Control of γ-Glutamyl Transpeptidase Expression by Glucocorticoids in the Rat Pancreas

CORRELATION WITH GRANULE FORMATION*

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Glucocorticoids are known to promote the formation of zymogen granules in acinar cells of the exocrine pancreas in vivo as well as in vitro. To gain insight into the mechanism of this regulation, we studied the effects of glucocorticoids on the synthesis of two components of the secretory granule membrane, the glycoprotein 2 (GP-2) and the γ-glutamyl transpeptidase (GGT). It was demonstrated that following adrenalectomy, degranulation of pancreatic acinar cells is accompanied by a sharp decrease in GGT and GP-2 synthesis as measured by mRNA and protein accumulation. The decline of GGT synthesis was prevented by glucocorticoid replacement therapy, whereas GP-2 synthesis could be maintained with either glucocorticoid or estradiol treatment. These in vivo observations were corroborated and extended in an in vitro study using AR42J pancreatic cells. With this cell line, it was demonstrated that dexamethasone induces the formation of zymogen granules and the accumulation of a specific GGT transcript (mRNA III) by decreasing its degradation rate. At the same time, the GP-2 mRNA level was not modified by the hormonal treatment. These data demonstrate that glucocorticoids exert a positive control on the GGT expression in pancreatic cells at a post-transcriptional level. GGT, an enzyme of the glutathione metabolism, could play a significant role in protein packaging in secretory cells.

In eukaryotic cells, newly synthesized secretory proteins are sequestered in the rough endoplasmic reticulum and delivered to the Golgi apparatus. At the trans-side of the Golgi, most of the secretory proteins are packaged into secretory granules, a storage compartment. Upon stimulation, the granules move to the cell apex and release their content by exocytosis (1, 2).

Despite a considerable amount of progress in our understanding of the intracellular trafficking of secretory proteins, the mechanisms by which they are packaged and maintained in an osmotically inactive form in the zymogen granule (ZG) is unknown. Possible clues for the elucidation of these processes come from experiments which reported that adrenalectomy of male rats caused a marked depletion of ZG in the pancreas acinar cells, an effect which was reversed by treatment with the synthetic glucocorticoid, triamcinolone (3, 4). It was also found that a dexamethasone treatment of the AR42J rat pancreatic cells induces the appearance of ZG (5). These observations led us to the hypothesis that glucocorticoids were directly involved in the packaging of secretory proteins and ZG formation.

Glucocorticoids could influence the packaging of secretory proteins and ZG formation through the ZG membrane protein content. The latter membrane is a highly specialized membrane; it contains, roughly, 10 major proteins and shows a rather limited spectrum of polypeptides (6, 7), with a significant overlapping composition to membranes from other exocrine glands, irrespective of the nature of stored proteins (8). The glycoprotein 2 (GP-2) is one of the major components of this membrane; it is a glycosyl phosphatidylinositol-anchored protein (9, 10) that is released in the pancreatic ducts upon cleavage with a phosphatidylinositol-phospholipase. The released GP-2 polymerizes and forms a fibrillar network (11, 12). The physiological role of GP-2 is not yet defined, but this fibrillar network may form a barrier to colonization of the pancreatic duct by intestinal bacteria. In addition, recent studies have shown that GP-2 shares some antigenic determinants and structural homology with a phospholipase A2 (13). γ-Glutamyl transpeptidase (GGT) is another glycoprotein component of the secretory granule membranes, which has been used as a marker to follow their isolation by subcellular fractionation (8). In the exocrine pancreas, we localized GGT, which is a type II membrane protein, in the ZG membrane, the apical membrane and pancreatic juice (14) where it is associated with "pancreasomes" (12, 15). GGT hydrolyzes glutathione, which may affect the disulfide bridges of the proteins with high cysteine content, like GP2, along the secretory pathway (16). Biochemical and immunocytochemical observations have indicated that both GP-2 and GGT are continuously released by the pancreas acinar cell in the pancreatic juice, and, hence, a substantial fraction of these proteins do not follow its host membrane that recycles after exocytosis (11, 12, 15). This implies a de novo synthesis of these proteins and their insertion into the recycled membrane as new granules form. If these proteins are essential for protein packaging and ZG formation, therefore, one could postulate an increase in their rate of synthesis when ZG formation is induced by glucocorticoids (4).

The study of the expression of these two very different mem-

line; kb, kilobase; bp, base pair(s); GSH, glutathione; GSSG, glutathione disulfide.
brane proteins is now greatly facilitated as a result of cloning and sequencing of their respective cDNAs (10, 17). In the rat, 
GGT is encoded by a single copy gene (18) transcribed into 
several mRNA species (mRNAs I to V) according to the tissues 
(19–23). The GP-2 protein is also encoded by a single copy gene, 
but only one species of mRNA is transcribed (10, 24). In this 
work, we show that in acinar cells, glucocorticoid treatment induces in parallel ZG formation and GGT expression. The 
GGT regulation occurs after a post-transcriptional level since 
the glucocorticoids induce the accumulation of the GGT mRNA III transcript by decreasing its degradation. We also provide evi-
dence that ZG formation can be induced independently of GP-2 expression.

**EXPERIMENTAL PROCEDURES**

**Animals and Treatments—**Male Sprague-Dawley rats, weighing approx-
imately 200 g, were used for these experiments. The animals were 
maintained on 0.9% saline drinking solution in addition to standard 
chow. They were divided in four groups of 10 animals, 1) sham-oper-
ated, 2) adenaledenized, 3) adenaledenized with a dexamethasone 
replacement therapy, 4) adenaledenized with an estradiol replace-
ment therapy. The experiments lasted 5 days starting with surgery on 
day 0. The following day, the rats on a replacement therapy received the hormone and the animals remained immobile. The rats were sac-
rificed on the 5th day. For the glucocorticoid replacement therapy, the 
animals were given 0.09 mg of dexamethasone in propylene glycol 
(Organon, Toronto, Canada), and for the estradiol replacement therapy, the animals received 0.042 mg of estradiol in propylene glycol (Sigma) 
as daily subcutaneous injections. The rats were decapitated and the 
pancreases quickly excised. Small pieces of tissues chosen at random in 
different regions of the gland were immediately fixed for morphological 
observations or used for GGT assay or RNA preparation. Livers were 
also excised from 18-day-old fetuses and from adult rats. Kidneys were 
obtained from adult rats and mammary glands from lactating female 
rats.

**Cell Culture—**The AR42J pancreatic carcinoma cells (CRL 1492) 
were obtained from the ATCC (Rockville, MD). The cells were plated at 
a density of 1.5 × 10^6 cells per 10-cm dishes and grown for 4 days in 
improved Ham’s F12 medium (Life Technologies, Inc., Cambridge, 
U. K.), supplemented with 10% fetal calf serum, 200 units/ml penicillin, 
50 μg/ml streptomycin, and 0.5 μg/ml fungizone, before different peri-
ods of treatment by 10 or 100 nm dexamethasone. In some experiments, 
the AR42J cell line RUI-38486 (gift from Rousset-Uclaf, France) was 
added to the dexamethasone-treated cells at a 0.5 μg/ml concentration. 
Cells were lysed directly into 1.5 ml of 4 M guanidinium thiocyanate per 
dish for RNA extraction or washed twice in cold PBS and scrapped for 
GGT assay, Western blot analysis, or nuclei purification.

**Electron Microscopy—**Cell pellets and pieces of pancreas tissues were 
processed for electron microscopy as described previously (4).

**GGT Assay—**Tissues were homogenized in water with a Potter-
Elvehjem, and AR42J cells were washed, scraped, and suspended in 
PBS. The GGT activity was determined according to Orlowski and Meister (25) and expressed as unit/mg protein. One unit corresponds to 
1 μmol of product formed per min at 37 °C. Proteins were assayed 
according to Bradford (26).

**Western Blot Analysis of GP-2—**Pancreases were homogenized with a 
Polytron homogenizer (Brinkman Instruments) in 45 mM Tris saline 
buffer, pH 7.6, and centrifuged at 700 × g for 15 min in a Beckman J-A 20 rotor. Supernatants were decanted and centrifuged for 60 min at 
100,000 × g in a Beckman 50 Ti rotor. The pellets were suspended in 
5 mM Tris-HCl, pH 7.6, washed, and resuspended in PBS; pellets were 
suspended in 1% SDS and boiled for 5 min. Proteins were estimated 
according to Bradford (26). Samples (100 μg) were resuspended in loading buffer and separated by SDS-polyacrylamide gel electrophoresis according to Laemmli (27). Proteins were stained with Coomassie Blue and GP-2 immunoblotting carried out as described 
previously (18).

**RNA Preparation—**Total RNA was extracted from rat tissues or 
AR42J cells using the guanidinium thiocyanate procedure (29). Poly(A)^+ 
RNA were obtained by two successive purifications on an oligo(dT) 
column as described previously (19), or using the microspin oligo(dT) 
column (RNA-Flash from Bioprobe Systems, Montrouge-sous-bois, 
France) when the amount of total RNA was less than 2 mg.

**Northern Blot Analysis—**The poly(A)^+ RNA samples were resolved on 
an 1.5% agarose denaturing gel, transferred onto a nylon membrane 
(Hybond N, Amersham, France), and hybridized to GGT and GP-2 
probes as described previously (19). Blots were also hybridized to a 
β-actin probe to assess the amount of sample loaded on the gel.

The labeled GGT complementary RNA (cRNA) probes, which hybrid-
ize specifically to the GGT mRNA I (cRNA-17), mRNA II (cRNA-12), 
mRNA III (cRNA-3), or to all the GGT mRNA types (cRNA-139A), were 
synthesized by in vitro transcription of the GGT plasmids pGEM-17, 
FL2, or pGEM4. The cRNA probes described for FL2 (10, 24) and 
Sadm (FL2 and pGEM4-139) prior to their transcription from the T7 
promoter in the presence of [α-32P]UTP, except for FL2 which was 
transcribed from its SP6 promoter. The GP-2 cRNA probe was obtained 
from a GP-2 recombinant plasmid (10) kindly provided by Dr. C. 
Roussel-Uclaf. The coding sequence from the nucleotide 174 to the nucleotide 1607, was subcloned into pGEM-4 (Promega, Madison) and digested 
by EcoRI and BamHI. The recombinant plasmid was then linearized by 
EcoRI and transcribed from its T7 promoter. The β-actin probe was 
obtained by labeling the 800-bp HindIII-KpnI cDNA fragment (30), using 
the megaprime labeling kit (Amersham, France) in the presence of 
50 μCi of [α-32P]UTP.

**Run-on Experiments—**Nuclei were prepared as described previously 
(31). Nascent RNA transcripts were labeled with [α-32P]UTP, isolated, 
and quantitated according to Antras et al. (32). The labeled RNA (10 × 
10^6 cpm) was hybridized to an Hybrid membrane where either 10 μg of 
control pGEM-3Zf(-) DNA (Promega, Madison) or 3 μg of GP-2 cDNA, 
2 μg of amylase cDNA, and 1.7 μg of a 470-bp GGT genomic sequence 
were immobilized. This latter 470-bp GGT sequence was obtained 
from the plasmid pGEM-III (22); it contains the 58 nucleotides at the 
3’end of the GGT mRNA III leader exon and extends 412 nucleotides 
downstream in the first intron. The 0.9-kb amylase cDNA sequence 
deduced into the plasmid pBR322 (33) was kindly provided by Dr. R. J. 
MacDonald. After hybridization, the blots were autoradiographed, 
and densitometric quantification of the signals was carried out using a 
Laser densitometer Ultrascan XL (Pharmacia Biotech, Inc.).

**Measurement of the GGT mRNA III Degradation Rate—**The AR42J 
cells were incubated for 48 h in the presence or in the absence of 100 nM dexamethasone prior to the addition of actinomycin D (1.25 μg/ml) for 
different periods of time. The total RNA was extracted, and aliquots 
(10–40 μg) were used to assay the amount of GGT mRNA III-specific 
sequences by an RNAase protection assay using the Ambion kit (Clini-
sciences, Montrouge, France). The cRNA-3 probe (362 bases) was trans-
scribed from the GGT plasmid FL2 linearized by EcoRI. The probe was 
purified on a 5% acrylamide, 8 × urea gel and eluted; 250,000 cpm of 
labeled cRNA were coprecipitated with each RNA sample. The pellets 
were resuspended in 80% deionized formamide, 100 mM sodium citrate, 
pH 6.4, 1 mM EDTA. Hybridization was performed overnight at 55 °C. 
The hybridization products were digested by the RNase A and T1 for 30 min at 37 °C, treated as described in the kit procedure, and loaded on a 5% acrylamide, 8 × urea gel. The gel was run for 2 h at 50 watts, dried, and autoradiographed. Densitometric quantification was performed using a Laser densitometer Ultrascan XL (Pharmacia). Films were exposed for different periods of time in order to measure the intensity of the signal in a linear range.

**RESULTS**

**Morphological Examination—**As illustrated by representa-
tive micrographs (Fig. 1, A–D), a decrease in the number of ZG 
was observed in the pancreatic acinar cells of adrenaledenized 
rats (Fig. 1B) as compared with the number found in cells from 
control animals (Fig. 1A). There is no noticeable difference 
between the pancreases of the adrenaledenized group (Fig. 
1B) and those of animals receiving estradiol as a replacement 
therapy (Fig. 1C). In contrast, the animals of the adrenaleden-
zied group that received a replacement therapy with dexam-
ethasone (Fig. 1D) exhibited an increase in the number of ZG 
in the pancreas, as compared with the controls. In control 
AR42J cells, there were only few small size granules (Fig. 2A). 
Dexamethasone treatment induces the appearance of ZG of 
different sizes (Fig. 2B).

**Effect of Glucocorticoids on GGT Activity and GP-2 Protein 
Level in Vivo and in Vitro—**In adrenaledenized rats, the pan-
creatic GGT activity (102 ± 21 milliunits/mg protein) is 
decreased by about 50% as compared with sham-operated ani-
mals (210 ± 35 milliunits/mg protein). Administration of 
dexamethasone results in a significant (p < 0.001) recovery of
the GGT activity (142 ± 25 milliunits/mg protein), whereas an estradiol replacement therapy is without effect on GGT activity (93 ± 20 milliunits/mg protein) that remains at the level found in adrenalectomized animals. The influence of glucocorticoids on GGT activity was also measured in vitro in the pancreatic adenocarcinoma AR42J cells. Incubation of these cells for 24 h, in the presence of $10^{-8}$, $10^{-7}$, and $10^{-6}$ M dexamethasone, enhances the GGT activity from 5 milliunits/mg protein (basal level) to 12, 17, and 17 milliunits/mg protein, respectively; this indicates that the dexamethasone effect is maximal at $10^{-7}$ M. Culture of AR42J cells at $10^{-7}$ M dexamethasone for different periods of time up to 72 h reveals that, after a latent period of 12 h, the GGT activity increases sharply reaching a value that represents 20 times the basal level after a 3-day treatment (Fig. 3). In experiments where the anti-glucocorticoid RU-38486 was added to the culture medium, the increase in GGT activity was minimal (4 milliunits/mg protein) compared with the basal level (2 milliunits/mg protein). Treatment with estradiol was without effect on GGT activity (data not shown). These in vivo and in vitro data show that GGT activity in the pancreas acinar cells is under the control of glucocorticoids. The higher amplitude of the GGT regulation observed in AR42J cells as compared with the pancreas in vivo might be explained by differences in the hormone concentration in contact with pancreatic cells. In vitro, AR42J cells were treated with $10^{-7}$ M dexamethasone, a concentration that induces granulation of these cells as well as maximal induction of GGT activity. The glucocorticoid concentration achieved in the pancreas in vivo during the hormonal therapy might be lower.

GP-2 protein level was measured by the Western blot technique on protein extracts prepared from pancreas and AR42J cells. The general pattern of the proteins appears to be similar among the different samples (Fig. 4). Immunoblotting with GP-2 polyclonal antibodies reveals a single band in control pancreatic extracts. This band exhibits an apparent molecular weight of 78,000 that corresponds to the glycosylated GP2.
polypeptide cannot be detected in the pancreas of adrenalectomized animals, but it reappears following a glucocorticoid replacement therapy (Fig. 4). No GP-2 polypeptide can be detected in AR42J protein extracts even when granule formation has been stimulated by glucocorticoids (Fig. 4).

Effects of Glucocorticoids on the GGT and GP-2 mRNA Levels in Vivo and in Vitro—Northern blot analysis of pancreatic mRNA prepared from animals with different glucocorticoid status was carried out with the cRNA probe 139A that recognizes all the different GGT mRNA species (20). Hybridization of this probe to the poly(A') RNA from sham-operated animals (Fig. 5A) reveals a major band which co-migrates with the 2.4-kb GGT band detected in the fetal liver. The 2.4-kb GGT band almost completely disappears in samples from adrenalectomized rats. Administration of dexamethasone to adrenalectomized animals not only prevents the disappearance of this GGT band but also increases its intensity as compared with controls. In contrast, the estradiol replacement therapy does not significantly affect the GGT mRNA level in the pancreas of adrenalectomized rats. A second hybridization of these mRNA samples with an actin probe shows comparable mRNA loading in the four pancreas samples (Fig. 5B). The mRNA samples were further analyzed by hybridization to the cRNA-3 probe specific for the 5'-untranslated sequence unique to the GGT mRNA III (Fig. 5C). This hybridization firmly identifies the 2.4-kb band as the GGT mRNA III previously found in fetal liver and lactating mammary gland (22). The mRNA III accumulation is under a positive glucocorticoid control since the effect of adrenalectomy is largely compensated by a dexamethasone therapy, whereas estradiol exhibits only a slight effect. The band corresponding to a low molecular weight poly(A') RNA revealed by the cRNA-3 probe in the fetal liver sample (Fig. 5C) has been observed in earlier studies (22). This 1.6-kb poly(A') RNA is not detected by the GGT probe 139A corresponding to the GGT coding sequence, and the length of this message is shorter than the GGT reading frame. Therefore, this message cannot encode a GGT polypeptide. The interpretation is that a piece of sequence in the GGT mRNA III-specific 5'-untranslated region, used as the probe, may be highly homologous to a sequence present in an unidentified RNA species expressed in the fetal liver. A second GGT band was also observed in pancreas samples from sham-operated animals (Fig. 5A). This band corresponds to a GGT mRNA species that migrates in the gel at a slightly higher rate than the GGT mRNA III. Hybridization to the cRNA-12 probe identifies this mRNA as the GGT mRNA II species that migrates at 2.2 kb (19) (Fig. 5D). It is not expressed in the pancreas of adrenalectomized animals and is not restored by glucocorticoid replacement therapy.

To determine whether the GGT mRNA III accumulation in pancreatic cells was under a direct control by glucocorticoids, GGT mRNA accumulation was measured in the AR42J cells. When cultured in the absence of hormone, these cells express the 2.4-kb GGT mRNA at a low level, as revealed by the hybridization to the cRNA-139A probe (Fig. 5A). Addition of 100 nM dexamethasone increases the GP-2 mRNA to a level that was also observed in pancreas samples from sham-operated rats (Fig. 5A). This signal is markedly decreased in the adrenalectomized group, whereas treatment with dexamethasone increases the GP-2 mRNA to a level that is higher than in the control group. Replacement therapy with estradiol also prevents a decrease of GP-2 mRNA level in adrenalectomized rats. A control hybridization with the actin probe
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Effects of Glucocorticoids on the mRNA Degradation—To determine whether glucocorticoids exert their regulatory action at a post-transcriptional level through a stabilization of the mRNAs, mRNA stability was assessed by measuring the decay in GGT mRNA accumulation following an actinomycin D treatment of AR42J cells (Fig. 8). As shown in a representative experiment (Fig. 8A), in control cells a marked and rapid decrease in the GGT mRNA level occurs a few hours after transcription arrest. A densitometric analysis of this autoradiogram revealed that 82% of the GGT mRNA had disappeared within 2 h (data not shown). In contrast, following a 48-h dexamethasone treatment, the GGT mRNA level is not significantly affected 4 h after actinomycin D treatment. Analysis of the GGT mRNA decay curves, obtained by densitometric quantification of the signals on autoradiograms from 6 experiments (Fig. 8B), reveals a much faster degradation rate in untreated as compared with glucocorticoid-treated cells. In the absence of glucocorticoids, the GGT mRNA decay curve shows a sharp
increase, since 82% of the mRNA transcripts had disappeared within 8 h. In contrast, the GGT mRNA III level decreases by only 34% in dexamethasone-treated cells (Fig. 8B). These results clearly show that glucocorticoids increase the GGT mRNA III level in pancreatic cells by preventing its rapid degradation.

**DISCUSSION**

To gain insight into the mechanisms of ZG formation in pancreatic acinar cells, we investigated the influence of glucocorticoids and estradiol on the expression of GGT and GP-2 proteins in the rat pancreas in vivo and in vitro, using the AR42J pancreatic carcinoma cell line. Adrenalectomy caused a marked depletion of zymogen granules that was prevented by glucocorticoid replacement therapy (Fig. 1) as described previously (4). In adrenalectomized animals we observed a decrease in the GP-2 mRNA level that cannot be related to a specific effect of glucocorticoids since it can also be prevented by estradiol (Fig. 6). The amount of GP-2 protein in pancreas is correlated to the GP-2 mRNA level under different glucocorticoid status (Fig. 4). In AR42J cells, however, glucocorticoid treatment, which promotes extensive granulation of these cells (Fig. 2), induces neither the GP-2 mRNA nor the GP-2 protein level (Figs. 4 and 6). These data show that granule formation can occur independently of GP-2 synthesis. This fact is corroborated by the absence of GP-2 mRNA in the lactating mammary gland (data not shown), an organ very active in protein secretion. In a recent study, Dittie` and Kern (34) reached similar conclusions based on the following observations. 1) GP-2 mRNA was undetectable in fetal pancreas during the differentiation period, including when ZG formation takes place. 2) GP-2 mRNA synthesis does not occur in parotid and adrenal glands. 3) Glucocorticoids are without effect on GP-2 mRNA accumulation in AR42J cells.

In contrast to GP-2, GGT expression is clearly under a direct positive control of glucocorticoids. This is supported by a decrease of both enzyme activity and mRNA level in adrenalectomized animals and by prevention of these effects with dexamethasone but not with an estradiol replacement therapy (Fig. 5). This direct effect of the hormone can be confirmed in vitro in AR42J cells that respond to dexamethasone by a marked increase in the GGT activity (Fig. 3). Northern blot analysis clearly showed that the increase in mRNA induced by glucocorticoids, observed both in vivo and in vitro, results from an accumulation of the GGT mRNA III (Fig. 5), transcribed from GGT promoter III on the GGT gene (22). The induction of the GGT expression by glucocorticoids is not due to a direct effect of the hormone receptor complex onto the palindromic target sequence, previously identified on the GGT promoter III (22), or to the participation of glucocorticoid-regulated factors that could act at the level of the GGT gene. In fact, no hormonal effect can be observed on the transcription rate of the GGT gene. In a recent study, Dittie` and Kern (34) reached similar conclusions based on the following observations. 1) GP-2 mRNA was undetectable in fetal pancreas during the differentiation period, including when ZG formation takes place. 2) GP-2 mRNA synthesis does not occur in parotid and adrenal glands. 3) Glucocorticoids are without effect on GP-2 mRNA accumulation in AR42J cells.

In conclusion, this study shows that ZG formation induced by glucocorticoids is closely linked to GGT expression. The localization of this enzyme in the granules of the pancreas and other secretory glands (8), as well as its induction in the lactating mammary gland (45), suggests that glucocorticoids may play a significant role in the packaging process by regulating the expression of GGT in these glands. The glucocorticoids appear now to have a well integrated function interacting at different levels of the secretory process, synthesis of secretory proteins as reported for amylase (35) or casein (46), increase in the number of cholecystokinin receptors (47), and also synthesis of protein components of secretory vesicles, as demonstrated in this study.

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