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# A pertussis toxin-sensitive GTP-binding protein plays a role in the $G_0$-$G_1$ transition of rat hepatocytes following establishment in primary culture

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Acute spontaneous c-*myc* gene expression and sustained increase of a GTP-binding protein(s) (G-protein) which is sensitive to islet-activating protein (IAP), pertussis toxin, occurred early during primary culture of adult rat hepatocytes. Following these earlier events, DNA synthesis was demonstrated in response to EGF and insulin. Addition of IAP immediately after plating of primary cultures inhibited c-*myc* expression and the hormone-induced DNA synthesis. Addition at 24 h or later following cell inoculation, however, produced only weak effects on DNA synthesis, even though the IAP-sensitive G-proteins were completely inactivated. We conclude that the IAP-sensitive G-protein(s) plays a role in the earlier process(es) of the $G_0$-$G_1$ transition, which is essential for the initiation of growth factor-dependent DNA synthesis.

Gene, c-*myc*; DNA synthesis; GTP-protein; Islet-activating protein; (Rat hepatocyte)

## 1. INTRODUCTION

Adult rat hepatocytes can initiate DNA synthesis and proliferate in response to EGF and insulin with a short lag time when cultured at low cell density [1]. At a high cell density of near confluence, however, induction of DNA synthesis requires a relatively longer preculture time [1]. Thus, the duration of the lag time depends on the cell density. This lag time is considered to be the time needed for the cells to acquire competence to respond to EGF and insulin. The biochemical events involved in this period of the hepatic cell cycle, however, are poorly understood.

Recent publications have shown that a GTP-binding protein(s) (G-protein) is involved in the signal-transduction systems of some growth factors which induce DNA synthesis [2-4]. We therefore aimed at elucidating the possible role of G-protein(s) in hepatocyte growth. For this purpose, we utilized islet-activating protein (IAP), pertussis toxin, which is known to ADP-ribosylate and inactivate G-protein(s) such as inhibitory G-protein, $G_i$, and, hence, to be useful for the identification of processes mediated by IAP-sensitive G-protein(s) [5]. We measured c-*myc* expression as an early event correlating with DNA synthesis reinitiation, since the expression of the proto-oncogene is known to occur in vivo, at an early stage of liver regeneration after hapatectomy [6]. The results indicate that an IAP substrate G-protein(s) is involved in the mechanism of c-*myc* expression and thus, induces 'competence' at an early stage of the hepatic cell cycle.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

IAP was purified as described [7]. [$\alpha$-$^{32}$P]NAD (31–48 Ci/mmol), [*methyl*-$^{3}$H]thymidine (20 Ci/mmol) and [$\alpha$-$^{32}$P]dCTP (3000 Ci/mmol) were purchased from New England Nuclear. The sources of all other reagents used for preparation and incubation of hepatocytes, cell culture, ADP-ribosylation of membrane proteins, and other purposes were those described in [8–10].

### 2.2. *Preparation and primary culture of hepatocytes*

Hepatocytes were isolated from tissues of male Wistar-derived Donryu rats (150–200 g) fed ad libitum according to [8]. Freshly prepared hepatocytes were suspended in William's E medium containing 5% fetal calf serum, 1 $\mu$M dexamethasone, 100 U/ml aprotinin, 100 U/ml penicillin G, and 100 $\mu$g/ml streptomycin and maintained at a density of $5 \pm 10^4$ cells/cm$^2$ (unless otherwise specified) on collagen-coated plastic petri dishes at 37°C under 5% $CO_2$ and 95% air [10]. Where indicated, the culture medium was supplemented with 100 ng/ml (unless otherwise specified) IAP. The medium was changed after the first 3 h and then every 2 days.

### 2.3. *DNA synthesis*

Insulin (0.1 $\mu$M) and EGF (20 ng/ml) were added to monolayer cultures at various times after plating. At 24 h after addition of these hormones, [$^{3}$H]thymidine (1 $\mu$Ci/well) was added to cultures, and the cells were further cultured for 4 h. Cells were washed once with cold phosphate-buffered saline and three times with 10% trichloroacetic acid. Cells were solubilized with 0.1 N NaOH, 2% $Na_2CO_3$ and 0.1% SDS. The radioactivity in the alkaline extract was measured in a liquid scintillation counter.

### 2.4. *RNA preparation and Northern blot hybridization*

RNA was isolated by the guanidium isothiocyanate/CsCl method as in [11] and 20 $\mu$g RNA was fractionated by electrophoresis in a 1.2% agarose/6.0% formaldehyde gel. RNA was transferred to a nitrocellulose filter according to the Southern technique [12]. RNA blots were pre-hybridized with 50 $\mu$g/ml of sonicated and denatured salmon sperm DNA in hybridization buffer described previously [13] for 6 h at 45°C, followed by hybridization with $^{32}$P-labeled c-*myc* probes ($5 \times 10^8$ cpm/$\mu$g) for 16 h at 45°C in a solution as described [14,15]. $^{32}$P-labeled probes were prepared by random primed DNA labeling (Boehringer Mannheim) of the *Cla*I/*Eco*RI fragment of human c-*myc* gene (including most of the third exon). The filters were washed four times for 15 min at room temperature in 200 ml of 2 × SSC, 0.2% SDS and twice for 25 min at 51°C in 200 ml of 0.1 × SSC, and exposed to X-ray film at −80°C for 5 days. The intensity of the c-*myc* band was estimated by densitometry.

### 2.5. *ADP-ribosylation of membrane proteins*

Preparation of crude plasma membranes and ADP-ribosylation of the cholate extract of the membranes were carried out as in [10].

## 3. RESULTS AND DISCUSSION

In the experiments shown in fig.1A, hepatocytes were cultured at $1.25 \times 10^4$ cells/cm$^2$ ('low cell density') or $5 \times 10^4$ cells/cm$^2$ ('high cell density') for 3 h (a) or 48 h (b), and then EGF and insulin were added together to the culture medium. At 24 h after the addition of these growth factors, DNA synthesis was measured. As shown previously [1], at high cell density, [$^{3}$H]thymidine incorporation was very low when the growth factors were added at 3 h, whereas it was remarkably high when added at 48 h after plating. In contrast, at low cell density, appreciable DNA synthesis was observed even when the growth factors were added at 3 h. The DNA synthesizing activity was further increased when the growth factors were added at 48 h of cultivation, and the value was almost identical to that of the high cell density culture.

Fig.1B shows the time-dependent change in the amount of IAP-sensitive G-protein estimated by IAP-catalyzed [$^{32}$P]ADP-ribosylation during primary culture without addition of growth factors. In the low cell density culture, the content of IAP substrate G-protein was gradually increased with an about 6 h lag, and reached the maximum at 72 h after plating. The maximal value was 4.5 times the initial value. In the high cell density culture, the IAP-sensitive G-protein content decreased for the first 6 h, probably via ADP-ribosylation of the protein by endogenous ADP-ribosyltransferase [9]. Thereafter, although the content gradually increased, the value at 72 h after plating barely reached 2-fold the initial value. Thus, an increase in IAP-substrate G-protein content was also cell-density-dependent.

The results mentioned above suggest that the appearance of EGF- and insulin-dependent DNA synthesis is associated with an increase in the content of IAP-substrate G-protein. To determine whether the G-protein actualle plays a role(s) in the induction of growth factor-dependent DNA synthesis, the effect of IAP treatment on DNA synthesis was studied. Hepatocytes were cultured at high cell density in the presence of graded doses of IAP, and at 48 h, EGF and insulin were added to the culture medium. As shown in fig.2, when the IAP dose was increased, EGF- and insulin-induced DNA synthesis was decreased. The minimum level corresponded to about 60% of the control in cells without IAP treatment. Basal DNA synthesizing activity was not appreciably affected by IAP treatment (fig.2A).

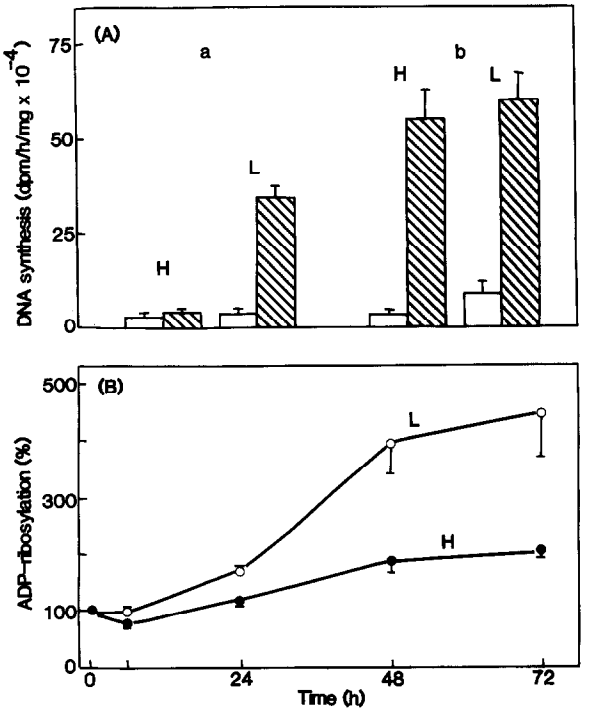


Fig.1. Density-dependent induction of DNA synthesis (A) and increase in content of IAP substrate G-protein (B). (A) Hepatocytes were cultured at $1.25 \times 10^4$ cells/cm$^2$ (L) or $5 \times 10^4$ cells/cm$^2$ (H) for 3 h (a) or 48 h (b), then EGF and insulin (hatched column) or their vehicle (open column) were added. After a further 24 h culture, DNA synthesis activity was measured. (B) Cells were cultured at (L;○) or at (H;●) for the indicated time, then membranes were prepared therefrom. Cholate extracts of membranes were ADP-ribosylated with [$\alpha$-$^{32}$P]NAD and activated IAP. The content of ~41 kDa IAP-substrate protein was expressed as percentage of the control value obtained just before the initiation of culture.

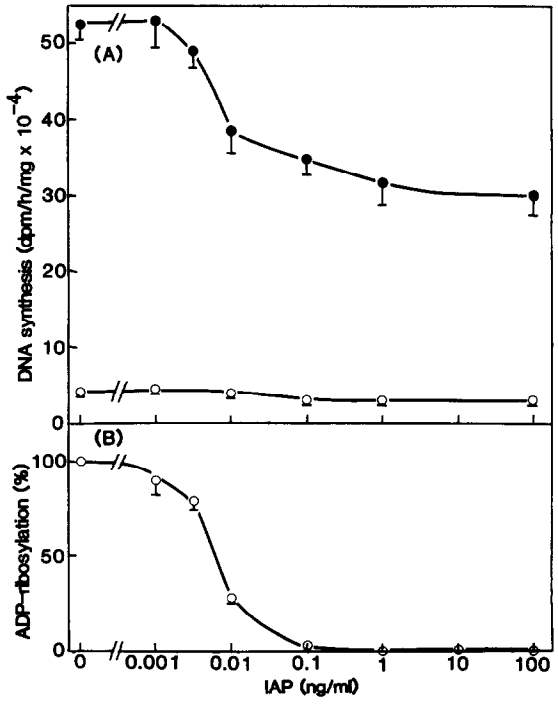


Fig.2. Inhibition by IAP of EGF- and insulin-induced DNA synthesis, as correlated with ADP-ribosylation of the ~41 kDa membrane protein. Hepatocytes were cultured in the presence of graded doses of IAP. In (A), after 48 h, cells were further cultured for 24 h with (●) or without (○) EGF and insulin, and then DNA synthesis activity was measured. In (B), 72 h after plating, membranes were prepared from cells, for [$^{32}$P]ADP-ribosylation. Other experimental conditions and data presentation same as in fig.1.

We measured [$^{32}$P]ADP-ribosylation of an aprox. 41 kDa protein in the mebrane fractions prepared from IAP-treated cells (fig.2B). The $^{32}$P labeling represented the IAP-sensitive G-protein which remained after ADP-ribosylation by prior exposure of cells to IAP. The depression of the response to thw growth factors was associated with a decrease in the IAP-sensitive G-protein content, although significant DNA synthesis was still shown even after the membrane G-protein was totally ADP-ribosylated and inactivated. These results suggest that the ~41 kDa protein is somehow involved in the process(es) which leads to DNA synthesis.

To answer the question pertaining to which stage in the cell cycle would be affected by IAP, the toxin was added at varying times after plating. EGF and insulin were always added at 72 h. In these experiments, the completeness of inactivation of IAP-sensitive G-protein was confirmed by IAP-catalyzed labeling. As shown in fig.3, when IAP was added at the beginning of culture, EGF- and insulin-induced DNA synthesis was reduced by about 40% (cf. fig.3A, a with b) in accordance with the results shown in fig.2. The degree of inhibition, hoewever, was decreased with the length of delay in IAP addition (fig.3A, b–d). As confirmed by IAP-catalyzed labeling, in all cases, the IAP-substrate ~41 kDa protein was completely ADP-ribosylated before EGF and insulin were added (fig.3B). Thus, the ~41 kDa protein cannot be directly involved in the signal transduction of EGF and insulin for

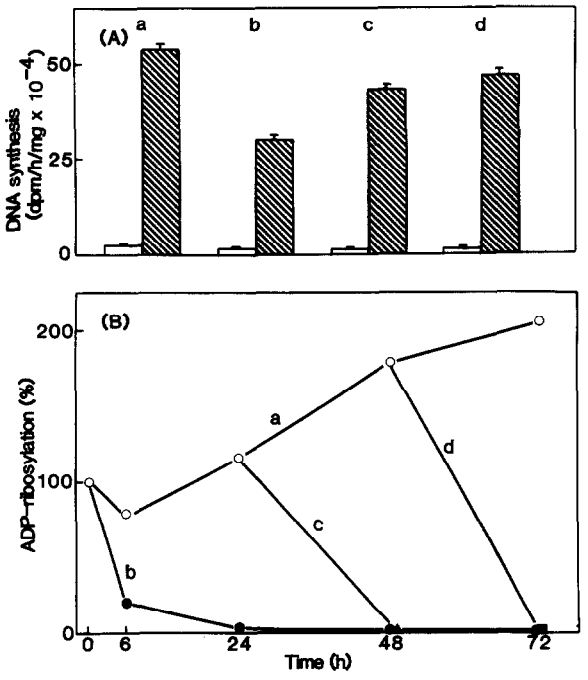


Fig.3. Change in degree of IAP inhibition of DNA synthesis with time of IAP addition. In hepatocyte cultures, IAP was not added (a), added at the time of plating (b), at 24 h (c) and at 48 h (d). In (A), EGF and insulin were added at 72 h, and then DNA synthesis during further 24 h culture was measured. In (B), membranes were prepared at the indicated time for measuring [$^{32}$P]ADP-ribosylation. Other experimental conditions and data expression as described for fig.1.

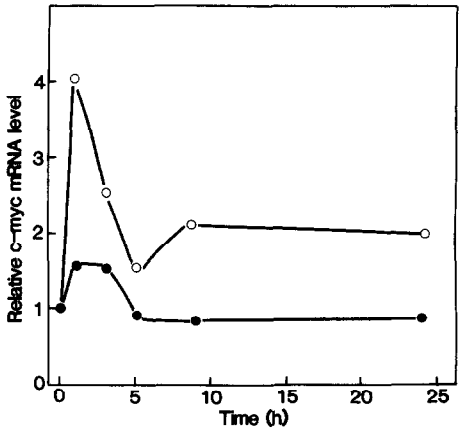


Fig.4. Inhibition of c-*myc* mRNA expression by IAP. Hepatocytes were cultured for the indicated times with (●) or without (○) IAP. Northern blot hybridization was performed for measuring c-*myc* mRNA as described in section 2.

DNA synthesis. If this were the case, the IAP-sensitive G-protein might play a role(s) in an early stage, probably for the $G_0$-$G_1$ transition rather than the exertion of EGS- and insulin-responsive $G_1$ phase.

The idea that an IAP-substrate G-protein(s) functions at an earlier stage of the cell cycle was further supported by measuring c-*myc* expression. Expression of this proto-oncogene has been shown to be responsible for DNA synthesis as an event occurring during the $G_0$-$G_1$ transition in many types of cells [16]. As shown in fig.4, c-*myc* mRNA was also induced at the earlier stage of hepatocyte culture in accordance with a previous observation (Makino, R. and Hayashi, K. personel communication; Nakamura, T. personel communication). The c-*myc* mRNA was increased about 4-fold within the first 1 h, decreased to about half-maximum by the 5th hour of cultivation and remained at that level for at least a further 20 h. When IAP was added at the beginning (the time of plating), c-*myc* level fell; it reached the maximum at 1 h but the level was lower than the half-maximum level attained by untreated cells and returned to basal level at 5 h.

Summarizing all the findings, we suggest that an IAP-substrate G-protein(s) functions at a certain early process(es) of the $G_0$-$G_1$ transition, proximal to c-*myc* expression and this provides preferable conditions for DNA synthesis in response to EGF and insulin. On the other hand, the mechanisms of IAP-substrate G-protein action in the transition from the $G_0$ to $G_1$ stage, including the induction of c-*myc* expression, and the regulatory mechanism of the G-protein content remain to be elucidated.

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