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Synthesis of the growth hormone-regulated rat liver anti-protease GHR-P^{63} is inhibited by acute inflammation

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Synthesis of the growth hormone-regulated anti-protease GHR-P^{63} by rat hepatocytes was strongly reduced during acute inflammation. This decrease was detected 8 h after the onset of inflammation and reached a maximum after 24 h. A decrease in the GHR-P^{63} mRNA level measured by in vitro translation and by hybridization mainly accounted for the alteration of GHR-P^{63} synthesis. Besides this major pretranslational mechanism, inflammation also interfered with GHR-P^{63} synthesis at a posttranslational level. This was indicated by the production of abnormal immunoprecipitable species at early stages of the acute-phase response.

Anti-protease; Inflammation; Hepatocyte; (Rat)

1. INTRODUCTION

Acute inflammation is rapidly followed by dramatic changes in liver synthesis and blood concentrations of a group of proteins called acute-phase proteins [1–3]. There are both positive and negative acute-phase proteins. In the rat, hepatic rates of synthesis of α_1-acid glycoprotein [4], α_2-macroglobulin [4], β-chain of fibrinogen [1,2], transferrin [4], α_1-proteinase inhibitor [4], angiotensinogen [5] and T-kininogen [6] were shown to be increased during the acute-phase response. Conversely, rates of synthesis of albumin, transthyretin, α_2 u-globulin [7,8] and that of the phosphoprotein PP^{63} [9] were diminished. Although the details of biochemical mechanisms are not yet fully understood, it has been established that variations in gene expression (i.e. pretranslational control) mainly accounted for the changes in liver production of these proteins [8]. Two types of effectors have so far been implicated. The first is represented by protein factors of the monokine family: hepatocyte stimulating factor [10,11] and interleukin-1 [12,13] and the second by glucocorticoid hormones [7].

We recently described an anti-protease secreted by rat hepatocytes whose synthesis was totally abolished by hypophysectomy (Le Cam et al., submitted). Here, we show that this protein (GHR-P^{63}) is also affected by acute inflammation, in a negative manner.

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Abbreviation: GHR-P^{63}, growth hormone-regulated 63-kDa protein

2. MATERIALS AND METHODS

2.1. *Chemicals*

L-[^{35}S]Methionine (1300 Ci/mmol), [α-^{32}P]-dCTP (3000 Ci/mmol) and the nick-translation DNA labelling kit were from Amersham, England. The mRNA-dependent rabbit reticulocyte lysate translation system was bought from Promega-Biotec (Madison, WI) and used according to the instructions provided by the supplier. Protein A-Sepharose CL-4B was from Pharmacia, Triton

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X-405 from Fluka and tunicamycin from Boehringer, Mannheim.

2.2. *Isolation of hepatocytes, incubation conditions and preparation of samples for analysis*

Hepatocytes were isolated from adult male rats (150–200 g) fed ad libitum by using the procedure in [14]. Inflammation was induced by a single subcutaneous injection of turpentine (0.5 ml/100 g) in the dorsal region. The time of injection was chosen so that liver cell isolation could be performed between 8 a.m. and 11 a.m., regardless of the duration of inflammation (0–24 h). Cell suspension viability ranged from 90 to 95% and was similar for control and treated animals. The procedures used to incubate hepatocytes for labelling with [^{35}S]methionine and to prepare the samples (cellular extracts and incubation media) for analysis were as described [9,15].

2.3. *Immunoprecipitation of GHR-P^{63}*

Precursor and mature forms of the protein present in cellular lysates, cell incubation medium and translation products were immunoprecipitated using the indirect immunoprecipitation procedure in [9]. Monospecific GHR-P^{63} antibodies were obtained as described elsewhere (Le Cam et al., submitted).

2.4. *Isolation of RNA, in vitro translation and Northern blot analysis*

Total cellular RNAs were extracted from freshly isolated hepatocytes (50×10^6) by the method of Chirgwin et al. [16] and used for mRNA in vitro translation [15]. The amounts of RNAs obtained from hepatocytes prepared at various times after the onset of inflammation were comparable and ranged from 30 to 40 μg/10^6 cells. Total RNAs were fractionated by electrophoresis in a 1% agarose gel that contained 1 M formaldehyde and transferred to nitrocellulose paper [17]. They were then hybridized to a ^{32}P-labelled nick-translated GHR-P^{63} cDNA probe (620 bp, spec. act. $1.5–2 \times 10^8$ cpm/μg DNA) (Le Cam et al., submitted).

2.5. *Polyacrylamide gel electrophoresis*

Proteins were analyzed by one-dimensional [18] and two-dimensional [19] gel electrophoresis as in [15]. The sizes of the precursors indicated on figures have been deduced from cDNA sequence analysis (Le Cam et al., submitted).

3. RESULTS

3.1. *GHR-P^{63} synthesis in inflamed rat hepatocytes*

To analyze the changes elicited by acute inflammation in the capacity of hepatocytes to synthesize

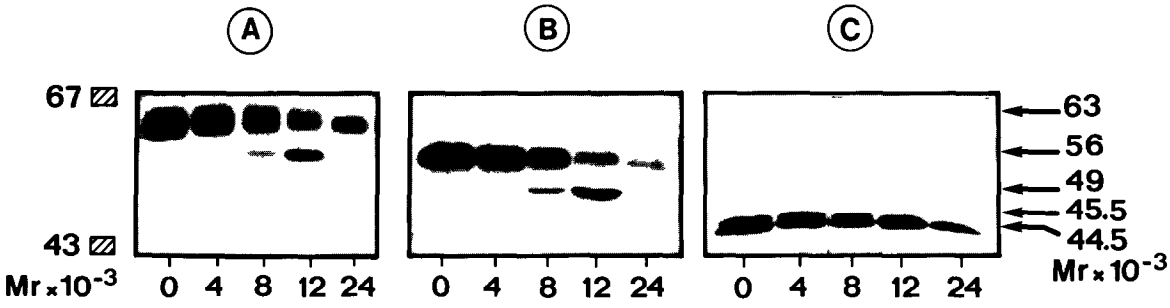

Fig.1. Variations in amounts of immunoprecipitable GHR-P^{63} related species synthesized by hepatocytes during inflammation. Hepatocytes isolated from rats treated with turpentine for various times were preincubated for 30 min without (A,B) or with tunicamycin (1 μg/ml) (C) before labelling for 90 min with [^{35}S]methionine (200 μCi/ml). GHR-P^{63} was then immunoprecipitated from incubation medium (A) and from cellular extracts (B,C) and analyzed by one-dimensional gel electrophoresis. Migration of the M_r markers (bovine serum albumin, M_r 67000; ovalbumin, M_r 43000) is shown on the left, and the size of the various GHR-P^{63} species on the right. Within each compartment (i.e. intracellular and extracellular), the same number of trichloroacetic acid-precipitable counts were used for immunoprecipitation, for all inflammatory conditions. The autoradiograms therefore visualize the actual variations in relative rates of synthesis of each GHR-P^{63} related species.

and to secrete GHR-P[63], the various ^{35}S-labelled immunoprecipitable forms were resolved by one-dimensional gel electrophoresis (fig.1). Both quantitative and qualitative modifications were observed. A decrease in the amounts of the 63-kDa mature protein which was the only species normally secreted by the cells was detected after 8 h turpentine treatment (panel A). The magnitude of this effect increased with the duration of inflammation to reach a maximum (≈80%) after 24 h. Concomitantly, a 59-kDa protein, barely detectable in the incubation medium of control cells, was secreted by inflamed rat hepatocytes (panel A). The amount of this 59-kDa species released in the medium increased up to 12 h and declined thereafter. Production of the major intracellular precursor form of GHR-P[63] (≈56 kDa) varied according to the same kinetics as that of the 63-kDa secreted species, during inflammation (panel B). Similarly to what was observed for secreted material, a new intracellular 49-kDa species was produced by inflamed cells with a maximum after 12 h inflammation. Two-dimensional gel analysis revealed that the 'abnormal' intracellular (≈49 kDa) and extracellular (≈59 kDa) proteins generated by inflamed rat hepatocytes displayed the same charge heterogeneity and had isoelectric points similar to those of their normal counterparts (unpublished).

In previous studies we found that tunicamycin treatment of hepatocytes to block *N*-glycosylation reactions [20] caused the accumulation of two unglycosylated intracellular precursors similar in size to those generated by in vitro mRNA translation (Le Cam et al., submitted). Accordingly, the amount of these precursors present in the cell can be taken as an index of the level of mRNA actually translated by the cell machinery. The data presented in panel C show that following inflammation, their production decayed in a time-

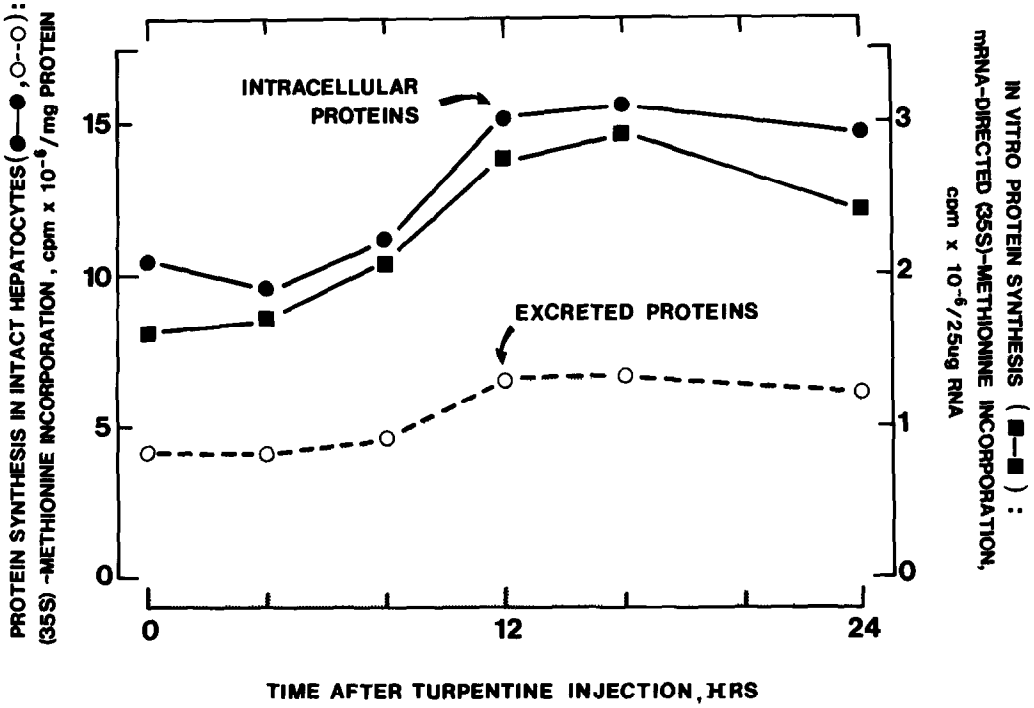

Fig.2. Analysis of the total protein synthesis capacity of hepatocytes and of the in vitro translational activity of mRNAs at various times after turpentine injection. Hepatocytes were labelled for 90 min with [^{35}S]methionine (200 μCi/ml) and the radioactivity precipitated by 10% (w/v) trichloroacetic acid associated with intracellular (●—●) and secreted proteins (○---○) was measured. 25 μg cellular RNAs extracted from isolated hepatocytes were used for in vitro mRNA translation. Trichloroacetic acid-precipitable ^{35}S-labelled material present in translation products was measured (■—■). All values are the mean of triplicate determinations.

dependent manner comparable to that found for the normal intracellular and secreted forms of the protein. In addition, at the latest time (i.e. 24 h turpentine treatment) the highest molecular mass precursor was affected more dramatically than the smallest one.

To rule out the possibility that variations in GHR-P^{63} synthesis could merely reflect a decrease in overall protein synthesis capacity, we measured [^{35}S]methionine incorporation in proteins in intact cells and in in vitro mRNA translation products (fig.2). A 40–50% increase in trichloroacetic acid-precipitable counts associated with intracellular proteins was observed after 8–12 h inflammation and persisted over 24 h. The same type of variation was detected for secretory proteins which represented 25–30% of the proteins synthesized by the cells regardless of the duration of turpentine treatment. Consistent with these observations, the translational activity of the whole mRNA population extracted from hepatocytes tested in an in vitro system followed the same evolution (fig.2).

3.2. *Effect of inflammation on the level of translatable and hybridizable GHR-P^{63} mRNA*

Regardless of the duration of turpentine treatment of the rat, in vitro translation of GHR-P^{63} mRNA in a heterologous system gave rise to the same two precursor polypeptides (≈46.7 and 45.5 kDa), in a roughly constant ratio (fig.3A). Both the kinetics and magnitude of the decay in relative amounts of GHR-P^{63} mRNA present in hepatocytes and monitored by this method closely correlated with those found for the capacity of intact cells to synthesize the protein (see fig.1). By Northern blot analysis, a single 1.8 kb mRNA species shown to encode GHR-P^{63} precursors was found in control hepatocytes (Le Cam et al., submitted). During inflammation this mRNA was strongly diminished in a time-dependent manner similar to that reported above for cellular synthesis of GHR-P^{63} (fig.3B). Another mRNA of 2.2 kb, virtually undetectable in control cells, was induced during inflammation. This species was unlikely to be related to the 1.8 kb mRNA species since it did not hybridize to a shorter (230 bp) GHR-P^{63} cDNA probe we recently obtained (unpublished).

4. DISCUSSION

Our present results show that the rat liver protein GHR-P^{63} is an acute-phase protein. The decreased capacity of hepatocytes to produce the

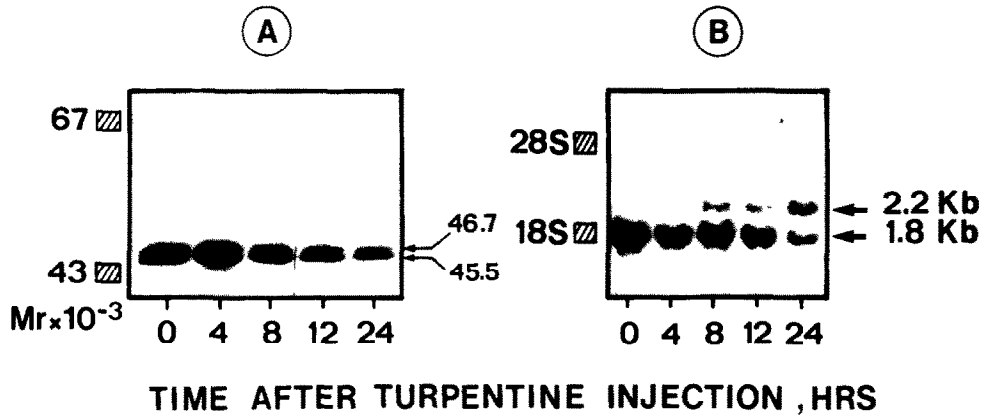

Fig.3. Variations in translatable and hybridizable GHR-P^{63} mRNA during inflammation. (A) In vitro translation. mRNAs contained in 25 μg total RNAs were translated in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine. For all inflammatory conditions tested, the same number of trichloroacetic acid-precipitable counts (i.e. 10^6) was used for immunoprecipitation of GHR-P^{63} precursors which were resolved by one-dimensional gel electrophoresis. Migration of M_r markers (as in fig.1) is indicated on the left and the size of the two precursors is shown on the right. (B) Northern blot analysis. 25 μg total RNAs were fractionated by electrophoresis on a 1% agarose gel under denaturing conditions. After transfer to nitrocellulose paper, they were hybridized to a ^{32}P-labelled GHR-P^{63} cDNA probe at 42°C for 16 h. The migration of 28 S and 18 S ribosomal RNAs indicated on the left was determined on samples run concomitantly, transferred to nitrocellulose and stained with methylene blue.

protein during inflammation resulted from specific impairment in its rate of synthesis. This appeared to involve mainly a pretranslational control mechanism which is similar to what has been described for other acute-phase proteins [8]. Indeed, the decrease in GHR-P^{63} mRNA level measured by in vitro translation and by hybridization was sufficient to account for the diminution in the capacity of hepatocytes to synthesize the protein. Nonetheless, the mRNA present in inflamed cells appeared qualitatively identical to the species found in control hepatocytes since the precursors immunoprecipitated from translation products generated in a heterologous in vitro system were analogous. Besides this major effect on mRNA level, inflammation was found to affect GHR-P^{63} synthesis posttranslationally, mainly at early stages. Generation of intracellular and secreted forms of the protein that were abnormal with respect to molecular masses suggested that glycosylation of precursors was somewhat impaired.

Considering that GHR-P^{63} is an anti-protease (Le Cam et al., submitted), the finding that it is a negative acute-phase protein may appear paradoxical. Indeed, the major anti-proteases produced by the liver and affected by acute inflammation (e.g. α_1-antitrypsin, α_1-antichymotrypsin, α_1-proteinase inhibitor, α_2-macroglobulin [4] and mouse contrapsin [21]) were all shown to be positively regulated. At the moment, no explanation can be offered for this difference. Clearly, GHR-P^{63} gene expression in liver is tightly controlled, both positively and negatively. Inflammation is a situation where negative control presumably exerted by monokines [10,11,13] appears to prevail. Conversely, the effect of hypophysectomy on its synthesis (Le Cam et al., submitted) along with our recent observation that growth hormone and glucocorticoids could induce the protein both in vivo and in vitro (Pagès et al., in preparation) suggest that these hormones exert a positive control. GHR-P^{63} appears to represent a remarkable model to study the regulation of gene expression in liver.

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