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

Stephen S. Macintyre
Case Western Reserve University at MetroHealth Medical Center, Cleveland, Ohio 44109-1998

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 luminal 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 ($K_d = 1 \text{ nM}$, $B_{max} = 1 \text{ pmol CRP/mg microsomal protein}$) as well as a much lower affinity ($K_d = 140 \text{ nM}$) 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...
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 Williams’ medium E containing 1 mM dexamethasone, 1 nM hormone-synthesizing 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 were 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-[35S]methionine (>88% 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 µg/ml) 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 volume 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 aliquots as well as the remaining lysate, were taken to determine the ER as the compartment in which CRP is retained. Both biochemical and immunochemical methods demonstrate specific binding of CRP to the luminal 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 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 immunochemical methods demonstrate specific binding of CRP to the luminal 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 hepatocytes. 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.

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 125I-labeled CRP or rabbit albumin (prepared as described below) in the homogenization buffer added to unlabeled hepatocytes before homogenization. The homogenate was centrifuged 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 model 1085 gamma counter. Homogenization-induced cell breakage and leakage of pulse-labeled proteins (10 min, 1500000 g) 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 supernatant to final concentrations of 10 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 [125I]-methionine, pulse-labeled proteins and adsorption of radiiodinated proteins to membranes were initially examined in four cultures of hepatocytes 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, each containing 5 x 10⁷ cells, yielded ~5 mg protein in total microsomes, 400 µg in smooth 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 (4) except that the assay 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 diphospbo-D-[U-14C]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 mouse 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, Plainview, 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. Radio-labeled CRP and albumin present in these same supernates were specifically immunoprecipitated as described previously (47). Transferase 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 radioactive activity 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 with water and stained bands corresponding to added carrier proteins were dissolved in 30% H2O; and the radioactivity determined as described (47). All immunoprecipitations were carried out under conditions of antibody excess as determined by precipitin curves employing radiodinated specific antigens.

To investigate the possible association of newly synthesized CRP with BiP (GRP), 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 [125I]-methionine as described above, four dishes of cells were rinsed with Hanks' buffered saline and were scraped in 3 ml of 20 mM Heps, pH 7.4, 0.15 M NaCl, 1 mM MgCl2, 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 CaCl2. After 15 min at 4°C the lysate was centrifuged at 12,000 g and 1 ml of the supernate was incubated as described for determination of 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 Heps, pH 7.4, 0.15 M NaCl, 0.1% BSA and the suspension was incubated for 1 h at 4°C with rotation. The Sepharose was pelleted by microcentrifugation (model 59A; Fisher Scientific, Fair Lawn, NJ) and washed twice with 1 ml of 20 mM Heps, pH 7.4, 0.4 M NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC and once with 1 ml of 20 mM Heps, 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 Heps, 0.15 M NaCl, 0.1% Chaps, pH 7.4, the blots were probed with 125I-CP (prepared as below) at a concentration of 0.5-1 µCi/ml (6-9 x 10⁶ cpm/ml) in the same buffer, containing 1.5 mM CaCl2, and 1.0% BSA. After incubation at 4°C for 2 h, the blots were washed in three changes of 20 mM Heps, 0.15 M NaCl, 0.1% Chaps, 1.5 mM CaCl2, and 1.0% BSA, pH 7.4, dried, and subjected to autoradiography. Western blots probed with anti-BiP (I:5,000 dilution of hybridoma culture medium) were then incubated with 1:3,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit (Pierce Chemical 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 radiiodinated exactly as described (11), except that equilibrium and kinetic binding studies 125I-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 Heps, pH 7.4, and was dialyzed exhaustively against 0.15 M NaCl, 20 mM Heps, pH 7.4. Specific radioactivities ranged from 6 to 9 x 10⁶ cpm/µg and CRP concentrations...
Microsomal Binding Assay

each sample's volume was 250/~1 and contained 10-15/zg microsomal pro-
tein 20 mM Hepes, 1.5 mM CaCl2, 1 mM MgCl2, 1.0% BSA, 0.035% DOC
Sepbarose 2B as described above. Incubation buffer included 0.15 M NaCl,
4~ samples were layered over discontinuous sucrose gradients contain-
ning 1.0% Triton X-100, 0.5% DOC, and 10 mM sodium citrate in order
to dissociate CRP by calcium-dependent interactions.

CRP (500-750 ~g/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 rain, 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 subjected to gel fil-
tration on Sephacryl S200 (Pharmacia Fine Chemicals, Piscataway, NJ).

Preparation of Samples for EM

Rough microsomes were prepared and permeabilized as described above in
0.33 M sucrose, 20 mM Hepes, pH 7.4. Microsomes containing microsomal
protein were dialyzed 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 CaCl2, 1 mM MgCl2, 1.0% BSA,
pH 7.4, and biotinylated CRP (2 ~g/ml). Control incubations included bi-
otinylated 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 lay-
ered on discontinuous sucrose gradients containing 30.0~1 of 1.9 M sucrose and 70.0% 20% sucrose, 0.15 M NaCl, 20 mM Hepes, 1.5 mM CaCl2, 1 mM MgCl2, pH 7.4, and were centrifuged at
38,000 rpm for 1 h at 4°C. The tops of the gradients were carefully as-
rated, 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 dia-
lyzed against 0.15 M NaCl, 20 mM Hepes, 1.5 mM CaCl2, 1 mM MgCl2, 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 CaCl2, 1 mM MgCl2, 1.0% BSA, 0.05% DOC,
and peroxidase-conjugated Streptavidin at a final dilution of 1:40 of that sup-
plied (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 inter-
face was collected and diluted to 5 ml with 0.25 M sucrose, pH 7.0, and glutar-
dehyde was added to a final concentration of 1.0%. After incubation for 1 h
at 4°C, samples were layered over discontinuous sucrose gradients contain-
ing 2 ml 2M sucrose and 4 ml 20% sucrose and were spun at 38,000 rpm
for 60 min (SW50.1 rotor). Material at the 1.9 M sucrose interface was col-
lected 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 mi-
crosomes 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 ~1 1.0% H2O2, samples were incubated for 15 min at 20°C and the reac-
tion was terminated by the addition of 9 mi of ice-cold 0.25 M sucrose. Sam-

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 CaCl2, 1 mM MgCl2, 1.0% BSA, 0.035% DOC
(except as indicated in Fig. 2), pH 7.4. In typical competitive binding assays,
capillary electrophoresis was 250 ~1 and contained 0.5-15 ~g microsomal
protein and 50-100 ng [3H]CRP. After incubation for 3 h at 4°C, duplicat-
ate 100-~l aliquots were layered over 200 ~1 15% sucrose, 0.15 M NaCl,
20 mM Hepes, 1.5 mM CaCl2, 1 mM MgCl2, pH 7.4, in 0.4-ml poly-
propylene centrifugation tubes. After microcentrifugation for 45 min at
11,000 g (model 59A; Fisher Scientific, Fair Lawn, NJ), 10-~1 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, 0.5% of
[3H]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. Addi-
tional proteins tested for competition included rabbit albumin and transferr-
in (Cappel, Cooper Biomedical), histones (calf thymus from U.S. Bio-
chemical 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 micro-
osomal binding, microsomes were preincubated with trypsin (Sigma Chemi-
ical Co.) at a concentration of 100 ~g/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 permeabi-
lized microsomes.

In the formal equilibrium binding studies incubations included paired
samples containing [3H]CRP ranging from 26 to 2.6 ~g (8.3 × 10-10
-8.3 × 10-10 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 em-
ployed. Data obtained were subjected to Scatchard analysis (63) and these
results were further interpreted employing the nonlinear curve fitting pro-
gram 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 concentra-
tion approximating that determined to be half-maximal saturation (from
equilibrium binding studies). Paired samples, one containing a 50-fold ex-
cess 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 deter-
mined from an incubation in which both labeled and unlabeled CRP were
coincubated with microsomes. Analysis of kinetic binding studies was per-
formed 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 tran-
sit time of ~120 min (68). Results of subcellular fraction-
ation of pulse–chase labeled hepatocytes prepared from an animal exhibiting a minimal response to turpentine injection (animal #1) (serum CRP level 16 ~g/ml; in vitro secretion
rate of 7.1 ng CRP/10 ~ cells/h) are shown in Fig. 1. After a 10-min pulse with [35S]methionine, the majority of each radio-
dabeled protein was, as expected, recovered in the ER-
derived fractions. After 75 min of chase, little albumin re-
ained 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 dis-
tributed 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
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.

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 [35S]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

| Protein | Rough | Smooth | Golgi |
|---------|-------|--------|-------|
| Albumin | 13    | 12     | 0.8   |
| Transferrin | 40 | 59     |       |
| CRP     | 12    | 35     |       |

* Data represent the secretion of proteins into culture medium during an 18-h incubation, expressed as CRP as a percentage of albumin secretion.

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 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 76–8.9% 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...
The population of total microsomes, binding assays were performed on detergent-permeabilized subcellular fractions. In two experiments, mean specific binding of 125I-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 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 125I-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 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 125I-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 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 125I-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 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 125I-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
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.

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 125I-CRP. As seen in Fig. 4 a, competition by unla-lebelled CRP (closed circles) indicated a $K_i$ (concentration at 50% inhibition) of $\sim 10^{-5}$ 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 any appreciable degree, as judged by the lack of competition by these proteins for binding of 125I-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 those 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 125I-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 at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a). Nonspecific binding was determined from an incubation containing labeled and unlabeled CRP at timed intervals thereafter (Fig. 5 a).
two sites ($p = 0.005$ versus a one-site fit) with dissociation rate constants ($k'$) of $3.5 \pm 2.5 \times 10^{-2}$ (±SEM) and $2.6 \pm 0.33 \times 10^{-4}$ 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 $7.5 \times 10^4$ and $7.9 \times 10^4$ M$^{-1}$ min$^{-1}$. From these data, calculation of equilibrium dissociation constants ($K_d = k'/k$) resulted in estimates of a $K_d = 3.3 \times 10^{-10}$ M for a high affinity site and a $K_d = 4.7 \times 10^{-7}$ 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 $\sim 3$ 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 $1.14 \times 0.83 \times 10^8$ and $7.29 \times 2.2 \times 10^4$ M$^{-1}$, yielding $K_d$ values of $8.8 \times 10^{-10}$ M for the high affinity site and $1.4 \times 10^{-7}$ 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 $1.5 \times 10^{-7}$ 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 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 x 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 $\sim 6,000$–$8,000$ 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.
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 anti-human BiP under conditions of ATP depletion and in the presence of 1.5 mM CaCl2, an 80-kD band, presumably BiP, was identified, but no detectable CRP was coprecipitated. Similarly, anti-CRP did not coprecipi-

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.

Figure 6. Scatchard plots of equilibrium binding studies. Permeabilized rough microsomes from two control rabbits (SA) and two animals undergoing the acute phase response (SB) were incubated with increasing concentrations of labeled CRP (range of 26 ng–2.5 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 (SA) demonstrates saturability of specific binding with increasing concentrations of added labeled CRP.
pared from an SDS gel (Fig. 7 a) loaded with samples of half-life of CRP of 18 h (47).

microsomes, but not in the other samples. When the Golgi microsomes from a stimulated animal (Fig. 7, lane 2) was

CRP-binding band present in the lysate of unstimulated rough microsomes (Fig. 4 c). The binding of rabbit CRP to rough microsomes (Fig. 7, lane 2) and 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 anti-human 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 2) was probed with radiiodinated 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, "Aquaeous") 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 luminal 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                     | <400                      |
| 46        | 20 ± 5                        | 86                        |
| 119       | 45 ± 4                        | 91                        |
| 171       | 63 ± 8                        | >953                      |

* 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.

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 Heps, 0.15 M NaCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 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, 0.05% 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 $K_d$ 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; $B_{max} = 1$ 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 nucleoRNP and contains a nuclear localization signal (18).
and Debra Rzewnicki for technical assistance; and Thomas Massella for the EM.

This work was supported by grant No. AR34313 from the National Institutes of Health.

Received for publication 31 January 1992 and in revised form 23 April 1992.

References

1. Anderson, J. K., R. M. Stroud, and J. E. Volanakis. 1970. Studies on the binding specificity of human C-reactive protein for phospholipid. Fed. Proc. 29:1495.

2. Andres, D. A., I. M. Dickerson, and J. E. Dixon. 1990. Variants of the carboxyl-terminal KDEL sequence direct intracellular retention. J. Biol. Chem. 265:5952-5955.

3. Andres, D. A., J. D. Rhodes, R. L. Meisel, and J. E. Dixon. 1991. Characterization of the carboxyl-terminal sequences responsible for protein retention in the endoplasmic reticulum. J. Biol. Chem. 266:14277-14282.

4. Aronson, N. N., and O. Touster. 1974. Isolation of rat liver plasma membrane fragments in isotonic sucrose. Methods Enzymol. 31:90-102.

5. Bach, B. A., H. Gerwurz, and A. P. Osmand. 1977. C-reactive protein in the rabbit: isolation, characterization and binding affinity to phosphocholine. Immunochimistry. 14:215-219.

6. Ballou, S. P., and I. Kushner. 1992. C-reactive protein and the acute phase response. In Advances in Internal Medicine. Vol. 37. G. Stollerman, J. T. LaMont, J. Leonard, and M. Siperstein, editors. Mosby Year Book, New York. Pp. 35-336.

7. Ballou, S. P., J. Buniel, and S. S. Macintyre. 1989. Specific binding of human C-reactive protein to human monocytes in vitro. J. Immunol. 142:2708-2713.

8. Bartles, J. R., H. M. Feracci, B. Stieger, and A. L. Hubbard. 1987. Bio genesis of the rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. J. Cell Biol. 105:1241-1251.

9. Bonafacino, J. S., P. Cosson, and R. D. Klausner. 1990. Colocalized transmembrane determinants for ER degradation and subunit assembly explain the intracellular fate of TCR chains. Cell. 63:503-513.

10. Bonafacino, J. S., R. L. Rubin, and R. K. Klausner. 1991. Role of potentially charged transmembrane residues in targeting proteins for retention and degradation within the endoplasmic reticulum. EMBO (Eur. Mol. Biol. Organ.) J. 10:2783-2793.

11. Booth, C., and G. L. E. Koch. 1989. Perturbation of cellular calcium induces secretion of luminal ER proteins. Cell. 59:729-737.

12. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256:1028-1036.

13. Carey, D. J., and C. B. Hirschberg. 1980. Kinetics of glycosylation and N-linked glycosylation and heavy chain-binding protein association with virus G protein in cells and in vitro. J. Biol. Chem. 255:4348-4354.

14. de Silva, A. M., W. E. Balch, and A. Helenius. 1990. Quality control in the endoplasmic reticulum: folding and misfolding of vesicular stomatitis virus glycoprotein in cells and in vitro. J. Cell Biol. 111:857-866.

15. Dorn, A. J., D. G. Bole, and R. J. Kaufman. 1987. The relationship of C-reactive protein to histones and chromatin. Proc. Natl. Acad. Sci. USA. 84:141-145.

16. Du Clos, T. W., L. T. Zlock, and I. Kushner. 1992. C-reactive protein and the acute phase response. In Advances in Internal Medicine. Vol. 37. G. Stollerman, J. T. LaMont, J. Leonard, and M. Siperstein, editors. Mosby Year Book, New York. Pp. 35-336.

17. Du Clos, T. W., L. T. Zlock, and R. L. Rubin. 1988. Analysis of the binding of C-reactive protein to histones and chromatin. Proc. Natl. Acad. Sci. USA. 85:1417-1421.

18. Du Clos, T. W., L. T. Zlock, and R. L. Rubin. 1989. Analysis of the binding of C-reactive protein to histones and chromatin. J. Immunol. 142:4266-4270.

19. Du Clos, T. W., C. Mold, and R. F. Stump. 1990. Identification of a polypeptide sequence that mediates nuclear localization of the acute phase protein C-reactive protein. J. Immunol. 145:3869-3875.

20. Du Clos, T. W., L. T. Zlock, and L. Mnern. 1991. Definition of a C-reactive protein binding determinant on histones. J. Biol. Chem. 266:2161-2171.

21. Dui, J. L., and Y. Argon. 1990. A single amino acid substitution in the variable region of the light chain specifically blocks immunoglobulin secretion. Proc. Natl. Acad. Sci. USA. 87:1835-1839.

22. Fleischer, S., and C. Ernster. 1976. Subcellular fractionation of rat liver. Methods Enzymol. 31:6-41.

23. Fried, E. D., and M. Edelman. 1965. C-reactive protein: a molecule composed of subunits. Proc. Natl. Acad. Sci. USA. 54:558-565.

24. Gotschlich, E. C., and G. M. Edelman. 1967. Binding properties and specificities of C-reactive protein. Proc. Natl. Acad. Sci. USA. 47:706-712.

25. Gotschlich, E. C., and G. M. Edelman. 1965. C-reactive protein: a molecule composed of subunits. Proc. Natl. Acad. Sci. USA. 54:558-565.

26. Hagejohr, S. M., M. Srinivasan, and M. Green. 1991. Analysis of the retention signals of two resident luminal endoplasmic reticulum proteins by in vitro mutagenesis. J. Biol. Chem. 266:6015-6018.
56. Paganelli, K. A., A. S. Stern, and P. L. Kilian. 1987. Detergent solubiliza-
      tion of the interleukin 1 receptor. *J. Immunol.* 138:2249-2253.
57. Pelham, H. R. B. 1989. Control of protein exit from the endoplasmic reticu-
      lum. *Annu. Rev. Cell Biol.* 5:1-23.
58. Robey, P. A., K. D. Jones, T. Tanaka, and T.-Y. Liu. 1984. Binding of
      C-reactive protein to chromatin and nucleosome core particles. *J. Biol.
      Chem.* 259:7311-7316.
59. Rodriguez, B. E., G. Kreibich, and D. D. Sabatini. 1978. Spatial orienta-
      tion of glycoproteins in membranes of rat liver rough microsomes. I. Lo-
      calization of lectin-binding sites in microsomal membranes. *J. Cell Biol.*
      78:874-893.
60. Rose, J. K., and R. W. Doms. 1988. Regulation of protein export from the
      endoplasmic reticulum. *Annu. Rev. Cell Biol.* 4:257-288.
61. Sambrook, J. F. 1990. The involvement of calcium in transport of secretory
      proteins from the endoplasmic reticulum. *Cell.* 61:197-199.
62. Samols, D., S. S. Macintyre, and L Kushner. 1985. Studies of translatable
      mRNA for rabbit C-reactive protein. *Biochem.* 227:759-765.
63. Scatchard, G. 1949. The attraction of proteins for small molecules and ions.
      *Ann. N.Y. Acad. Sci.* 51:660-672.
64. Scheele, G. A., G. E. Palade, and A. M. Tartakoff. 1978. Cell fractionation
      studies on the guinea pig pancreas. Redistribution of exocrine proteins
      during tissue homogenization. *J. Cell Biol.* 78:110-130.
65. Siboo, R., and E. Kulisek. 1978. A fluorescent immunoassay for quantifica-
      tion of C-reactive protein. *J. Immunol. Methods.* 23:59-67.
66. Stafford, F. J., and J. S. Bonifacino. 1991. A permeabilized cell system
      identifies the endoplasmic reticulum as a site of protein degradation. *J.
      Cell Biol.* 115:1225-1236.
67. Strous, G. J. A., and H. F. Lodish. 1980. Intracellular transport of secre-
      tory and membrane proteins in hepatoma cells infected by vesicular stoma-
      titis virus. *Cell.* 22:709-717.
68. Supattapone, S., P. F. Worley, J. M. Baraban, and S. H. Snyder. 1988.
      Solubilization, purification, and characterization of an inositol trisphos-
      phate receptor. *J. Immunol.* 135:1530-1534.
69. Swift, A. M., and C. E. Machamer. 1991. A golgi retention signal in a
      membrane-spanning domain of coronavirus-E1 protein. *J. Cell Biol.*
      115:19-30.
70. Szul, E. S., K. E. Howell, and G. E. Palade. 1985. Biogenesis of the poly-
      meric IgA receptor in rat hepatocytes. I. Kinetic studies of its intracellular
      forms. *J. Cell Biol.* 100:1248-1254.
71. Tebo, J. M., and R. F. Mortensen. 1990. Characterization and isolation of
      a C-reactive protein receptor from the human monocytic cell line
      U-937. *J. Immunol.* 144:231-238.
72. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of
      proteins from polyacrylamide gels to nitrocellulose sheets: Procedure
      and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
73. Vaux, D., J. Tooze, and S. Fuller. 1990. Identification by anti-idiotypic
      antibodies of an intracellular membrane protein that recognizes a mamma-
      lian endoplasmic reticulum retention signal. *Nature (Lond.)* 345:495-
      502.
74. Volanakis, J. E., and M. H. Kaplan. 1971. Specificity of C-reactive protein
      for choline phosphate residues of pneumococcal C-polysaccharide. *Proc.
      Soc. Exp. Biol. Med.* 136:612-614.
75. Volanakis, J. E., and K. W. A. Wirtz. 1979. Interaction of C-reactive pro-
      tein with artificial phosphatidylcholine bilayers. *Nature (Lond.)* 281:
      155-157.
76. Wilstrom, L., and H. F. Lodish. 1991. Nonlysosomal, pre-Golgi degrada-
      tion of unassembled asialoglycoprotein receptor subunits: a TLCK- and
      TPCK-sensitive cleavage within the ER. *J. Cell Biol.* 113:997-1007.
77. Williams, D. B., S. J. Sweidler, and G. W. Hart. 1985. Intracellular trans-
      port of membrane glycoproteins: two closely related histocompatibility
      antigens differ in their rates of transit to the cell surface. *J. Cell Biol.*
      101:725-734.
78. Yeo, K. T., J. B. Parent, T. K. Yeo, and K. Olden. 1985. Variability in
      transport rates of secretory glycoproteins through the endoplasmic reticu-
      lum and Golgi in human hepatocyte cells. *J. Biol. Chem.* 260:7896-7902.
79. Young, N. M., and R. E. Williams. 1978. Comparison of the secondary
      structures and binding sites of C-reactive protein and the phosphorylch-
     oline-binding murine myeloma proteins. *J. Immunol.* 121:1893-1898.
80. Zahedi, K., J. M. Tebo, J. Siripont, G. F. Klimo, and R. F. Mortensen.
      1989. Binding of human C-reactive protein to mouse macrophages is me-
      diated by distinct receptors. *J. Immunol.* 142:2384-2392.