Hemopexin Is a Developmentally Regulated, Acute-phase Plasma Protein in the Chicken*

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Identity has been established between chicken hemopexin and α1-globulin "M," a plasma protein known for the hormone responsiveness of its synthesis in monolayer cultures of embryonic chicken hepatocytes (Griepinger, G., Plant, P. W., Liang, T. J., Kalb, R. G., Amrani, D., Mosesson, M. W., Hertzberg, K. M., and Pindyk, J. (1983) Ann. N. Y. Acad. Sci. 408, 469–489). Identification was based on immunological cross-reactivity, electrophoretic behavior on sodium dodecyl sulfate-polyacrylamide gels, heme-binding capacity, and pattern of cleavage by proteolytic enzymes. Electromunoassays were used to investigate plasma protein levels, particularly those of hemopexin, in the acute-phase response and embryonic development. Acute-phase plasma protein production, elicited by injection of chickens with turpentine, bore many similarities to the pattern of hepatocellular plasma protein synthesis produced in response to the addition of specific hormones in culture. The response of the stressed chickens included elevated levels of hemopexin and fibrinogen (5- and 2-fold, respectively) accompanied by a 50% drop in albumin. Hemopexin levels of developing chick embryos were measured for several days before and after hatching. Onset of hemopexin production occurred around the time of hatching, and was followed by a steep increase (more than 1000-fold over 4 days). Similarly, it was not until the 12th h of culture that hepatocytes isolated from both early and late stage chicken embryos began to produce hemopexin, although, from their initiation in culture, they secreted a number of other plasma proteins in quantity. After 12 h, hepatocellular output of hemopexin rapidly accelerated. This precocious induction ex vivo required no hormonal or macromolecular medium supplements. These observations indicate that the embryonic chicken hepatocyte culture system will provide a useful model for studying the regulation of hemopexin biosynthesis in hepatic development and the acute-phase response.

Expression of a variety of hepatic genes is confined to specific phases of development, but only in certain cases are the factors that affect this timing understood (1–5). Studies with hepatocytes derived from chicken embryos suggest that they may provide an ex vivo system in which to clarify the critical factors involved in the transition from fetal to adult hepatic protein synthesis. Once established in monolayer culture, these embryonic cells begin to develop certain characteristics typical of adult hepatocytes, including a lack of cell division (6, 7) and adult levels of glycogen deposition (8), UDP-glucuronol transferase activity (9), and cytochrome P-450 (10). Moreover, synthesis of several plasma proteins, not produced by the embryo, is induced. Five such plasma proteins were demonstrated by crossed immunoelectrophoresis of spent culture medium using anti-adult chicken serum absorbed with embryonic serum (11). The most prominent of these is an α1-globulin which we termed "M." Chicken embryo hepatocyte culture systems have also been shown to express the adult form of antithrombin-III (12).

The fortuitous presence of anti-M antibodies in rabbit anti-albumin (Cohn Fraction V) enabled us to devise a reagent for monitoring secreted M in the hepatocyte cultures (13). It was found that accelerated production of this protein began at about 12 h of culture and continued thereafter (7, 11).

An intriguing feature of this culture system is that viable monolayers, which become induced for M synthesis, are established even in the absence of serum or hormonal supplementation of the medium (7). It has also been found that M production is highly responsive to a number of hormones, including insulin (7), glucocorticoids (14), and thyroid hormones (15). The stimulatory effect was even greater when the individual hormones were combined (14), with the secretion of M accounting for 5–10% of total plasma protein output under such conditions (6, 14).

To identify the function of α1-globulin M, we have purified it and compared it with known chicken plasma proteins. In this study, we demonstrate that M is the heme transport protein, hemopexin, and that, in addition to its being a developmentally regulated protein, it is a major participant in the acute-phase response of the chicken. Our findings suggest that, by experimental manipulation of the conditions of culture, it may be possible to discern the factors responsible for triggering major changes in hemopexin production in pathological states and during hepatic development.

EXPERIMENTAL PROCEDURES

Reagents—3,3',5-Triiodo-~thyronine (thyroid hormone or, simply, T3) (sodium salt) was obtained from Sigma as were insulin.

1 The abbreviations used are: T3, 3,3',5-triiodo-L-thyronine (thyroid hormone); HEPES, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Hx, hemopexin.

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(bovine pancreas, crystalline; 24.3 units/mg) and hemin (type 1).
Dexamethasone was purchased from Steroids, Wilton, NJ. SDS
(Sequanal grade, catalogue No. 28364) was purchased from Pierce.
Tunicamycin was from Behring Diagnostics.

Methods—Primary monolayer cultures of hepatocytes from 16-
doay-old chicken embryos were prepared as described (7, 16).
They were maintained in modified Ham’s F12 medium with un-
formed or serum supplement so that the cells were cultured from
the onset in a chemically defined medium, free of added macromo-
cules. Cultures derived from younger (12-day) or older (19-day)
embryos were prepared in a similar fashion with the aim of obtaining
similar cell densities. Culture medium was replaced with an equal
volume of fresh medium every 24 h unless otherwise indicated.

Hepatocyte monolayers in 35-mm diameter dishes were radiola-
labeled, as described previously (17), with [35S]methionine (specific
activity, ~1000 Ci/mmol) for 2 h in methionine-containing medium
(0.1 mM) buffered with 26 mM HEPES and supplemented with 150
units/ml Trasylol.

Hemopexin was affinity-purified from pooled adult chicken plasma
by the method of Tsutsui and Mueller (18) using a heme-agarose
peak was observed (not shown). The homogeneity of the
hemopexin, is highly glycosylated is indicated by the greater mo-
tivy of the polypeptide immunoprecipitated from the me-
sibly of the polypeptide immunoprecipitated from the me-
theelopexin, transferrin, and retinol-binding protein—and/or
in the first-dimension gel was
corresponding to M/hemopexin in the first-dimension gel was
by two-dimensional peptide mapping in which the single band
was

RESULTS AND DISCUSSION

The protein known as \( \alpha_1 \)-globulin M (11) was isolated from
spent medium of chicken embryo hepatocyte cultures. Puri-
ification was accomplished by immunoadsorbent column chro-
matography, using bound anti-M IgG prepared from rabbit
anti-albumin (Cohn Fraction V) antiserum that had been
previously made monospecific by absorption with embryonic
chicken serum (see "Experimental Procedures"). In analysis of the eluted protein by crossed immunoelectrophoresis with
antisera against most chicken plasma proteins, only one
peak was observed (not shown). The homogeneity of the
preparation was corroborated by electrophoresis on SDS-
polyacrylamide gels, where a single band (M, 49,500) was
obtained (Fig. 1, lane 2). When the protein was reduced with
dithiothreitol and alkylated prior to electrophoresis, its
mobility decreased, resulting in an apparent molecular weight
of 62,000 (lane 4). This dramatic shift suggests that M may be a
tightly folded protein constrained by internal disulfide bonds.

When purified \( \alpha_1 \)-globulin M and its antisera were tested against
a number of known chicken plasma proteins—including
\( \alpha_1 \)-acid glycoprotein, antithrombin III, haptoglobin, hem-
opexin, transferrin, and retinol-binding protein—and/or
their corresponding antisera (not shown), cross-reaction
was found only with hemopexin. A single band, co-migrating
with M on SDS-polyacrylamide gels, both before and after treat-
ment with dithiothreitol, was immunoprecipitated when anti-
hemopexin IgG was incubated with metabolically labeled
plasma proteins from spent hepatocyte culture medium (Fig.
1, compare lanes 5 and 7 with lanes 4 and 2, respectively).

The parallel electrophoretic behavior of the two proteins
synthesized in culture—one immunopurified with anti-M, the
other immunoprecipitated with anti-hemopexin—strongly
supports the contention that M is hemopexin. Of note, the
mobility of hemopexin purified by passing adult chicken
plasma over a heme-affinity column was slightly greater than
that of the hepatocyte culture-derived molecules (Fig. 1,
compare lanes 1 and 3 with 2 and 4), possibly as a result of either
a reduction in carbohydrate content or a slight degree of
proteolysis. That hepatocyte hemopexin, like plasma hemo-
pexin, is highly glycosylated is indicated by the greater mo-
bility of the polypeptide immunoprecipitated from the me-
dium of tunicamycin-treated hepatocytes (Fig. 1, lanes 6 and
8; see also Ref. 26).

Identification of \( \alpha_1 \)-globulin M as hemopexin was extended by
two-dimensional peptide mapping in which the single band
weakly corresponding to M/hemopexin in the first-dimension gel was
subjected to limited proteolysis with Staphylococcus aureus
V8 protease; the fragments thus generated were then sepa-

2 The plasma protein previously named "C" (11, 42) has recently
been identified as transferrin ("prealbumin") (S. Lee and G. Grien-
ger, unpublished observation).
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FIG. 1. Electrophoretic analysis of α1-globulin M and hemopexin. Affinity-purified hemopexin and immunopurified α1-globulin M were compared on a 7.5–11.4% gradient SDS-polyacrylamide gel with hemopexin immunoprecipitated from the medium of cultures that were labeled with [35S]methionine in the presence and absence of tunicamycin (Tu). A photograph of the Coomassie Blue-stained, purified proteins (Panel A) is displayed alongside an autoradiograph of the immunoprecipitated, metabolically labeled proteins (Panel B). To obtain the labeled proteins, hepatocyte monolayers were exposed at 3 and 24 h of culture to fresh medium containing 10 nM Tu, 1 nM dexamethasone, and 35 nM insulin. Treatment of monolayers with tunicamycin was initiated 3 h before radioactive labeling by adding the inhibitor to the medium (5 μg/ml) and maintaining it during the labeling period. Under these conditions, N-glycosylation of fibrinogen is completely inhibited (21). The cells were labeled at 48 h in the presence and absence of tunicamycin with 1.5 and 1.0 μCi of [35S]methionine, respectively, per 0.4 ml of medium. Two hours later, spent media were collected, and 0.01 ml was immunoprecipitated with anti-hemopexin IgG and electrophoresed. Samples separated in lanes 3, 4, 5, and 6 were reduced and alkylated prior to electrophoresis and are marked with an asterisk (*). Lanes 1 and 3*, hemopexin (5 μg) affinity-purified from chicken plasma; lanes 2 and 4*, α1-globulin M (2 μg) immunoadsorbed from hepatocyte cultures (see “Experimental Procedures”); lanes 5* and 7, immunoprecipitated hemopexin from untreated cultures; lanes 6* and 8, immunoprecipitated hemopexin from tunicamycin-treated cultures. The numbers on the left and right side of the panels indicate molecular mass in kilodaltons.

rated in the second-dimension gel. Immunoadsorbed M and immunoprecipitated hemopexin from the cultures gave rise, by this procedure, to peptide maps which were virtually indistinguishable from each other or from that of hemopexin affinity-purified from adult chicken plasma (Fig. 2, compare tracks 1–3). Nonglycosylated hemopexin from tunicamycin-treated cells had a much different partial peptide map (track 4), except in the lowest region of the gel, suggesting that normally all but the smallest fragments bear carbohydrate residues.

The function of hemopexin in the blood stream is that of a heme transport molecule, preventing the urinary excretion of heme and facilitating the conservation of iron (27–29). Chicken plasma-derived hemopexin binds heme in an equimolar ratio as does hemopexin from other species (19). Titration of the heme-binding capacity of the immunoadsorbed hepatocyte M preparation also indicated a molar ratio of heme to protein of 1:1 (Fig. 3). Intersection of the titration curve with the ordinate (0 addition of heme) further suggests that 62% of the M molecules purified from spent culture medium contain heme.

The binding of heme, it has been found, protects plasma hemopexin to a degree from proteolytic attack by trypsin (19, 31, 32). Under our assay conditions, for example, heme-hemopexin (i.e. plasma-derived protein, saturated with heme) remained largely intact, with about 20–30% of the molecules suffering only a small loss of approximately 7,000 daltons (Fig. 4, compare lanes 2 and 3). The apoprotein, in contrast, was proteolyzed to at least half a dozen lower molecular weight fragments, ranging in size from 40,000 to 14,000 (lanes 4 and 5). Similar patterns were generated with α1-globulin M isolated from culture. Treatment of fully loaded heme-M produced two high molecular weight bands (lanes 6 and 7), co-migrating with those obtained with heme-hemopexin. However, due to the partially (62%) loaded nature of the M preparation as isolated (see Fig. 3), its treatment generated a profile that was a composite of the higher and lower molecular weight bands (lanes 8 and 9).

Thus, on the basis of immunological cross-reactivity, electrophoretic behavior on SDS-polyacrylamide gels, heme-binding capacity, and pattern of cleavage by proteolytic enzymes, we have concluded that α1-globulin M is hemopexin. Henceforth, we will drop the term α1-globulin M in referring to this protein.

Clinical studies indicate that serum hemopexin increases 10–100-fold in humans from the fetal to the adult level (33). In mice, it has been shown that hemopexin synthesis increases as part of the acute-phase response to injury or stress (34, 35). However the agents and mechanisms involved in the regulation of hemopexin synthesis in vivo have yet to be defined. Because of the demonstrated hormone sensitivity of hemopexin production in chicken embryo hepatocyte cultures (14), we have begun to investigate this system's potential as a model for studying the regulation of hemopexin synthesis. In this context, we have characterized the acute-phase and developmentally altered hemopexin levels that occur in the
peak 4; was immunopurified from spent culture medium as described under “Experimental Procedures” and titrated with heme as follows: Hemin (type I) was dissolved in 0.1 N NaOH and diluted with 50 mM sodium phosphate, 100 mM NaCl, pH 7.4. The concentration of heme was measured spectrophotometrically in 40% dimethyl sulfoxide using a millimolar extinction coefficient of 180 at 400 nm (30). The binding of heme to M/hemopexin was determined by adding increasing amounts of heme to two cuvettes, one containing phosphate buffer alone and the other containing the protein solution (44.7 pg in 1.0 millimolar extinction coefficient of 180 at 414 nm). For determining the molar ratio of binding, a molecular weight of 62,000 has been used for M/hemopexin as derived from Fig. 1, which compares well with the one estimated by gel filtration (19). After extrapolation to 0 AOD411, it follows that when saturation is reached, 0.70 nmol of heme is bound to 0.72 nmol of protein.

Chicken and compared them with the changes in hemopexin production elicited in culture. Prior to this report, the acute-phase response of the chicken had been characterized only in terms of changes in the electrophoretic profile of serum proteins (36).

To assess the acute-phase response, blood samples were withdrawn from chickens (3.5 months) for several days following subcutaneous injection with turpentine. This standard method of experimentally eliciting the acute-phase response brought about dramatic differences in the plasma protein profile, as illustrated by crossed immunoelectrophoresis (Fig. 5). Secretion of a few plasma proteins (e.g. “Z”) was not affected. Hepatocellular production of the remainder evidenced either a temporary enhancement (e.g. hemopexin, peak 6; fibrinogen, peak 21) or a temporary reduction (e.g. albumin, peak 4; transthyretin, peak 2). The 5-fold increase in hemopexin was accompanied by a nearly 2-fold elevation of fibrinogen and a more than 50% drop in albumin (Fig. 6). These apparently compensating changes, which have also been noted in the acute-phase response of other animal species (37-39), reached their maximum 2-3 days following injection of the irritant.

It has been shown for other species that many of the changes originate in altered rates of hepatic biosynthesis of these proteins (34, 35, 38-41); however, no single agent has yet been shown to mimic completely the pattern of acute-phase plasma protein production by its action on liver cells in culture. Using as a base line the low level output of plasma proteins by the chicken embryo hepatocytes cultured in the absence of hormones, serum, or other macromolecular supplement, we have measured the effects of the controlled addition of physiological concentrations of several hormones, including thyroid hormones, glucocorticoids, and insulin, on plasma protein production. In each case, production of hemopexin (M) was as much or more responsive to stimulation than that of the other plasma proteins assayed, whether the hormones were added individually or in combination (14). All three together, for example, elicited a 7.2-fold increase in hemopexin secretion as compared with 2.7-, 2.6-, and 1.4-fold stimulation for that of fibrinogen, albumin, and transferrin, respectively. Interestingly, the pattern of production elicited by the combination of only glucocorticoids and thyroid hormones most closely resembled that seen in acute-phase chickens: 5.3- and 4.8-fold enhancements of hemopexin and fibrinogen, respectively, with no increase in albumin. A direct link between these culture conditions and the hormonal status of the traumatized animal has not been established; however, further studies along these lines may ultimately resolve the critical balance of factors upon which the hormonal milieu in vivo is built in both normal and pathological states.

Our previous studies have shown that although hemopexin is present in normal adult chicken serum, it is absent from the serum of 17-day-old chicken embryos (11). To pinpoint the induction of hemopexin in vivo, serum hemopexin levels of developing chicks were evaluated daily before and after hatching (Fig. 7A). No hemopexin was detected by electroimmunoassay in samples of undiluted chicken embryo serum taken throughout the 5 days prior to hatching. An abrupt change occurred on the day of hatching; however, initiating a steady rise in the level of hemopexin over the 4 subsequent days to achieve a concentration of 150 µg/ml or half the level...
FIG. 5. Crossed immunoelectrophoresis of proteins in chicken plasma before and during the acute-phase response. Four chickens (3.5 months old) were injected subcutaneously with turpentine (0.5 ml per kg of body weight) in the scapular area. Blood samples were drawn from the wing vein and collected in heparin, and plasma was prepared. Plasma samples, drawn from one representative chicken on day 0, day 3, and day 10 following turpentine injection, were analyzed by crossed immunoelectrophoresis. Samples (3 μl) were placed in the appropriate well in the lower left corner of each panel. Electrophoresis in the first dimension was performed from left to right and in the second dimension from bottom to top. The second-dimension gel (antibody-containing) contained a mixture of antisera, similar to that used previously (42), which recognizes most chicken plasma proteins. Immunoplates were stained with Coomassie Blue. In this assay, the amount of each plasma protein is reflected by the intensity and area of its respective peak (11). Several peaks have been identified with the use of specific antisera or purified antigens and are numbered according to Ref. 42. Peak 2, transthyretin (previously termed "prealbumin C"); peak 4, albumin; peak 6, hemopexin; peak 17, α1-antitrypsin; peak 19, transferrin; peak 21, fibrinogen; peak 25, chicken immunoglobulins.

FIG. 6. Hemopexin, fibrinogen, and albumin levels during the acute-phase response. Hemopexin, fibrinogen, and albumin were quantified by individual electroimmunoassays of plasma samples drawn from the turpentine-injected chickens of Fig. 5 on the days indicated. Values, plotted as percent of normal plasma level, represent measurements derived from four animals (mean ± S.D.).

FIG. 7. Hemopexin and albumin levels in the serum of chick embryos and young hatchlings. Blood was taken, as described under "Experimental Procedures," from chick embryos and young hatchlings on the days indicated, and serum was prepared. Hemopexin and albumin were determined by electroimmunoassay of serum samples. The dashed line indicates average time of hatching. Days numbered to the left of the line correspond to the length of time the fertilized eggs were incubated, whereas those to the right represent the age of the hatchling chick. Values, plotted as μg/ml of serum, represent measurements derived from four animals (mean ± S.D.). A, hemopexin; B, albumin.

found in fully grown chickens (3.5 months old). Based on the limit of detection of our electroimmunoassay (0.05 μg/ml), the serum hemopexin level of the 4-day-old chicks represents a more than 1000-fold increase over embryonic levels. During the period of most dramatic change for hemopexin, albumin levels increased by only a factor of three (Fig. 7B), in accord with the findings of others (43).

Developmental induction of hemopexin production at hatching bears some similarities to the changes observed in rat liver enzyme activities around the time of birth (reviewed in Ref. 1). Based on rapidity and magnitude, the elevated levels of hemopexin most likely reflect an increase in hemopexin mRNA. In this context, we note that there is a rapid accumulation of mRNA for contrapsin (a plasma protein of the anti-chymotrypsin family) on a comparable scale in the developing mouse embryo liver just prior to birth (3, 4).
hemoproteins and to establish their relationship to the precocious induction of UDP-glucuronyl transferase in these cells. At the end of the 1st and 2nd day of culture, respectively.

The induction of hemopexin in chick embryos was induced to a degree \( \text{ex vivo} \) by placing embryonic chicken liver cells in culture (11). Although the cultured cells produced a number of plasma proteins in quantity (6, 7), their secretion represented a derepression after the constraint of the embryonic environment has been removed. Preliminary experiments suggested that the constraint could be imposed by a hormonal factor such as glucagon. Addition of physiological concentrations of glucagon alone to the medium, early in culture, strongly inhibited hemopexin output. 4

In conclusion, our studies have shown that cultured chicken embryo liver cells mimic hepatic behavior with respect to hemopexin production in terms of both its induction and its high degree of sensitivity to stimulation. Using this model system, it may be possible to dissect the mechanisms underlying these phenomena and to establish their relationship to hematocyte development and the acute-phase response in the intact animal.

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