of ER retention, assembly of subunits, and degradation of improperly assembled complexes appear to be tightly coupled (9, 10).

While much evidence indicates that the process of quality control within the ER can prevent secretion or accumulation of abnormal proteins, the extent to which ER retention may also serve to posttranslationally regulate the intracellular trafficking of normal secretory proteins is not clear. It is widely recognized that different secretory proteins exit the ER at varying rates (22, 41, 44, 66, 79) and we have previously reported that the efficiency of secretion of an individual

protein, C-reactive protein (CRP[1]), varies markedly under differing physiologic conditions (47). CRP, a homopentamer composed of nonglycosylated 24-kD subunits associated by noncovalent forces (24, 29), is a plasma protein whose rate of synthesis by the hepatocyte increases by several hundredfold or more during the systemic acute phase response to tissue injury (39). In studies of the synthesis and secretion of CRP by rabbit primary hepatocyte cultures prepared from animals stimulated in vivo to undergo the acute phase response, the half-time for secretion of newly synthesized CRP was found to decrease markedly from as much as 18 h in control hepatocytes to as little as 75 min in cells from acute phase animals (47), while the kinetics of albumin secretion determined in the same cultures, were rapid (half-time of 30–45 min) and did not vary during the acute phase response. Results of studies in which a fusion gene consisting of the rabbit CRP gene linked to the mouse metallothionein promoter was transfected into HeLa cells (29) indicated that the default rate of rabbit CRP secretion was rapid and suggested that changes in its transit time were due to its specific retention within unstimulated rabbit hepatocytes, rather than to facilitated export in cells from stimulated animals. However, CRP does not contain carboxy-terminal sequences reported to result in ER retention (29). Further, assembly of CRP does not appear to be rate-limiting, since in control as well as stimulated cells >90% of antigenically detectable CRP labeled in a 10-min pulse is pentameric as judged by its size on gel filtration, its differential reactivity with structure-specific antibodies, and its ability to bind phosphocholine (29, 46, 47, and our unpublished observations). Despite its prolonged intracellular half-life in unstimulated hepatocytes, CRP is not degraded (47).

In the present report, pulse–chase subcellular fractionation experiments identify the ER as the compartment in which CRP is retained. Both biochemical and immunohistochemical methods demonstrate specific binding of CRP to the lumenal face of detergent-permeabilized rough microsomes, but not to Golgi subfractions. Kinetic and equilibrium binding studies identify a high affinity CRP binding site which is present in hepatic rough microsomes from normal rabbits, but is not detected in microsomes from animals undergoing the acute phase response. Finally, nitrocellulose blots probed with radiolabeled CRP demonstrate a 60-kD band, distinct from BiP, which is detected in extracts of control rough microsomes, but not in Golgi fractions or rough microsomes from stimulated animals. Together, these findings are consistent with the hypothesis that CRP is specifically retained within the ER by a novel mechanism which is downregulated during the acute phase response.

Materials and Methods

Animals and Cell Cultures

Primary hepatocyte cultures were prepared from male New Zealand White rabbits (obtained from Howard Gutman, Madison, OH) by an in situ collagenase (Type I; Sigma Chemical Co., St. Louis, MO) perfusion technique as described previously (46). Acute tissue injury was induced in some rabbits by the intramuscular injection of 1 ml turpentine in each thigh 18–24 h before cell preparation. Blood obtained from the marginal ear vein at the time of sacrifice was used for serum CRP determinations by radial immunodiffusion as described (45). Initial cell suspensions were plated in 100 × 15 mm plastic culture dishes (Lux Scientific, Lab-Tek Division, Miles Laboratories, Naperville, IL) at a density of 8–9 × 10^6 cells per dish. After a 1.5-h attachment period (70–90% efficiency), cells were maintained in serum-free William's medium E containing 1 μM dexamethasone and insulin (0.02 U/ml) as described previously (47). Cell counts (90–95% survival after attachment) were performed on Trypan blue–treated dishes under phase-contrast microscopy as described (46).

In the pulse–chase studies, cells were allowed to acclimate to culture conditions for 20–22 h. Medium was removed, the dishes rinsed twice with 5 ml of warm Hanks' buffered saline, 5 ml per dish of medium lacking methionine (RPMI-1640-Select-amine kit; Gibco Laboratories, Grand Island, NY) was added, and the cells were incubated for 30 min before the addition of 500 μCi/dish of L-[^{35}S]methionine (>888 Ci/mmol; NEN Research Products, Boston, MA). After a 10-min incubation period, medium was removed, the dishes rinsed twice with 5 ml Hanks' buffered saline, and 5 ml of William's medium E containing unlabeled methionine (300 mg/liter) was added to each dish. After 75 min of chase incubation, the cells were harvested and processed as described below. Medium from a replicate dish which had received no medium change other than the rinse after cell attachment was used to determine rates of extracellular accumulation of both CRP and albumin employing RIAs as described below.

Subcellular Fractionation

For the preparation of subcellular fractions from cultured cells, medium from a minimum of 10 culture dishes per time sampling was removed and the dishes were rinsed in ice-cold homogenization buffer consisting of 0.25 M sucrose, 20 mM Hepes, 10 mM KCl. Cells were scraped from the dishes in a total of 6 ml of homogenization buffer and were homogenized in a glass Dounce Type homogenizer with 15 passes of a tight glass pestle followed by 10 passes of a Teflon pestle. Microsomal subfractions corresponding to rough, smooth and Golgi were prepared by Carey and Hirschberg's modification (13) of the technique of Fleischer and Kervina (21) and all operations were at 4° C. A 1-ml aliquot of the lysate was removed and both the aliquot as well as the remaining lysate, were centrifuged at 11,000 *g* for 10 min. The 1-ml portion was used to estimate homogenization-induced leakage of pulse-labeled proteins as described below. 8 ml of 60% sucrose (wt/wt) was added to 4 ml of the remaining 11,000 *g* supernate, the resulting solution placed in a Beckman SW 28 tube, and the sample overlaid with 6.5 ml each of 38.7, 29, and 8.2% sucrose. After centrifugation at 25,000 rpm for 60 min, the 38.7/29 and 29/8.2 interfaces were pooled, diluted with water to 0.25 M sucrose and material representing the Golgi fraction was harvested at 110,000 *g* for 60 min. 4.8 ml of water followed by 1.6 ml of 0.15 M CsCl was added to 10 ml of the residual 43% sucrose layer and three 5-ml aliquots of the resultant solution were placed in tubes for the Beckman 50.1 rotor. These were overlaid with 5 ml each of 1.3 M sucrose, 15 mM CsCl and the samples were centrifuged at 45,000 rpm for 2 h to pellet the rough microsome fraction. The material at the interface was diluted with water to 0.25 M sucrose and centrifuged at 110,000 *g* for 60 min to yield the smooth microsome pellet.

For use in the initial microsomal binding assays (see below) subcellular fractions were prepared from whole rabbit liver by the same procedure except that homogenization was performed as described (13), employing motor driven graded Teflon pestles with clearances of 0.026 and 0.012 inches in a Potter-Elvehjem homogenizer. In subsequent binding assays employing purified rough microsomes, subcellular fractionation was performed as described previously (71). In those cases where unfractionated microsomes were studied, the initial 11,000 *g* supernate was centrifuged at 110,000 *g* for 60 min to produce a total microsome pellet.

For phase partitioning of microsomal proteins, Triton X-114 (Sigma Chemical Co., St. Louis, MO) was precondensed as described previously (12) and was considered to be 11.4% by weight. Pelleted rough microsomes (1.5 mg total protein) were homogenized in 750 μl ice cold 0.5% Triton X-114 in 20 mM Hepes, 0.15 M NaCl, pH 7.4, and incubated for 30 min at 4° C. Following centrifugation at 11,000 rpm for 30 min at 4° in a microcentrifuge, the clear supernatant was layered over 6% sucrose, 0.06% Triton X-114, incubated at 37° for 10 min, and centrifuged for 20 sec in the microcentrifuge at room temperature. The aqueous and detergent phases were saved and the sucrose discarded. The aqueous phase was washed twice at 37° C with 100 μl of 11.4% Triton X-114, centrifuging at room temperature as before and the final aqueous phase was stored frozen. The detergent phase was mixed with 750 μl cold 20 mM Hepes, 0.15 M NaCl, pH 7.4, layered over 500 μl 6% sucrose, 0.6% Triton X-114, warmed to 37° C for 10 min and centrifuged at room temperature as before. The aqueous phase and su-

1. *Abbreviations used in this paper*: CRP, C-reactive protein; DOC, sodium deoxycholate.

crose layer were discarded and sufficient 20 mM Hepes, 0.15 M NaCl, pH 7.4, was added to the detergent phase to yield a volume equal to that of the aqueous phase.

Estimation of Cell Breakage and Leakage and Adsorption of Pulse-labeled Proteins

Homogenization-induced leakage of pulse-labeled proteins and adsorption of leaked proteins to microsomal fractions were estimated employing a strategy described previously (65). Adsorption was determined in initial control experiments by including trace amounts of ^{125}I-labeled CRP or rabbit albumin (prepared as described below) in the homogenization buffer added to unlabeled hepatocytes before homogenization. The homogenate was centrifuged as usual at 11,000 *g* for 10 min and the resulting postmitochondrial supernate was centrifuged at 143,000 *g* for 60 min. The distribution of added radioactivity in the initial pellet, the microsomal pellet, and the soluble supernate was determined by counting in a Nuclear Chicago model 1085 gamma counter. Homogenization-induced cell breakage and leakage of pulse-labeled proteins (10 min, [^{35}S]methionine) were estimated using the 1 ml aliquot of lysate referred to in the section above. The 11,000 *g* pellet from this sample was suspended in 5 ml of lysis buffer (10 mM Tris, 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate) while the supernate was centrifuged at 143,000 *g* to produce a microsomal pellet and a soluble supernate. The microsomal pellet was suspended in 5 ml lysis buffer, and Triton X-100 and sodium deoxycholate were added to the supernate to final concentrations of 1.0 and 0.5%, respectively. Labeled CRP and albumin were specifically immunoprecipitated (see below) from each of these three samples, using 10% of available sample for albumin and 80% for CRP. The immunoprecipitates were subjected to SDS-PAGE and specific protein bands quantitated as described previously (47). The proportion of labeled protein in the initial pellet, as a percentage of the total of the three fractions, was considered to reflect the presence of whole cells, since adsorption was found to be negligible. Leakage of labeled proteins was estimated from the radioactivity in the soluble supernate as a percentage of the radioactivity present in the microsomal pellet plus that present in the soluble supernate.

Leakage of [^{35}S]methionine, pulse-labeled proteins and adsorption of added radioiodinated proteins to membranes were initially examined in four primary hepatocyte cultures prepared from two control rabbits and two animals stimulated by intramuscular turpentine injection. Adsorption of both CRP and albumin to the 11,000 *g* pellet and to total microsomes was found to be minimal (range of 2.8–3.9% of input) in all four cultures and notably less than previously reported for exocrine pancreas (65), possibly due to the more dilute homogenate in this procedure.

Leakage of pulse-labeled proteins was considerable, presumably owing to the relatively harsh conditions of homogenization found to be required to disrupt the hepatocytes. Leakage of albumin ranged from 56 to 71% and did not differ in cultures from control compared to stimulated animals. Interestingly, while leakage of CRP determined in the two cultures from stimulated cells was 52% and 58%, leakage from control cultures was less (31 and 38%), consistent with our previous work suggesting that CRP may be preferentially retained within normal hepatocytes and that retention is decreased during the acute phase response (30). In the pulse-chase studies, leakage of individual proteins was determined from the percentage of radioactivity present in the 11,000 *g* supernate which remained soluble following the 143,000 *g* centrifugation and no corrections were made for adsorption of leaked proteins. In a typical preparation, 10 culture dishes (5×10^7 cells) yielded ~1.5 mg protein in total microsomes, 400 μg in rough microsomes, 180 μg in smooth microsomes, and 150 μg in Golgi fractions.

Enzyme and Immunoassays

The efficacy of the fractionation procedure was assessed by determination of specific marker enzyme activities. Glucose-6-phosphatase activity was determined exactly as described previously (4) except that the A_{660} of samples was determined 3 h after the addition of the 1-amino-2-naphthol-4-sulfonic acid reagent. Galactosyl transferase activity was measured as described previously (8) except that uridine diphospho-D-[U-^{14}C]galactose (Amersham Corp.. Arlington Heights, IL) was employed at a specific activity adjusted to 4.3 mCi/mmol. Sample values were obtained by subtracting values obtained with exogenous substrate (Trypsin inhibitor type III-O; Sigma Chemical Co.) from those determined without substrate. For both enzyme assays, determinations were made on at least two different volumes of sample membranes. Distribution of marker enzyme specific activities within the homogenate as compared to the three subcellular fractions indicated that the Golgi fraction was enriched 17-fold in galactosyl transferase activity and the rough microsome fraction was enriched 3.4-fold in glucose-6-phosphatase activity, values in reasonable agreement with those reported previously (27 and 3.4-fold, respectively) for fractions prepared from murine whole liver (13).

CRP and albumin contained in subcellular fractions were quantitated by radioimmunoassay as described previously (45, 47). Membrane pellets were suspended in 5 ml lysis buffer, sonicated (model 185E Sonifier; Heat Systems-Ultrasonics, Plainville, NY) with the small probe at 30 W for 30 s on ice, and the 12,000 *g*, 15 min supernate was used in the RIAs. Radiolabeled CRP and albumin present in these same supernates were specifically immunoprecipitated as described previously (47). Transferrin was precipitated employing 10 μg carrier rabbit transferrin and 50 μl goat antirabbit transferrin (both from Cappel, Cooper Biomedical, Malvern, PA). Volumes of supernate used for immunoprecipitation were 2 ml for CRP, 1 ml for albumin, and 0.5 ml for transferrin. This strategy allowed for adequate radioactivity to be recovered for each protein within fractions from the two chase times and was based upon individual differences in methionine composition, relative rate of synthesis and transit time. Portions of the initial cell homogenates were adjusted to 1% Triton X-100 and 0.5% deoxycholate (DOC) to determine total intracellular immunoprecipitable proteins. These samples combined with immunoprecipitates from culture medium were used to determine the efficacy of the 75-min chase incubation. Immunoprecipitates were washed with lysis buffer and were subjected to SDS-PAGE on 12.5% gels. After autoradiography, gels were rehydrated in water and stained bands corresponding to added carrier proteins were excised, dissolved in 30% H_2O_2 and the radioactivity determined as described (47). All immunoprecipitations were carried out under conditions of antibody excess as determined by precipitin curves employing radioiodinated specific antigens.

To investigate the possible association of newly synthesized CRP with BiP (grp78), pulse-labeled cell lysates were incubated with rat monoclonal antihuman BiP (generous gift of Dr. David Bole) under ATP-depleting conditions. After a 30-min incubation with [^{35}S]methionine as described above, four dishes of cells were rinsed with Hanks' buffered saline and were scraped in 3 ml of 20 mM Hepes, pH 7.4, 0.15 M NaCl, 1 mM $MgCl_2$, 5 mM glucose, 10 U/ml hexokinase (Calbiochem-Behring Corp., La Jolla, CA), 1% NP-40 (Pierce Chemicals, Rockford, IL), 1 mM PMSF (Eastman Kodak Co., Rochester, NY) or in the same buffer containing 1.5 mM $CaCl_2$. After 15 min at 4° the lysate was centrifuged at 12,000 *g* and 1 ml of the supernate was used for immunoprecipitation of CRP as described above. 0.5 ml of the supernate was incubated with 100 μl anti-BiP and 50 μl protein A–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) made up as a 50% suspension in 20 mM Hepes, pH 7.4, 0.15 M NaCl, 0.1% BSA and the suspension was incubated for 1 h at 4° with rotation. The Sepharose was pelleted by microcentrifugation (model 59A; Fisher Scientific, Fair Lawn, NJ) and washed twice with 1 ml of 20 mM Hepes, pH 7.4, 0.4 M NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC and once with 1 ml of 20 mM Hepes, 0.15 M NaCl, pH 7.4 before boiling in SDS-PAGE sample buffer and analysis by autoradiography of 12.5% gels as described (47).

Nitrocellulose blots used for ligand probing were prepared by transferring samples separated on 10% SDS gels to nitrocellulose in 25 mM Tris, 190 mM glycine, 20% methanol buffer (73) employing a Genie electroblotter (Idea Scientific Co., Minneapolis, MN) and the blot was blocked in 1% gelatin overnight. After two washes in 20 mM Hepes, 0.15 M NaCl, 0.1% Chaps, pH 7.4, the blots were probed with ^{125}I-CRP (prepared as below) at a concentration of 0.5–1 μg/ml ($6–9 \times 10^6$ cpm/ml) in the same buffer, containing 1.5 mM $CaCl_2$, and 1.0% BSA. After incubation at 4° for 2 h, blots were washed in three changes of 20 mM Hepes, 0.15 M NaCl, 0.1% Chaps, 1.5 mM $CaCl_2$, and 0.1% BSA, pH 7.4, dried, and subjected to autoradiography. Western blots probed with anti-BiP (1:5,000 dilution of hybridoma culture medium) were then incubated with 1:3,000 dilution of alkaline phosphatase–conjugated goat anti-rat Ig (Pierce Chemicals Co., Rockford, IL) and developed with BCIP/NBT (Bio-Rad Laboratories, Richmond, CA) as per the manufacturer's instructions.

Protein Purification and Modification

The purification of rabbit CRP from acute phase rabbit serum and of CRP subunits from purified CRP was as described previously (45). CRP was radioiodinated exactly as described (45), except that for equilibrium and kinetic binding studies ^{125}I-CRP obtained after G200 chromatography was concentrated by affinity chromatography on a 0.25-ml column of phosphocholine-agarose (Pierce Chemical Co.). Bound material was eluted with 0.15 M NaCl, 25 mM sodium citrate, 20 mM Hepes, pH 7.4, and was dialyzed exhaustively against 0.15 M NaCl, 20 mM Hepes, pH 7.4. Specific radioactivities ranged from 6 to 9×10^6 cpm/μg and CRP concentrations

from 9 to 15 μg/ml. ^{125}I-CRP was stored frozen as aliquots, was used within 3–4 wk of preparation, and was microfuged for 45 min before use in binding assays. Estimates of CRP contained within isolated rough microsomes were made by RIA determinations of microsomes lysed in buffer containing 1.0% Triton X-100, 0.5% DOC, and 10 mM sodium citrate in order to dissociate CRP bound by calcium-dependent interactions.

CRP (500–750 μg/ml) was biotinylated employing a 30-fold molar excess of freshly prepared NHS-LC-biotin (Pierce Chemical Co.) in 0.15 M NaCl, 20 mM Hepes, pH 7.4. After incubation at 20° C for 60 min, glycine was added to a concentration of 100 mM and the sample dialyzed exhaustively against 0.15 M NaCl, 20 mM Hepes, pH 7.4. An aliquot of the resulting sample was radioiodinated as described above and was subjected to gel filtration on Sephacryl S200 (Pharmacia Fine Chemicals, Piscataway, NJ) as well as affinity chromatography on phosphocholine-agarose (Pierce Chemical Co.). Greater than 93% of radioactivity was present as native, functional CRP as judged by pentameric molecular size and ability to bind to phosphocholine. Greater than 70% of this material bound to Streptavidin-agarose (Pierce Chemical Co.) and resisted washing with 0.1% SDS as well as 1 mM EDTA, indicating the functional availability of biotin moieties. As a control, BSA was biotinylated in exactly the same manner.

Preparation of Samples for EM

Rough microsomes were prepared and permeabilized as described above in 0.05% DOC at a concentration of 2 mg protein/ml. Aliquots were incubated for 1 h at 4°C (final concentration of 1 mg protein/ml) in buffer containing 0.15 M NaCl, 20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.0% BSA, pH 7.4, and biotinylated CRP (2 μg/ml). Control incubations included biotinylated BSA in place of CRP, biotinylated CRP incubated in the absence of DOC, and biotinylated CRP incubated with DOC in the presence of a 15-fold excess of native CRP. To remove unbound CRP, samples were layered over discontinuous sucrose gradients (Beckman SW41 tubes) containing 3.0 ml of 1.9 M sucrose and 7.0 ml 20% sucrose, 0.15 M NaCl, 20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, and were centrifuged at 38,000 rpm for 1 h at 4°C. The tops of the gradients were carefully aspirated, rinsed with 0.15 M NaCl, 20 mM Hepes, pH 7.4, and the rough microsomes present at the 1.9 M sucrose interface were collected and dialyzed for 1 h at 4°C against 0.15 M NaCl, 20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4. Dialyzed samples were then incubated for 1 h at 4°C, under permeabilizing conditions, in buffer containing 0.15 M NaCl, 20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.0% BSA, 0.05% DOC, and peroxidase-conjugated Streptavidin at a final dilution of 1:40 of that supplied (Zymed Laboratories, San Francisco, CA). Free enzyme–conjugated Streptavidin was removed by centrifugation through discontinuous sucrose gradients and harvested as before. Material at the 1.9 M sucrose interface was collected and diluted to 5 ml with 0.25 M sucrose, pH 7.0, and glutaraldehyde was added to a final concentration of 1.0%. After incubation for 1 h at 4°C, samples were layered over discontinuous sucrose gradients containing 2 ml 2M sucrose and 4 ml 20% sucrose and were spun at 38,000 rpm for 1 h at 4°C (SW41 rotor). Material at the 2 M sucrose interface was collected and dialyzed at 4°C overnight versus 0.25 M sucrose, pH 7.0, in order to remove traces of glutaraldehyde and to allow for diffusion out of the microsomes of unreacted Streptavidin which might have been trapped within the microsomes in the previous step. The volumes of dialyzed samples were adjusted to 2 ml with 0.25 M sucrose and 0.5 ml of 1% 3,3′-DAB (Sigma Chemical Co.) in 50 mM Tris, pH 7.4, was added. After the addition of 10 μl 1.0% H_2O_2, samples were incubated for 15 min at 20°C and the reaction was terminated by the addition of 9 ml of ice-cold 0.25 M sucrose. Samples were cleared of debris by centrifugation at 1,500 *g* for 5 min, the supernates were layered over 2 ml 20% sucrose, and the samples were spun at 38,000 rpm for 1 h at 4°C (SW41). Pellets were cut into strips, postfixed with OsO_4 (1% in H_2O), and dehydrated through graded ethanol. After imbedding in Spurr, thin sections were cut and examined, without further staining, in a Jeol CX 100 II electron microscope.

Microsomal Binding Assay

Microsomal subfractions were prepared, permeabilized, and passed over Sepharose 2B as described above. Incubation buffer included 0.15 M NaCl, 20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.0% BSA, 0.035% DOC (except as indicated in Fig. 2), pH 7.4. In typical competitive binding assays, each sample's volume was 250 μl and contained 10–15 μg microsomal protein and 50–100 ng ^{125}I-rabbit CRP. After incubation for 3 h at 4°C, duplicate 100-μl aliquots were layered over 200 μl 15% sucrose, 0.15 M NaCl, 20 mM Hepes, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4, in 0.4-ml polypropylene centrifugation tubes. After microcentrifugation for 45 min at 11,000 *g* (model 59A; Fisher Scientific, Fair Lawn, NJ), 10-μl aliquots of the supernatant were removed to determine free radioactivity, the tubes were frozen in a solution of dry ice and isopropanol, and the tips cut off and counted to determine bound radioactivity. In control experiments, <0.005% of ^{125}I-CRP was recovered in the counted tips when microsomes were omitted from the incubation. Specific binding (usually ~85% of total binding) was defined as total binding less the radioactivity bound in the presence of at least a 50-fold excess of unlabeled CRP as competitor. Additional proteins tested for competition included rabbit albumin and transferrin (Cappel, Cooper Biomedical), histones (calf thymus from U.S. Biochemical Corporation, Cleveland, OH and H2A from Sigma Chemical Co.), as well as human CRP prepared from malignant ascites fluid using the same procedures used for rabbit CRP.

In experiments designed to determine the trypsin sensitivity of the microsomal binding, microsomes were preincubated with trypsin (Sigma Chemical Co.) at a concentration of 100 μg/ml for 30 min at 4°C in the presence of 0.035% DOC. After the addition of the trypsin inhibitor PMSF (Eastman Kodak Co.) to 1 mM, samples were incubated for an additional 15 min at 4°C. Controls consisted of samples treated in the same manner with trypsin which had been preincubated with PMSF before incubation with permeabilized microsomes.

In the formal equilibrium binding studies incubations included paired samples containing ^{125}I-CRP ranging from 26 ng to 2.6 μg (8.3×10^{-10} to 8.3×10^{-8} M) incubated in the presence or absence of a 60-fold excess of competing unlabeled CRP, except in the case of the lowest concentration of labeled CRP, in which a 120-fold excess of unlabeled CRP (representing 25 times the estimated concentration of half-maximal binding) was employed. Data obtained were subjected to Scatchard analysis (63) and these results were further interpreted employing the nonlinear curve fitting program LIGAND (53), modified for microcomputers (49), and carried out on a Macintosh II computer (Apple Computer, Cupertino, CA).

In kinetic binding studies, labeled CRP was added to at least a concentration approximating that determined to be half-maximal saturation (from equilibrium binding studies). Paired samples, one containing a 50-fold excess of unlabeled CRP, were further processed at timed intervals after the addition of labeled CRP. Experiments designed to determine dissociation kinetics was performed by preincubating labeled CRP with microsomes for 3 h, then adding a 50-fold excess of unlabeled CRP and separating bound from free radioactivity at timed intervals. Nonspecific binding was determined from an incubation in which both labeled and unlabeled CRP were coincubated with microsomes. Analysis of kinetic binding studies was performed with the aid of the nonlinear curve fitting program KINETIC (49).

Results

Kinetics of Transport of Pulse-labeled Proteins

Studies were carried out on cultures prepared from two animals manifesting notably different responses to inflammatory stimulus, as judged by serum CRP levels and rates of CRP secretion in vitro. In addition to CRP and albumin, transferrin was also studied since it has a relatively long transit time of ~120 min (68). Results of subcellular fractionation of pulse–chase labeled hepatocytes prepared from an animal exhibiting a minimal response to turpentine injection (animal #1) (serum CRP level 16 μg/ml; in vitro secretion rate of 7.1 ng CRP/10^6 cells/h) are shown in Fig. 1. After a 10-min pulse with [^{35}S]methionine, the majority of each radiolabeled protein was, as expected, recovered in the ER-derived fractions. After 75 min of chase, little albumin remained in any of the fractions, consistent with its half-time for secretion of ~30 min (47). The amount of labeled transferrin decreased substantially and was about equally distributed between the rough and smooth ER fractions, with a small amount in the Golgi fraction where its migration was somewhat slower, presumably due to the acquisition of sialic acid residues. In contrast, the majority of radiolabeled CRP was found to persist within the ER fractions after the 75-min chase period. Quantitation of the labeled proteins in the gel

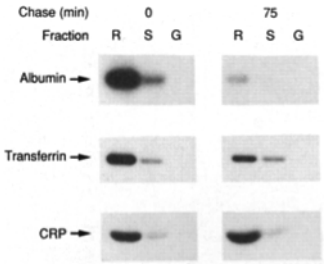

Figure 1. Chase of pulse-labeled proteins from subcellular fractions. Cells prepared from an animal manifesting a minimal response to inflammatory stimulus (serum CRP 16 μg/ml), were pulse labeled with [^{35}S]methionine for 10 min and subcellular fractions were prepared from 10 dishes immediately after the pulse and from 10 dishes after 75 min of chase. Radiolabeled albumin, transferrin, and CRP were specifically immunoprecipitated from portions of lysates of fractions and were subjected to SDS-PAGE followed by autoradiography. Panels are from portions of three gels used to analyze the immunoprecipitates of the three proteins present in rough (*R*), smooth (*S*), and Golgi (*G*) fractions. To achieve comparable autoradiographic results, the proportions of lysates used for immunoprecipitation of individual proteins (CRP = 40%, albumin = 20%, transferrin = 10%) was designed to account for differences in methionine composition, relative rates of synthesis, and intracellular transit times (see Materials and Methods for details). Exposure times were 3 d for transferrin and 5 d for albumin and CRP.

Table I. Changes in Pulse-Chase Kinetics and Relative Protein Content of Subcellular Fractions during the Acute Phase Response

Serum CRP level (μg/ml)	Animal 1 16	Animal 2 64
CRP Secretion (ng/10^6 cells/h)	7.1	22
CRP/Albumin Secretion (%)*	1.1	3.0
CPM Chased from Rough Microsomes (%)‡		
Albumin	85	82
Transferrin	40	59
CRP	12	35
CRP/Albumin (%)§		
Rough	13	8.9
Smooth	12	7.6
Golgi	0.8	2.9

* Data represent the secretion of proteins into culture medium during an 18-h incubation, expressed as CRP as a percentage of albumin secretion.
‡ Percentage decrease in radiolabeled protein present in the rough microsome fractions after the 75-min chase period.
§ Protein content of subcellular fractions expressed as CRP as a percentage of albumin. The data have been corrected for leakage in the two experiments (animal 1 and animal 2) for CRP (36 and 56%) and albumin (58 and 62%) and represent the means of the two determinations for each of the 0- and 75-min chase periods.

used for Fig. 1 demonstrated that 85% of radiolabeled albumin apparently exited from the ER-derived fractions during the 75-min chase, while labeled transferrin decreased by 40%, and CRP by only 12% (Table I). The efficacy of the chase conditions was determined by comparison of total immunoprecipitable protein (cell lysate plus culture medium) present at the end versus the beginning of the chase period and ranged from 111% for CRP to 105% for albumin (data not shown).

For comparison, chase kinetics of radiolabeled proteins in cells prepared from an animal showed a greater CRP response to turpentine injection (Table I, animal #2) (serum CRP of 64 μg/ml; in vitro secretion rate of 22 ng CRP/10^6 cells/h). While there was little difference (compared to animal #1) for albumin (82% of ER cpm lost during the 75-min chase), or for transferrin (50% increase in chased transferrin from 40 to 59%), a threefold increase in chase of radiolabeled CRP from 12 to 35% was observed. This finding corroborates our previous observations of a decrease in transit time for CRP during the course of the acute phase response (47), and is in agreement with the conclusions of most other workers that the rate-limiting step in the secretion of secretory proteins is exit from the ER (22, 44, 64, 78).

Steady-state Distribution of Proteins within Subcellular Fractions

It is possible that the increased rate of exit from the rough microsomal fraction observed in cells from the more responsive animal might be the result of an increase in the relative concentration of CRP within the ER, allowing it to better compete in a concentration-dependent fashion for a rate-limiting step common to other secretory proteins. To investigate this possibility, the concentrations of CRP and albumin were determined in isolated subcellular fractions employing specific RIAs. In each experiment, the finding that the concentrations of CRP and albumin in the rough fraction are very similar to those in the smooth microsomal fraction is consistent with the concept that these fractions are both derived from a continuous membranous network comprising the endoplasmic reticulum. In the minimally responsive animal (Table I, Animal #1), the mean ratios of CRP/albumin within the rough and smooth fractions were 13 and 12%, respectively. By contrast, the ratio within the Golgi fractions was only 0.8%, in good agreement with the observed ratio of rates of secretion of CRP and albumin in this animal, CRP being 1.1% that of albumin. Thus, upon arrival in the Golgi, the kinetic differential between the two proteins has been overcome. In contrast, the relatively high ratios of CRP/albumin found in the microsomal fractions are in agreement with our earlier proposal (47) that newly synthesized CRP equilibrates within an intracellular pool of preexisting CRP molecules, and identify the site of this pool as the ER.

The specific activities of CRP and albumin in the rough microsomal fraction from the 0-min chase sample were calculated from the observed radioactivity and protein concentration (by RIA) of the two proteins, correcting for methionine composition (20 residues per CRP pentamer [29] and 1 residue per rabbit albumin molecule [33]. The observed specific activities (cpm/pmol) were 120 for CRP and 770 for albumin, again suggesting that newly synthesized CRP molecules are diluted within a pool of unlabeled CRP molecules.

The ratio of CRP/albumin found in the Golgi fraction from the more responsive Animal #2 was 2.9%, again in good agreement with the ratio of rates of secretion into medium (3.0%; Table I). In this case, CRP represented 7.6–8.9% of albumin within the ER fractions, as compared to 12–13% in the cells from the less responsive animal. The observation that the relative concentration of CRP within the microsomal fractions was actually lower in the cells with a more rapid transit time for CRP indicates that exit of CRP from the ER is not simply a diffusion-dependent process driven by relative protein concentration. Instead, CRP appears to be specifically retained within the ER of the control hepatocyte.

Evidence for a Specific CRP Binding Site within Permeabilized Rough Microsomes

To investigate the interaction of CRP with the membranous

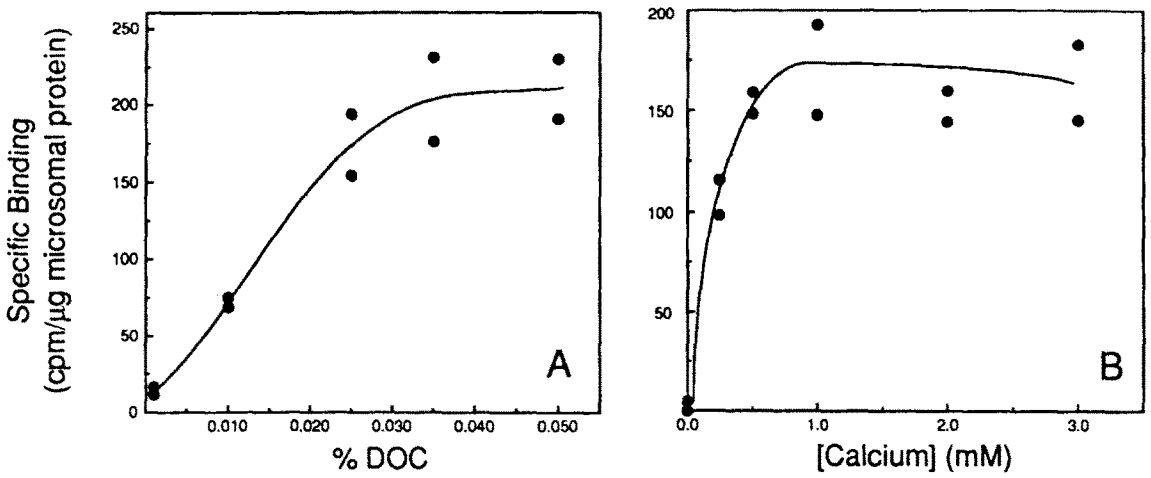

Figure 2. Effect of DOC concentration (*A*) and calcium concentration (*B*) on specific binding of CRP to isolated total microsomes. Aliquots of microsomes were diluted with high salt buffer, harvested by centrifugation, and resuspended in incubation buffer (containing 2.5 mM $CaCl_2$). DOC was added to the final concentrations indicated and the samples were incubated for 1 h at 4°C before chromatography on separate Sepharose 2B columns, each equilibrated in buffer of the appropriate DOC concentration. Fractions corresponding to the Vo of each column were pooled separately for protein determinations. 30 μg of microsomal protein from each sample was used per incubation in the CRP binding assay in the presence and absence of a 50-fold excess of unlabeled CRP. In *B*, microsomes were permeabilized in 0.035% DOC, subjected to Sepharose 2B chromatography, and incubated with labeled CRP at the concentrations of calcium indicated. Data represent specific binding in duplicate incubations.

structures of the secretory pathway, techniques were developed to assess the specific binding of CRP to microsomes isolated from rabbit liver. Since such interactions would be expected to occur on the lumenal surface of the vesicles, we took advantage of previous work demonstrating that isolated microsomes can be effectively permeabilized by low concentrations of deoxycholate (38, 58). Fig. 2 *a* demonstrates the effect of increasing concentrations of DOC on the specific binding of labeled CRP to total microsomes. While little binding was seen in the absence of DOC, detectable specific binding of CRP increased with increasing concentrations of detergent, reaching a maximum value at 0.035% DOC. The observed relationship between DOC concentration and binding activity is in excellent agreement with previous comprehensive studies demonstrating the relationship between DOC concentration and induced permeability of rough microsomes to macromolecules (38). In order to maximize the ability to detect CRP binding yet minimize release of phospholipids (38), subsequent binding studies were carried out in 0.035% DOC.

Binding of CRP to permeabilized microsomes was found to be calcium-dependent (Fig. 2 *b*), with maximum binding occurring at calcium concentrations in excess of 0.5 mM (in the presence of 1.0% BSA). This finding is not unexpected, in that CRP is known to bind calcium (25) and many of its other recognized binding properties are calcium-dependent (6). Subsequent studies were carried out in the presence of 1.5 mM calcium.

To investigate the distribution of CRP binding within the population of total microsomes, binding assays were performed on detergent-permeabilized subcellular fractions. In two experiments, mean specific binding of ^{125}I-CRP (ng bound/mg microsomal protein) was found to be 20.5 for rough microsomes, 14.8 for smooth microsomes, and was not detected in Golgi-derived microsomes. Thus, the localization of the binding activity to fractions derived from the ER correlates with the observation that the ER is the compartment in which CRP is retained as judged by the results of the pulse-chase and steady-state distribution experiments described above. In subsequent studies, purified rough microsomes were used in order to avoid the possibility of heterogeneity in the smooth ER fractions.

Identification of CRP Binding by EM

Since biotinylated CRP was found to be indistinguishable from native CRP in competitive binding studies of permeabilized rough microsomes (see below), it was considered to be a suitable ligand, when combined with peroxidase-conjugated Streptavidin, for EM studies. As seen in Fig. 3 *b*, DOC-permeabilized rough microsomes incubated with biotin-BSA did not accumulate any peroxidase reaction product when compared to untreated rough microsomes (Fig. 3 *a*). These findings indicate the absence of detectable nonspecific interaction between biotinylated proteins and permeabilized rough microsomes, as well as the absence of components within rough microsomes which either contain, or are capable of binding biotin. Examination of rough microsomes not treated with DOC before sequential incubation with biotin-CRP and peroxidase-conjugated Streptavidin revealed occasional microsomes which appeared to accumulate reaction product within the vesicle (Fig. 3 *c*). This may have been due to a small incidence of vesicles sufficiently damaged during preparation to allow entry of CRP and may correlate with the very low levels of binding seen in the absence of DOC (Fig. 1). As indicated by the arrow (Fig. 3 *c*), some degree of diffusion of reaction product was apparent, as evidenced by the presence of electron dense material in the portion of a negative vesicle which is adjacent to a positive vesicle. In detergent-permeabilized preparations incubated with biotinylated CRP followed by peroxidase-conjugated Streptavidin (Fig. 3 *d*), the majority of microsomes contained reac-

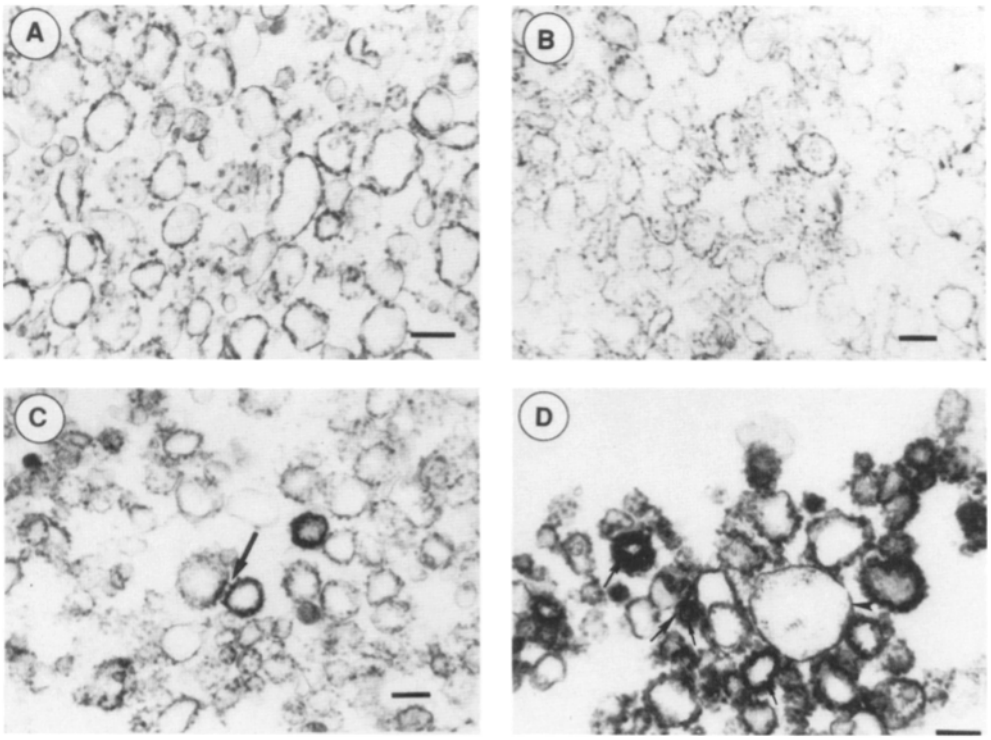

Figure 3. Electron microscopic localization of CRP binding to rough microsomes. Rough microsomes prepared as described in Materials and Methods were permeabilized with DOC (*B* and *D*), and incubated with biotinylated BSA (*B*), or biotinylated CRP (*C* and *D*), before incubation with peroxidase-conjugated Streptavidin followed by peroxidase substrate. The sample in *A* represents untreated rough microsomes and the sample in *C* represents rough microsomes not treated with detergent prior to incubation with biotinylated CRP. Bar, 0.5 μm.

tion product, occasionally nearly filling the vesicle (Fig. 3, *large arrows*) and/or appearing to preferentially deposit on the inner surface of the membrane (Fig. 3, *small arrows*). In contrast, a contaminating large smooth vesicle (Fig. 3, *arrowhead*) appeared to be negative. The observed detergent-dependence of CRP binding seen both in radioligand binding studies as well as by EM indicates that the detectable binding activity is present on the lumenal face of permeabilized rough microsomes.

Specificity of Binding of CRP to Permeabilized Rough Microsomes

The specificity of the interaction between rabbit CRP and permeabilized rough microsomes was investigated by assessing the ability of a variety of proteins to compete for the binding of ^{125}I-CRP. As seen in Fig. 4 *a*, competition by unlabeled CRP (*closed circles*) indicated a K_i (concentration at 50% inhibition) of $\sim 10^{-8}$ M, with specific binding being greater than 90% of total binding. In contrast, only minimal diminution in binding was observed when rabbit CRP subunits (Fig. 4 *a*, *open circles*) were employed as competitor, suggesting that pentameric structure is critical to the interaction of CRP with the membrane. Further, this observation suggests that the binding site does not function in the assembly of newly synthesized CRP subunits (the unique primary translation product [62]) into the native pentameric molecule.

Rabbit albumin and transferrin (Fig. 4 *b*) and human CRP (Fig. 4 *c*) did not appear to interact with the CRP binding site to an appreciable degree, as judged by the lack of competition by these proteins for binding of ^{125}I-CRP to permeabilized rough microsomes. Histones, tested because CRP has been shown to have a calcium-dependent, phosphocholine-inhibitable affinity for histones (17), also failed to compete with CRP for binding. Histones did, however, appear to interact with CRP in that concentrations in excess of 10^{-6} M (not shown) resulted in the precipitation of CRP, in agreement with a recent report (19). Biotinylated CRP was found to be indistinguishable from native rabbit CRP in its ability to compete for binding (Fig. 4 *b*), confirming its suitability as a probe for localization of the binding site by EM.

Since CRP has a known calcium-dependent binding capacity for the polar head group of phosphocholine (75) and binds to disrupted but not intact membranes (40, 50, 76), it is possible that these observations could represent binding of CRP to phosphocholine exposed on the inner surface of permeabilized microsomes. Phosphocholine was found to inhibit the binding of ^{125}I-CRP, but concentrations necessary for competition equivalent to that by CRP were 100-fold greater (Fig. 4 *d*), indicating it is unlikely that the binding site is simply phosphocholine itself. The effect of trypsin on the binding of CRP to microsomes also supports this conclusion. CRP binding to permeabilized microsomes that had been pretreated with trypsin, then quenched with PMSF, was found to be only 20% of the binding to permeabilized microsomes which had been preincubated with trypsin which had been premixed with PMSF (data not shown). Possible explanations for the effect of phosphocholine on the binding of CRP to rough microsomes are dealt with in the Discussion.

Kinetic and Saturation Binding Studies

Kinetics of dissociation of CRP from permeabilized rough microsomes isolated from two unstimulated (control) rabbits were determined by preincubating fixed amounts of labeled CRP with microsomes, then adding a 60-fold excess of unlabeled CRP and determining bound and free labeled CRP at timed intervals thereafter (Fig. 5 *a*). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP added simultaneously. Analysis (KINETIC [49]) of the dissociation kinetics indicated the presence of

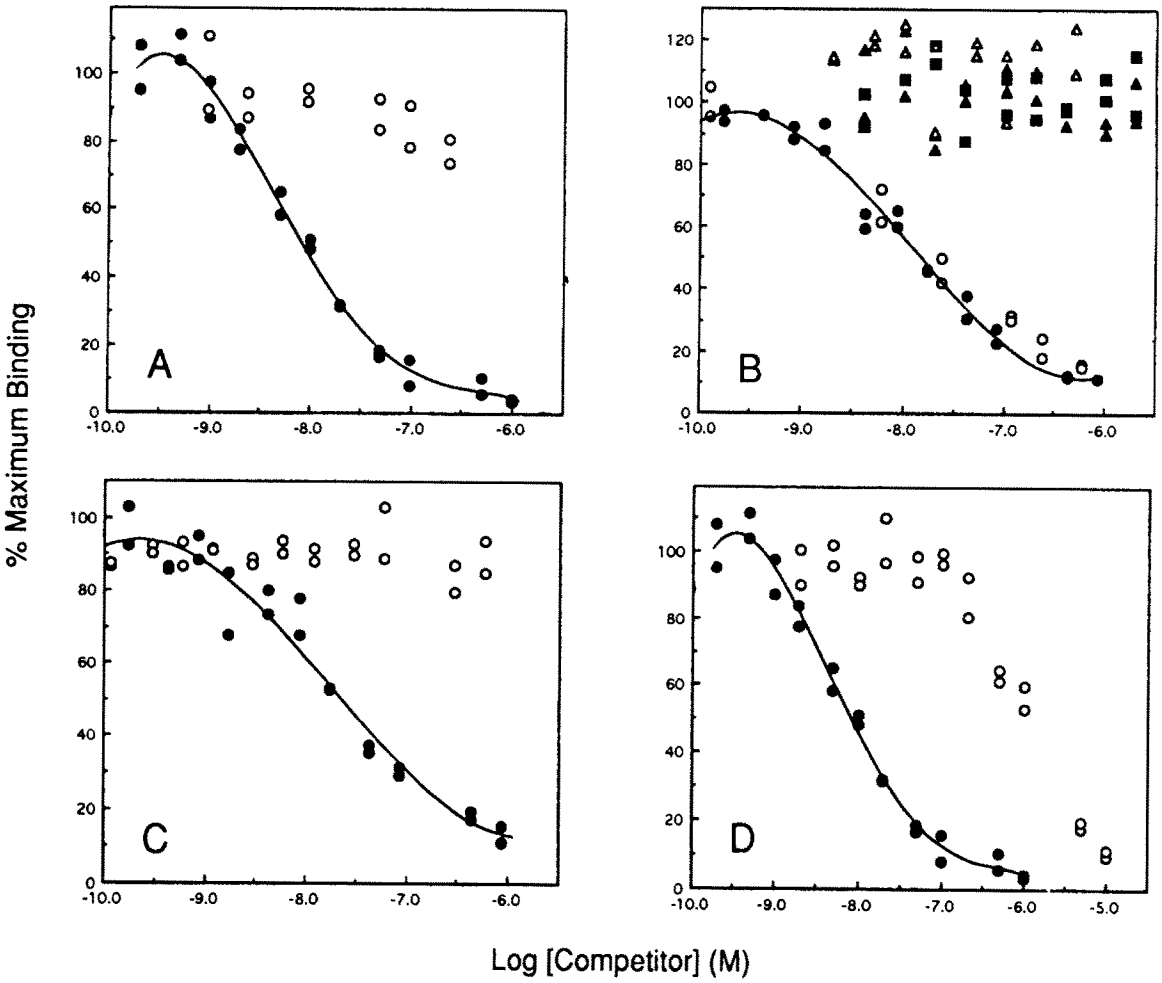

Figure 4. Specificity of binding of CRP to permeabilized rough microsomes. 8–10 μg of purified rough microsomes were incubated in the presence of 50–100 ng labeled CRP (5–9 × 10^5 cpm) and a variety of competing proteins. Solid circles and solid lines indicate the reference competition for binding obtained with purified unlabeled CRP coincubated at the indicated concentrations. Minimal competition was observed with CRP subunits (*A*, *open circles*). No competition was detectable (*B*) with rabbit transferrin (*solid squares*), rabbit albumin (*solid triangles*), or histones (*open triangles*), while competition by biotinylated rabbit CRP was indistinguishable from native CRP (*B*, *open circles*). Human CRP did not compete in the assay (*C*, *open circles*) and equivalent inhibition by phosphocholine (*D*, *open circles*) required concentrations ~100-fold greater than native CRP. Maximum binding ranged from ~6,000–8,000 cpm/250 μl incubation (2–3,000 cpm/100 μl centrifuged and counted) and specific binding represented 85–95% of total binding as shown. Reference competitive binding curves for unlabeled CRP (*solid circles* and *solid line*) are representative in *A* and *B* and were determined in side-by-side incubations in *C* and *D*.

two sites (p = 0.005 versus a one-site fit) with dissociation rate constants (k') of 3.5 ± 2.5 × 10^{-2} (±SEM) and 2.6 ± 0.33 × 10^{-4} min^{-1}. Similarly, analysis of association kinetics (Fig. 5 *b*) also suggested the presence of two sites (p = 0.001) with calculated association rate constants (k) of 7.5 × 10^4 and 7.9 × 10^6 M^{-1} min^{-1}. From these data, calculation of equilibrium dissociation constants (K_d = k'/k) resulted in estimates of a K_d = 3.3 × 10^{-10} M for a high affinity site and a K_d of 4.7 × 10^{-7} M for a second, lower affinity site.

Saturation binding studies were carried out employing increasing amounts of labeled CRP in paired incubations plus and minus at least a 60-fold excess of unlabeled CRP (see Materials and Methods). Studies of permeabilized rough microsomes from unstimulated rabbits suggested saturable binding was approached at concentrations of labeled CRP in excess of 20 nM (Fig. 6 *a*, *inset*), and allowed for estimation of a mean K_d (as judged by concentration of CRP at half saturation) of ~3 nM. Nonlinear curve fitting analysis (LIGAND [53]) again indicated a two-site fit was statistically superior (P < 0.02) (Fig. 6 *a*). The resulting association constants were 1.14 × ±0.83 × 10^8 and 7.29 × ±2.2 × 10^6 M^{-1}, yielding K_d values of 8.8 × 10^{-10} M for the high affinity site and 1.4 × 10^{-7} M for the low affinity site, values in acceptable agreement with those obtained from the kinetic binding studies.

For comparison, saturation binding studies were also performed with permeabilized rough microsomes prepared from two animals stimulated in vivo to undergo the acute phase response (Fig. 6 *b*). In this case, a single fit model resulted in an estimated K_d of 1.5 × 10^{-7} M, corresponding to the lower affinity site seen with microsomes from unstimulated animals, and the higher affinity site was not demonstrable. These data correlate with our previous observations that the half-time for secretion of CRP is markedly longer in normal hepatocytes than in cells from animals undergoing

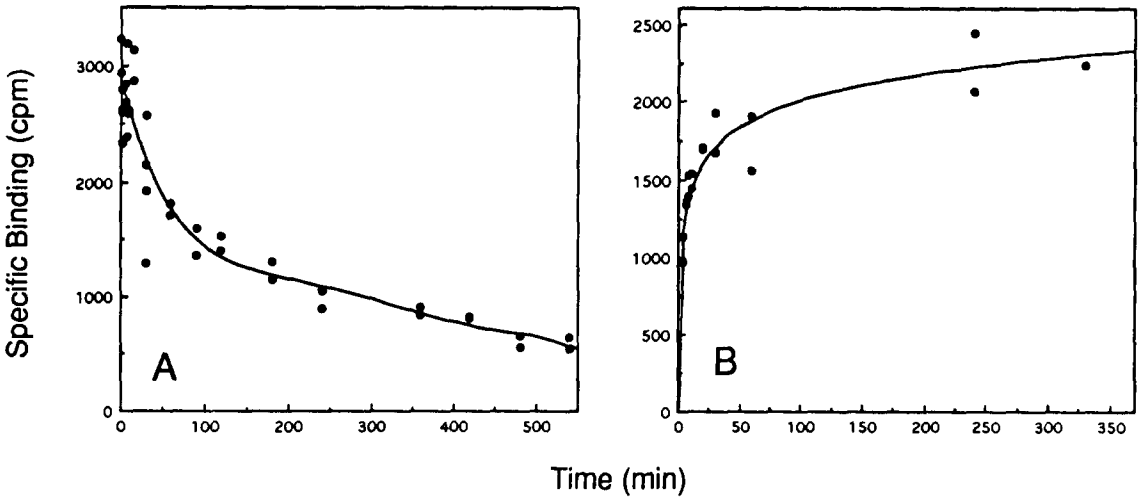

Figure 5. Kinetic analysis of binding of CRP to permeabilized rough microsomes. Dissociation (*A*) and association (*B*) binding studies were carried out on control rough microsomes. Individual 25-μl incubations contained 240 ng labeled CRP (= 7.7 nM) and 9 μg microsomal protein. Data represent means of duplicates from two experiments for each study.

the acute phase response (47) as a result of retention of CRP within the ER of the normal cell (Fig. 1 and Table I), and indicate that the decreased expression of the high affinity site could be the mechanism responsible for the observed differences in kinetics of CRP secretion.

Preliminary Characterization of the ER Binding Site for CRP

Since evidence suggests that the ER resident protein BiP (GRP78) plays a role in determining folding efficiency and exit of at least some secretory proteins from the ER (15, 20, 27 and reviewed in 23), we attempted to detect an interaction between newly synthesized CRP and BiP. When lysates of metabolically labeled hepatocytes were immunoprecipitated with rat monoclonal antihuman BiP under conditions of ATP depletion and in the presence of 1.5 mM $CaCl_2$, an 80-kD band, presumably BiP, was identified, but no detectable CRP was coprecipitated. Similarly, anti-CRP did not coprecipi-

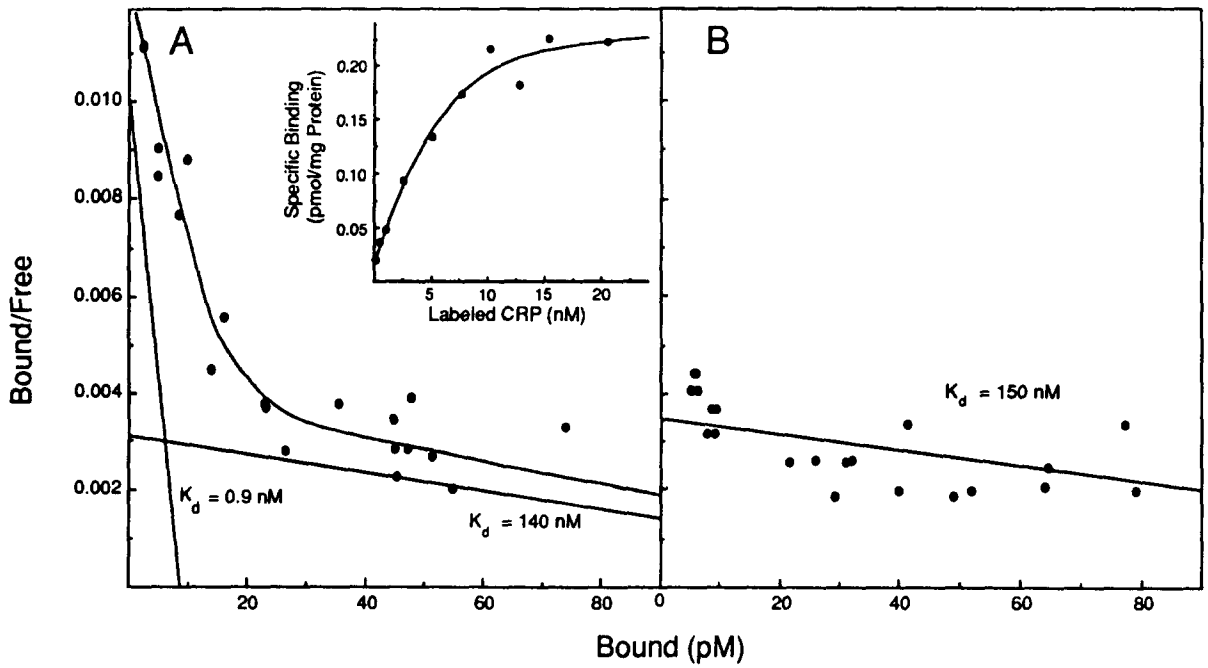

Figure 6. Scatchard plots of equilibrium binding studies. Permeabilized rough microsomes from two control rabbits (8A) and two animals undergoing the acute phase response (8B) were incubated with increasing concentrations of labeled CRP (range of 26 ng-2.6 μg) in the presence and absence of competing unlabeled CRP. Individual incubations contained 9–11 μg microsomal protein and, in the case of incubations from control microsomes, mean maximal specific binding in counted aliquots (100 μl) was 5,900 cpm, representing 86% of total bound CRP and 1.1% of total labeled CRP. Each plot represents the means of duplicates from two incubations. Inset (8A) demonstrates saturability of specific binding with increasing concentrations of added labeled CRP.

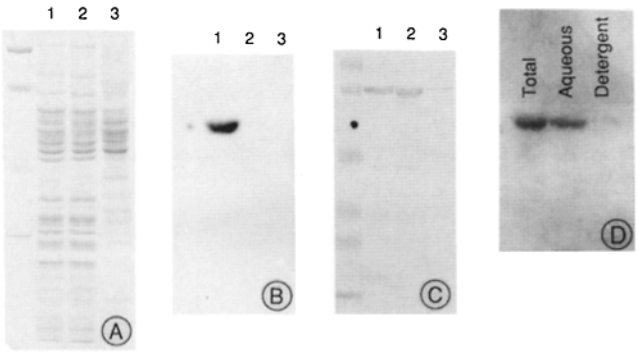

Figure 7. Identification of an ER lumenal protein capable of binding CRP. 75 μg protein from unstimulated rough microsomes (lane *1*), stimulated rough microsomes (lane *2*), and unstimulated Golgi membranes (lane *3*) were subjected to electrophoresis on a 10% SDS gel. After transfer to nitrocellulose, the gel was stained with Coomassie blue (*A*). The nitrocellulose blot was probed with radiolabeled CRP and an initial autoradiograph of the blot was used to align placement of a radioactive dye spot prior to a second autoradiographic exposure (*B*). The same blot was then incubated with rat antihuman BiP followed by alkaline phosphatase-conjugated rabbit anti-rat Ig before enzymatic color development (*C*). Molecular mass markers (in kD) are 97, 66, 45, and 31 in *A* and prestained markers of 110, 84, 47, 33, 24, and 16 in *C*. In *D*, rough microsomes from an unstimulated animal were suspended in 0.5% Triton X-114 and subjected to phase partitioning as described in Materials and Methods. Equivalent proportions of the total lysate, detergent phase, and aqueous phase were run on 10% SDS gels in the lanes indicated. After transfer to nitrocellulose, the blot was probed with radiolabeled CRP.

tate BiP (data not shown). Since we were unable to reproducibly coprecipitate other proteins with anti-BiP, we cannot conclude whether BiP may play a role in the retention of CRP within the ER. However, it is unlikely that an interaction between BiP and CRP would be undetected by these techniques if it were responsible for a stable intracellular half-life of CRP of 18 h (47).

In an initial approach to the direct identification of the CRP-binding site, nitrocellulose blots of electrophoretically resolved microsomal lysates were found to be suitable for the demonstration of a CRP binding band. A blot (Fig. 7 *b*) prepared from an SDS gel (Fig. 7 *a*) loaded with samples of rough microsomes (Fig. 7, lane *1*) and Golgi membranes (Fig. 7, lane *3*) from an unstimulated rabbit, and rough microsomes from a stimulated animal (Fig. 7, lane *2*) was probed with radioiodinated CRP and demonstrates a 60-kD CRP-binding band present in the lysate of unstimulated rough microsomes, but not in the other samples. When the same blot was subsequently probed with anti-BiP (Fig. 7 *c*), a different band, 80 kD in size, was identified in equivalent amounts in the rough microsomal samples, but greatly diminished in the Golgi sample, findings that are consistent with the expected size and distribution of BiP and indicate that BiP expression does not change significantly during the acute phase response. Thus, the expression of the 60-kD CRP-binding band, a protein distinct from BiP, correlates well with the results of the binding of CRP to permeabilized microsomal subfractions, as judged by both subcellular localization as well as a decrease in activity in microsomes from stimulated animals. When rough microsomes were extracted with Triton X-114 (12), virtually all of the 60-kD CRP-binding activity present in total lysate (Fig. 7 *d*, "Total") was found to partition into the aqueous phase (Fig. 7 *d*, "Aqueous") indicating that the 60 kD material is likely not a transmembrane protein. Together, these findings suggest that the 60 kD CRP-binding site is a lumenal ER protein, possibly localized to the ER via interaction with the KDEL or a homologous retrieval system (57).

Discussion

These studies were undertaken to investigate the mechanisms underlying our previous observation that the half-time for CRP secretion decreases markedly during the acute phase response (47) and the finding that a rapid and constant transit time in HeLa cells transfected with the rabbit CRP gene suggested that the observed differences in CRP secretion kinetics were due to differential intracellular retention of CRP within the normal rabbit hepatocyte (30). The major findings of the present studies are as follows. (*a*) The site of CRP retention is, not surprisingly, the endoplasmic reticulum. (*b*) Specific, detergent-dependent binding of CRP to microsomes is limited to ER-derived subcellular fractions. (*c*) Specific binding of CRP is greatly diminished in samples from animals undergoing the acute phase response and appears to involve an ER protein distinct from BiP. These data correlate well with our previous observations that the exit of CRP from the hepatocyte is regulated at a posttranslational level during the acute phase response.

Since CRP has a known calcium-dependent binding affinity for the polar head group of phosphocholine (75) and adheres preferentially to disrupted but not intact membranes (40, 50), it is possible that phosphocholine exposed by detergent treatment of rough microsomes might be an available ligand for CRP and confound the interpretation of the binding data. However, several lines of evidence indicate that the high affinity microsomal binding site is not simply exposed phosphocholine. The estimated affinity for CRP is ~500-fold greater than that for free phosphocholine (1). The high affinity site was not detected in Golgi fractions or rough microsomes from stimulated animals. Furthermore, no specific binding of CRP was found with permeabilized rough microsomes prepared from mice (data not shown), an unusual species in which CRP synthesis is minimal and does not change substantially during the acute phase response (66). Finally, human CRP, which has the same affinity for phosphocholine as does rabbit CRP (1, 5), did not compete for the binding of rabbit CRP to rough microsomes (Fig. 4 *c*).

Nevertheless, we did observe weak inhibition by phosphocholine of the interaction between CRP and rough microsomes. One possible explanation for this finding would be that phosphocholine is a constituent of the rough microsomal binding site for CRP and the greater apparent affinity of this site for CRP is due to additional protein structure. Such a phenomenon would be analogous to the observation that the affinity of the cation-dependent mannose-6-phosphate receptor for mannose-6-phosphate expressed in lysosomal enzymes is substantially greater than that for free mannose-6-phosphate (28). Alternatively, phosphocholine could be exerting an allosteric effect, since it is known that the interaction of phosphocholine with CRP results in a conformational change in CRP (80). Thus, phosphocholine added to the assay could bind to free CRP and result in a conformational

Table II. Relationship Between Serum CRP Level and CRP Content of Isolated Rough Microsomes: Effect of Detergent Permeabilization

Serum CRP	Rough microsomal CRP content*	CRP released by detergent‡
μg/ml	*ng CRP/mg microsomal protein*	%
<2	4.4 ± 0.5	<40§
46	20 ± 5	86
119	45 ± 4	91
171	63 ± 8	>95§

* Mean ± SEM of duplicate determinations employing two different volumes for assay.
‡ Proportion of CRP which was rendered soluble by DOC permeabilization, expressed as a percentage of total CRP content.
§ Represent estimates due to limiting sensitivity of the RIA in detecting the small amounts of CRP released from control microsomes and retained within microsomes from a highly responsive animal.
Rough microsomes prepared from one control animal (serum CRP <2 μg/ml) and from three stimulated rabbits were resuspended in 0.25 M sucrose, 20 mM Hepes, 0.15 M NaCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4. After removal of aliquots for protein determinations, DOC was added to a concentration of 0.035% and the samples incubated for 1 h at 4°C. Microsomes were harvested by microfugation for 45 min and the pellets were extracted with 0.15 M NaCl, 20 mM Tris, 1% Triton X-100, 0.5% DOC, 10 mM sodium citrate, pH 7.4, to release bound CRP. CRP present in both the initial supernate and the pellet extract was determined by RIA. Data have not been corrected for homogenization-induced leakage.

change which lessens the ability of CRP to bind, via another site, to the rough microsomal membrane. Indeed, the observed K_i of ~3 μM (Fig. 4 *d*) for phosphocholine in the binding assay is in agreement with what would be expected for the interaction between CRP and free phosphocholine, having a K_d of 5 μM (5). The lack of inhibition of microsomal binding by human CRP suggests that the effect of phosphocholine on the binding of rabbit CRP to rough microsomes is due to an allosteric effect of phosphocholine on the CRP molecule, although it remains possible that phosphocholine is a constituent of the binding site and that additional protein structure increases the affinity for rabbit CRP, but also sterically interferes with the interaction of human CRP with the phosphocholine moiety of the microsomal binding site.

Our conclusion that the expression of the ER binding site for CRP is regulated during inflammatory states is of significance, since it implies a physiological role for the binding. Thus, it is important to confirm that the observed changes in kinetics of secretion of CRP (47) are not simply the result of saturation of a constitutively expressed binding site. This is particularly true since endogenous CRP, if present in rough microsomes from stimulated animals, could have effectively decreased the true specific activity of the labeled CRP. This circumstance would preferentially obscure detection of higher affinity binding sites. However, Table II demonstrates that the Sepharose 2B chromatography step used in the preparation of permeabilized microsomes effectively removed endogenous CRP from stimulated samples. The maximum amount of endogenous CRP which could have been in the incubations was only 25 pg, representing only 0.1% of the amount of labeled CRP included in the incubations containing the lowest concentration of labeled CRP. Finally, the differences seen in CRP binding by nitrocellulose blot analysis of unstimulated versus stimulated microsomes reflect a direct assessment of binding site expression, since endogenous CRP is both dissociated and physically separated from the 60-kD CRP-binding band.

In addition to a high degree of specificity, the affinity of binding (K_d = 1 nM) detected in permeabilized rough microsomes is considerably greater than the affinities previously reported: 5 μM for phosphocholine (1), 0.8 μM for chromatin (17, 58), and 0.03–0.1 μM for surface receptors present on phagocytic cells (6, 51, 72, 81). The B_{max} determined for the high affinity site (0.88 pmol CRP/mg microsomal protein) is within the range of values reported for physiologically significant receptors, including receptors for IL-1; B_{max} = 0.5 pmol/mg membrane protein (56), for inositol trisphosphate; B_{max} = 5 pmol/mg protein (69), and for 5-hydroxytryptamine$_3$; B_{max} = 1 pmol/mg protein (48). On the basis of these data, the estimated density of the high affinity site within the ER would be the equivalent of a few thousand cell surface receptors per cell. A B_{max} of ~0.9 pmol (110 ng) CRP per mg microsomal protein is more than sufficient to account for the amount of CRP contained within rough microsomes isolated from animals synthesizing CRP at low rates (Table II). Accounting for homogenization-induced leakage, the amount of CRP within control microsomes represents ~7–10% of the B_{max}. The nature of the lower affinity site is at present of uncertain significance in that it was detected in microsomes prepared from both stimulated as well as control animals and the apparent affinity was only ~30-fold greater than the affinity of CRP for free phosphocholine.

The results presented here illustrate a novel mechanism which could effectively reroute the intracellular trafficking of a secretory protein under differing physiologic conditions. What might be the function of such a regulated retention mechanism for CRP? On the basis of previous findings (47) as well as the pulse-chase data and in vitro binding assays reported here, it is apparent that effective retention of CRP within the ER occurs preferentially in hepatocytes synthesizing CRP at relatively low rates. As a result, the cell accumulates a small pool of CRP within the ER. Since the retention (or retrieval) of CRP is calcium-dependent, this pool would be rapidly mobilizable in response to transient decreases in local calcium concentration resulting, for example, from signal transduction during the early acute phase response. While there is controversy regarding the effects of calcium ionophores on the fate of ER resident proteins (11, 43), local calcium fluxes within the ER appear to be of great potential physiologic significance (61). Whether a rapid secretory burst of intracellular CRP might play a role in the early acute phase response is presently unknown.

An alternative explanation for the retention of CRP would be that CRP has a function within the ER of the hepatocyte which is superseded during the acute phase response. While the majority of functions ascribed to CRP are related to its role as a major acute phase plasma protein, it is intriguing to note that CRP has been demonstrated to bind to chromatin (17, 58), histones (17, 19), and U1 snRNPs (16) and further, that it is structurally homologous to nucleoplasmin and contains a nuclear localization signal (18).

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