Secretion of C-reactive Protein Becomes More Efficient during the Course of the Acute Phase Response*

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We studied the kinetics of synthesis and secretion of the acute phase plasma protein, C-reactive protein, in primary hepatocyte cultures prepared from rabbits manifesting differing degrees of the acute phase response to inflammatory stimulus. In cultures prepared from progressively more responsive animals, rate of C-reactive protein secretion increased to a much greater degree than did intracellular C-reactive protein content, resulting in a progressive decrease in the ratio of intracellular content to rate of secretion. This ratio, which represents the time required to secrete the amount of C-reactive protein contained within the intracellular pool, decreased from 18 h in cultures from unstimulated rabbits to 2.5 h in cells from highly responsive animals. In contrast, these ratios for albumin were short and fell within a narrow range (0.8–2.1 h). In pulse-chase labeling experiments, the time required for secretion of 50% of pulse-labeled C-reactive protein varied markedly, ranging from well over 6 h in cells from a minimally responsive animal to about 75 min in cells from a highly responsive rabbit. In contrast, the half-time for secretion of albumin was consistently about 45 min in the same cultures. Taken together, these findings indicate that the process by which C-reactive protein is secreted becomes more efficient during the course of the acute phase response.

Recent studies have indicated that secretory proteins pass from the rough endoplasmic reticulum to Golgi at different and characteristic rates, possibly by a receptor-mediated process in which rate of transfer is determined by receptor affinity. We postulate that C-reactive protein secretion is regulated, during the course of the acute phase response, either by alterations in availability of specific receptors or by competition between different secretory proteins for a common receptor.

In eucaryotic cells, secretory proteins are synthesized on the membranes of the rough endoplasmic reticulum and are transferred to the Golgi apparatus (1–7). At the level of the Golgi, several divergent pathways have been identified which serve to transport proteins to a variety of destinations (8–11), as exemplified by the specialized pathways described for lysosomal hydrolases (12, 13), adrenocorticotropic hormone (14), and insulin (15). In contrast, most integral plasma membrane proteins and secretory proteins are believed to be transported to the cell surface via a common pathway (2, 5, 6).

Many plasma membrane and secretory proteins appear to reach the cell surface within 25–45 min after their synthesis (5, 16, 17), consistent with the theory that these proteins are transported in a coordinate fashion by bulk phase movement of luminal contents (5, 18). However, transferrin has been found to require a longer period of time to traverse the hepatic secretory pathway than did the other plasma proteins studied (17, 19–23). This observation raised the possibility that multiple secretory pathways may exist or that specific recognition signals may play a role in the secretory process. More recently, several plasma membrane and secretory proteins have been shown to require different and characteristic periods of time for intracellular transport (22–24); the rate-limiting step in transport has been reported to be at the level of the rough endoplasmic reticulum. These observations have been interpreted as suggesting that a membrane-bound receptor mechanism mediates transport of proteins from endoplasmic reticulum to Golgi at different rates dependent upon receptor affinity for individual proteins.

C-reactive protein (CRP) is an acute phase plasma protein composed of five identical nonglycosylated subunits (Mr = 21,000); hepatic synthesis of this protein increases by as much as several responses to tissue injury or infection (25, 26). To further clarify the mechanisms of plasma protein secretion, we undertook studies of the dynamics of secretion of CRP in hepatocyte cultures prepared from rabbits manifesting differing degrees of the acute phase response. Primary hepatocyte cultures prepared from rabbits undergoing the acute phase response have been shown to synthesize and secrete CRP for 24–48 h at constant rates which were in good agreement with the broad range of rates of hepatic CRP synthesis attained in vivo (26, 27). In the present study, the time required for intracellular transport of newly synthesized CRP was found to be prolonged compared to albumin and to differ markedly depending upon the overall rate of CRP secretion.

**MATERIALS AND METHODS**

**Preparation and Maintenance of Hepatocyte Cultures—**Hepatocytes were obtained from 2–3-kg New Zealand White rabbits 15–24 h following intramuscular injection of 0.5–1 ml of turpentine into each thigh (stimulated) or from unstimulated (control) animals. Hepatocytes were prepared by a modification of a collagenase perfusion technique (28) as described previously (26). Briefly, 2–5 × 10⁶ cells in 5 ml of Williams medium E (Gibco) containing 10% fetal calf serum were allowed to attach to 60-mm culture dishes (Lux Scientific) for 1–2 h at 37°C. Following attachment, culture medium was replaced with 5 ml of serum-free Williams medium E containing 1 μM dexamethasone (dexamethasone; SDs, sodium dodecyl sulfate; Tris/saline buffer, 0.01 M Tris, 0.15 M NaCl, 0.1% NaN₃ pH 7.4; 1, liter.

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†The abbreviations used are: CRP, C-reactive protein; SDS, sodium dodecyl sulfate; Tris/saline buffer, 0.01 M Tris, 0.15 M NaCl, 0.1% NaN₃ pH 7.4; 1, liter.
methasone and insulin (0.02 unit/ml). Synthesis and secretion of CRP and intracellular CRP content in such hepatocyte cultures have been shown to remain constant for at least 24 h of incubation (28).

**Extraction of Intracellular Proteins**—Culture dishes were rinsed twice with phosphate-buffered saline and the cells in each dish were scraped in a total of 2-4 ml of Tris/saline buffer containing 1% Triton X-100, 1% deoxycholate and stored frozen. Cell lysates were thawed and sonicated (model 185 E Sonifier; Heat Systems-Ultrasonics, Plainville, NY) with the small probe at 30 watts for 1 min at 4°C. The suspensions were then sedimented at 12,000 x g for 20 min before analysis of the resulting supernatants.

**Quantitation of CRP and Albumin**—The quantity of CRP present in culture medium or in intracellular extracts was determined by radioimmunoassay exactly as described previously (26). A similar radioimmunoassay was developed for albumin. Briefly, purified rabbit albumin (Sigma) was radiiodinated by the chloramine-T technique employing IODO-BEADS (Pierce) (29). Three beads were added to 0.1 ml of phosphate-buffered saline containing 25 μg of albumin and 1 mCi of sodium NaI (New England Nuclear). Following incubation at room temperature for 5 min, the liquid was removed, ovalbumin (Sigma) was added to a concentration of 0.1%, and the sample was dialyzed versus Tris/saline buffer for 4 h at room temperature. For the purification and characterization of the iodinated products, this was accomplished by chromatography on Sephadex G-200 (Pharmacia). The resulting ^125I-albumin (approximately 2 x 10^6 cpm/μg) was employed in a sequential, double antibody radioimmunoassay as described previously for CRP (28). The first antibody was goat anti-rabbit albumin (Cappel) and the second was rabbit anti-goat IgG covalently linked to Staphylococcus aureus Ms3502 (Ab-SORB beads; Immuno-Reagents, Sequestin, TX).

**Immunoprecipitation**—Radioiodelabeled CRP and albumin were immunoprecipitated using goat anti-rabbit CRP prepared previously (30) and goat anti-rabbit albumin (Cappel). Ten μg of rabbit albumin (Sigma) and acute phase rabbit serum containing 15 μg of CRP were employed as carrier proteins. All immunoprecipitations were carried out under conditions of antibody excess as determined by precipitin curves employing radioiodinated antigens. Radiolabeled proteins present in intracellular extracts were immunoprecipitated directly by the addition of appropriate carrier proteins and antiserum. In the case of radiolabeled culture medium, Triton X-100 and sodium deoxycholate were added, each to a final concentration of 1% (v/v), prior to immunoprecipitation. In the usual case, approximately 75% of the total volumes of intracellular extract and culture medium were used for precipitation of CRP and 10% for albumin. Following incubation at 4°C overnight, precipitates were washed three times with 5 ml of Tris/saline buffer containing 1% Triton X-100, 1% sodium deoxycholate, dissolved in 50 μl of sample buffer (31) and subjected to SDS-gel electrophoresis on 12.5% gels (31). Gels were stained with Coomassie blue to identify positions of carrier CRP and albumin.

**Secretion and Secretion Kinetics**—Intracellular proteins present in intracellular extracts were immnoprecipitated by precipitin curves employing radioiodinated antigens. Radiolabeled CRP and intracellular CRP content in such hepatocyte cultures have been determined in seven of the same cultures (Table I). Secretion rates tended to diminish in cells from the most responsive animals, in agreement with the reports of others (32-34). In contrast to the findings for CRP, changes in albumin secretion were accompanied by parallel changes in intracellular albumin content, resulting in a narrow range of residence times (Fig. 1). Regardless of the degree of the CRP response, residence times for albumin were short relative to the residence time (TRP) of the culture system.

**Analysis of Pulse-Chase Labeling Kinetics of CRP and Albumin**—To help distinguish between the mechanisms which could be responsible for the long residence times for CRP found in cells from unstimulated animals, we compared the kinetics of secretion of pulse-chase labeled CRP to those of albumin.

The secretion kinetics of [^35S]CRP from hepatocytes prepared from the most highly responsive animal studied are shown in Fig. 2. In this case, labeled CRP was detected in culture medium within 30 min postpulse. Including the 15-min pulse period, synthesis and secretion of CRP required a minimum of 45 min. About 50% of pulse-labeled CRP was secreted by 60-90 min. In contrast, in the same culture, approximately 50% of labeled albumin was secreted within about 30 min. While essentially all labeled albumin was secreted by 90 min of chase, labeled intracellular CRP was still detectable after 240 min.

Quantitative analyses of kinetics of CRP and albumin secretion were carried out in three additional hepatocyte cultures prepared from rabbits manifesting differing degrees of response to inflammatory stimulus (Fig. 3). The data have been expressed as intracellular and extracellular radioactivity as a percentage of total radioactivity (the sum of both), in order to allow for direct comparison of CRP to albumin and to eliminate differences in slopes due to differences in synthetic rates. The kinetics of albumin secretion were very similar in the four cultures, as illustrated in Figs. 2 and 3, with 50% of labeled albumin appearing in culture medium within 20-40 min of chase time. In contrast, secretion of pulse-labeled CRP was increasingly rapid in cultures prepared from progressively more responsive animals. Although radioactive CRP first appeared in culture medium within 20-30 min of chase in all cultures, time required for secretion of 50% of pulse-labeled CRP ranged from well over 6 h in cells prepared from a minimally responsive animal (Fig. 3C) to approximately 75 min in the culture from the most highly responsive animal (Fig. 2).

The data in Fig. 3C were re-examined in terms of absolute radioactivity present in intracellular and extracellular CRP. Over the course of the 6-h chase period, the decrease in labeled intracellular CRP amounted to 631 cpm/10^6 cells and the increase in radioactive extracellular CRP was 640 cpm/10^6 cells, indicating there was no degradation of newly synthesized intracellular CRP during this period. Similarly, no evidence of degradation of intracellular albumin was found.
Efficiency of C-reactive Protein Secretion

TABLE I

| Serum [CRP] (µg/ml) | Secretion rate (A) (ng/10^6 cells/h) | Intracellular content (B) (ng/10^6 cells) | Ratio (B/A) | CRP |
|---------------------|-------------------------------------|------------------------------------------|-------------|-----|
| 2                   | 1.2                                 | 22                                       | 18          |     |
| 2                   | 2.3                                 | 32                                       | 14          |     |
| 15                  | 8.3                                 | 84                                       | 10          |     |
| 46                  | 8.3                                 | 92                                       | 11          |     |
| 38                  | 23                                  | 160                                      | 7.7         |     |
| 55                  | 25                                  | 150                                      | 5.8         |     |
| 90                  | 56                                  | 140                                      | 2.5         |     |
| 118                 | 62                                  | 160                                      | 2.6         |     |
| 140                 | 65                                  | 340                                      | 5.2         |     |
| 180                 | 120                                 | 380                                      | 3.2         |     |

| Serum [Albumin] (µg/ml) | Secretion rate (A) (ng/10^6 cells/h) | Intracellular content (B) (ng/10^6 cells) | Ratio (B/A) | Albumin |
|------------------------|--------------------------------------|------------------------------------------|-------------|---------|
| 2                      | 1900                                 | N.D.                                     | 1.5         |         |
| 2                      | 1100                                 | 900                                      | 0.8         |         |
| 15                     | 330                                  | 690                                      | 2.1         |         |
| 46                     | 770                                  | 780                                      | 1.0         |         |
| 38                     | 470                                  | 430                                      | 0.9         |         |
| 55                     | 630                                  | 680                                      | 1.1         |         |
| 90                     | 1300                                 | 1000                                     | 0.8         |         |

**FIG. 1.** Relationship of CRP and albumin residence times to rate of CRP secretion by hepatocytes. Ratios of intracellular content to secretion rate (residence times) for CRP (closed circles), determined in 10 cultures, and albumin (open circles), determined in 7 cultures, have been plotted against CRP secretion rates in the same cultures. Data are from those listed in Table I.

**FIG. 2.** Electrophoretic analysis of pulse-chase labeled CRP and albumin. Hepatocytes prepared from a highly responsive animal were pulse-labeled for 15 min with L-[35S]methionine (100 Ci/ml) and chased with unlabeled methionine as described under "Materials and Methods." At the indicated intervals, intracellular (I) and extracellular (E) CRP and albumin were immunoprecipitated and subjected to SDS-gel electrophoresis as described under "Materials and Methods." The gel was soaked in 1 M sodium salicylate, 1% glycerol (35) and dried prior to fluorography on Kodak XRA-5 film.

**FIG. 3.** Kinetics of pulse-chase labeling of CRP and albumin. Hepatocyte cultures were prepared from rabbits manifesting minimal (C), moderate (B), and marked (A) responses to inflammatory stimulus and were pulse-labeled with L-[35S]methionine as described in Fig. 2. Intracellular (open circles) and extracellular (closed circles) radiolabeled CRP (left panel) and albumin (right panel) were immunoprecipitated at the indicated intervals of chase time. Following SDS-gel electrophoresis, radioactivity present in sections of gel was determined directly as described under "Materials and Methods." Data for each protein are expressed as a percentage of total (intracellular + extracellular) radioactivity in CRP or in albumin at each interval. Rates of CRP secretion found in each culture (determined as described under "Materials and Methods") are indicated in the left panel.
DISCUSSION

Our major findings were that both the ratio of intracellular CRP content to secretion rate (residence time) and the halftime for secretion of pulse-labeled CRP decreased in cultures prepared from animals showing progressively greater responses to inflammatory stimulus. In contrast albumin residence times and secretion kinetics were more rapid and did not change appreciably in the same cultures. Taken together, these observations indicate that secretion of CRP becomes more efficient during the course of the acute phase response; the average newly synthesized CRP molecule requires less time to traverse the secretory pathway in cultures from more responsive animals.

Several recent studies indicated that newly synthesized secretory proteins are distributed throughout a pool of proteins within the rough endoplasmic reticulum, and that exit from this pool, with concomitant transfer to the Golgi, is a random process which occurs at rates characteristic for individual proteins (22-24). Once proteins reach the Golgi, they appear to be transported to the cell surface at the same rate (22-24), within a common vesicle (36). Transfer from rough endoplasmic reticulum to Golgi has been postulated to be a receptor-mediated process, with the rate of transfer being proportional to the affinity of the receptor (or receptors) for individual proteins. Our findings are consistent with these observations; they are best explained by the existence of an expandable intracellular pool of CRP in which newly synthesized molecules are diluted prior to secretion.

In addition, because of the acute phase nature of CRP, we were able to study kinetics of secretion of a single protein over a wide range of secretory rates and intracellular content. We found that CRP traverses the secretory pathway at different rates under different physiologic conditions. This observation is not consistent with the hypothesis that rate of exit of a secretory protein from the intracellular pool is determined by receptor affinity for that protein. Receptor affinity for CRP would not be expected to change during the course of the acute phase response, except for the unlikely possibility that CRP or the receptor could be secondarily modified under these conditions. Instead, we propose that the availability of the hypothetical receptor plays a role in regulating the rate at which CRP is transported to the cell surface.

There are at least two mechanisms by which receptor availability might be regulated. If transport from RER to Golgi occurs via receptors specific for each protein, then differences in time required for transport would be related to numbers of specific receptors. In the case of CRP, this alternative would imply increased receptor synthesis during the course of the acute phase response. On the other hand, if multiple proteins share a common receptor, then competition between proteins for such a receptor would determine the rate of transport of each protein. In this model, rate of exit from the intracellular pool would be determined by the relative concentration of each protein within the intracellular pool, as well as by differential receptor affinity for each protein.

Since CRP is a polymeric protein, it is possible that exit from the intracellular pool might be dependent upon assembly of CRP subunits. However, we were previously unable to detect CRP subunits within hepatocytes cultured from either stimulated or control animals (26), suggesting that assembly of CRP subunits is not a rate-limiting process. Our data indicate that CRP exists within the intracellular pool as the native, pentameric molecule.

Other mechanisms which could be responsible for our observations were confronted. We concluded that the possibility of intracellular degradation (37) of CRP did not play a role in regulation of CRP secretion, since the decrease in pulse-labeled intracellular CRP was accompanied by an equivalent increase in secreted, labeled CRP. If endocytosis of secreted CRP by cultured hepatocytes occurred, it would increase the apparent size of the intracellular CRP pool relative to the rate of CRP secretion and would also influence the apparent kinetics of secretory CRP. However, we have previously shown that extracellular CRP present in hepatocyte cultures persists without detectable loss over a 24-h period (26), indicating it is unlikely that endocytosis of extracellular CRP was responsible for our present observations. Finally, while the finding of a long residence time for CRP in cultures prepared from unstimulated animals could theoretically be explained by a kinetically stable intracellular storage pool which is not secreted under our experimental conditions, the kinetics of secretion of pulse-labeled CRP were prolonged compared to albumin, a finding which is not consistent with this theoretical possibility.

Considering our findings and recent studies of other proteins (22-24), we propose that newly synthesized CRP molecules are distributed throughout an intracellular pool of CRP, most likely within the rough endoplasmic reticulum. Exit from this pool is a random process in which the probability that a given molecule will be transported out of the pool is dependent upon the availability of a hypothetical receptor. Such a receptor might be specific for CRP, in which case receptor number would increase during the course of the acute phase response. Alternatively, a common receptor might mediate the transport of multiple proteins. In this case, concentration of an individual protein within the intracellular pool as well as affinity of the receptor for that protein would determine the efficiency of intracellular transport.

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REFERENCES

1. Jamieson, J. D., and Palade, G. E. (1967) J. Cell Biol. 34, 577-596
2. Palade, G. (1975) Science (Wash., D.C.) 199, 347-358
3. Rothman, J. E., and Lenard, J. (1977) Science (Wash., D.C.) 195, 743-753
4. Davis, B. D., and Tai, P.-C. (1980) Nature (Lond.) 283, 433-438
5. Hubbard, S. D., and Ivatt, R. J. (1981) Annu. Rev. Biochem. 50, 555-583
6. Lodish, H. F., Braess, W. A., Schwartz, A. L., Strous, G. J. A. M., and Zilberstein, A. (1981) Int. Rev. Cytol. Suppl. 12, 247-306
7. Sabatini, D. D., Kreibich, G., Morimoto, T., and Adesnik, M. (1982) J. Cell Biol. 92, 1-22
8. Fahruah, M., and, Palade, G. E. (1981) J. Cell Biol. 91, 77s-105s
9. Tartakoff, A. (1980) Int. Rev. Exp. Pathol. 22, 227-249
10. Rothman, J. E. (1981) Science (Wash., D.C.) 213, 1212-1219
11. Mellman, I. (1982) Nature (Lond.) 299, 301-302
12. Gonzalez-Noriega, A., Grubb, J. H., Talkad, V., and Sly, W. S. (1980) J. Cell Biol. 85, 839-852
13. Fischer, H. D., Gonzalez-Noriega, A., Sly, W. S., and More, D. J. (1980) J. Biol. Chem. 255, 9608-9615
14. Gumbiner, B., and Kelly, R. B. (1982) Cell 28, 61-59
15. Gold, G., Landahl, H. D., Gishizky, M. L., and Grodsky, G. M. (1982) J. Clin. Invest. 69, 554-563
16. Choi, M. G., and Hynes, R. (1979) J. Biol. Chem. 254, 12050-12055
17. Strous, G. J. A. M., and Lodish, H. F. (1980) Cell 22, 709-717
18. Rothman, J. E., and Lodish, H. F. (1977) Nature (Lond.) 269, 775-780
19. Morgan, E. H., and Peters, T., Jr. (1971) J. Biol. Chem. 246, 3508-3511
20. Schreiber, G., Dryburgh, H., Millership, A., Matsuda, Y., Inglis, A., Phillipa, J., Edwards, K., and Maggs, J. (1979) J. Biol.
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21. Ledford, B. E., and Davis, D. F. (1983) J. Biol. Chem. 258, 3304–3308
22. Lodish, H. F., Kong, N., Snider, M., and Strous, G. J. A. M. (1983) Nature (Lond.) 304, 80–83
23. Fries, E., Gustafsson, L., and Peterson, P. A. (1984) EMBO J. 3, 147–152
24. Fitting, T., and Kabat, D. (1982) J. Biol. Chem. 257, 14011–14017
25. Macintyre, S. S., Schultz, D., and Kushner, I. (1982) Ann. N.Y. Acad. Sci. 389, 76–87
26. Macintyre, S. S., Schultz, D., and Kushner, I. (1983) Biochem. J. 210, 707–715
27. Chelladurai, M., Macintyre, S. S., and Kushner, I. (1983) J. Clin. Invest. 71, 604–610
28. Laishes, B. A., and Williams, G. M. (1976) In Vitro 12, 521–532
29. Markwell, M. A. K. (1982) Anal. Biochem. 125, 427–432
30. Kushner, I., and Kaplan, M. H. (1961) J. Exp. Med. 114, 961–974
31. Laemmli, U. K. (1970) Nature (Lond.) 227, 680–685
32. Jamieson, J. C., Morrison, K. E., Molasky, D., and Turchen, B. (1975) Can. J. Biochem. 53, 401–414
33. Fouad, F. M., Scherer, R., Abd-El-Fattah, M., and Ruhenstroth-Bauer, G. (1980) Eur. J. Cell. Biol. 21, 175–179
34. Baumann, H., Jahreis, G. P., and Gaines, K. C. (1983) J. Cell Biol. 97, 866–876
35. Chamberlain, J. P. (1979) Anal. Biochem. 98, 132–135
36. Strous, G. J. A. M., Willemsen, R., van Kerkhof, P., Slot, J. W., Geuze, H. J., and Lodish, H. F. (1983) J. Cell Biol. 97, 1815–1822
37. Bienkowski, R. S. (1983) Biochem. J. 214, 1–10