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18. In a typical experiment we use 0.5 to 1.0 ml of 1 to 3 m*M* cyt $c_{(ox)}$ in buffered aqueous solution (*p*H 5 to 7) and an Au or Pt electrode with an exposed area of ~ 0.5 cm^2 derivatized with the $(PQ^{2+/+})_{surf}$ system at 10^{-9} to 10^{-8} mole/cm^2. Electrolyte solutions are kept under Ar, since $(PQ^{+\cdot})_{surf}$ and cyt $c_{(red)}$ are sensitive to air. Derivatization of Au or Pt is effected by reacting pretreated [M. S. Wrighton, M. C. Palazotto, A. B. Bocarsly, J. M. Bolts, A. B. Fischer, L. Nadjo, *J. Am. Chem. Soc.* **100**, 7264 (1978)] surfaces with 1 to 3 m*M* CH_3CN solutions of **1** (*1*) under N_2 for 3 to 24 hours at 298 K. Electrochemistry was carried out with a three-electrode configuration in a two-compartment cell, using a saturated calomel electrode as reference and Pt as the counterelectrode. Cyclic voltammetry and other electrochemical measurements were carried out with a PAR model 173/175 potentiostat/programmer with a model 179 digital coulometer. Spectral measurements were made with a Cary 17 ultraviolet-visible-near infrared spectrophotometer.
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21. Electrodes with the $(PQ^{2+/+})_{surf}$ system at ~ 10^{-8} mole/cm^2 have generally been used. But since cyt $c_{(ox)}$ is mass transport-limited, it responds only to the $(PQ^{2+/+})_{surf}$ material available at the outermost surface. We assume that the coverage in this "monolayer" is ~ 10^{-10} mole/cm^2. We have consistently observed the same current density for cyt $c_{(ox)}$ reduction with electrodes having coverage in the range 7×10^{-10} to 3×10^{-8} mole/cm^2.
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22 September 1980

Insulin as a Potent, Specific Growth Factor in a Rat Hepatoma Cell Line

Abstract. *A line of rat hepatoma cells in culture which, in response to serum starvation, become arrested in the early G_1 phase of growth, can be stimulated by insulin alone to enter the cell cycle and traverse S phase. A half-maximum response is observed at 30 to 70 picomolar concentrations and the maximum response is essentially identical to that found with optimum serum concentrations.*

The ability of insulin to act as a growth factor has been investigated in a variety of cell types in culture including chick embryo fibroblasts (*1*), human fibroblasts (*2*), mouse 3T3 fibroblasts (*3*), rat liver cells (*4*), and baby hamster kidney cells (*5*). In these studies at least one of three limitations was noted. First, suprapysiological concentrations of insulin, ranging from 20 n*M* to 1 μM and higher, were required for a maximum response (*1*, *4*, *5*). Second, although in some of these reports a response was observed with concentrations as low as 10 n*M*, in no instance was a maximum effect obtained at this concentration. Indeed, even at significantly greater concentrations the response to insulin was not comparable to that found with optimum concentrations of serum (*2-4*). Third, in most of the reports, controls indicating the biological specificity of the response for insulin are lacking (*1*, *4*, *5*). This last criticism is particularly important when one considers the supraphysiological concentrations usually used and the report that an apparent growth effect could still be obtained by insulin preparations that had been autoclaved (*6*). This suggests that higher concentrations of insulin can produce "growth effects" in the absence of biologically active insulin molecules. In view of the observations that one or more of these three limitations applies to virtually all studies of the possible role of insulin as a growth factor, it is clear that the significance of insulin as a physiologically meaningful growth factor remains in doubt.

We have been using a clone of the H4-EII-C3 rat hepatoma cell line (H35) originally described by Pitot *et al.* (*7*). This

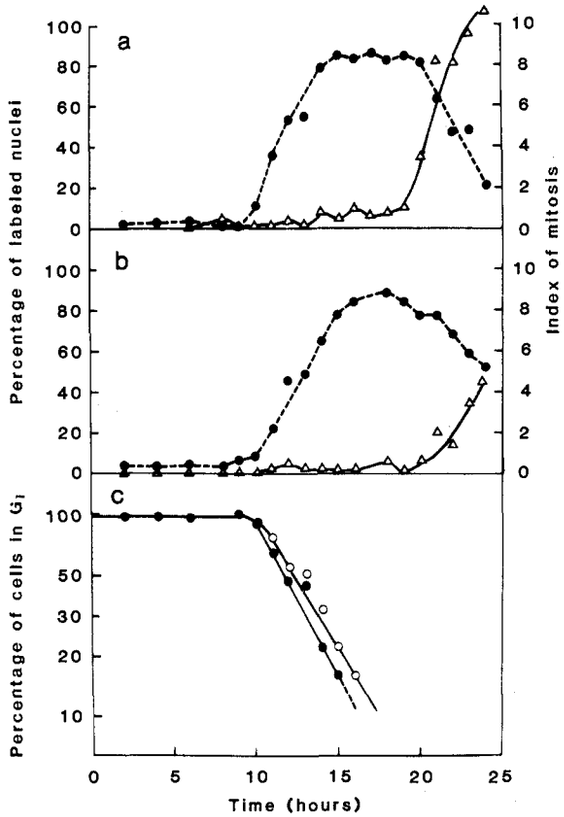

Fig. 1. Cells were grown in Dulbecco's modified Eagle's medium with 5 percent calf serum and 5 percent fetal calf serum in an atmosphere with 10 percent CO_2 at 37°C. Two days after subculture into 60-mm dishes the cells were washed once with balanced salt solution and fresh serum-free medium (SFM) was added. Cells remained in SFM for approximately 72 hours and then the medium was changed to (a) serum-containing medium or (b) SFM plus $10^{-9}M$ insulin. Every hour for 30 minutes, the cells were exposed to [^{3}H]thymidine (NEN, 0.5 μCi/ml, 0.2 μM). At the end of the labeling period, the cells were washed, fixed and dried, and then examined by autoradiography with NTB2 (Eastman Kodak). After development, the cells were stained with Giemsa (Harleco) and the dishes scored for the percentage of labeled nuclei and the mitotic index, with at least 1000 cells per dish being counted. The mitotic index is a reflection of cells counted in all phases of mitosis. Symbols: in (a) and (b), ●, percentage of labeled nuclei; △, index of mitosis; in (c), ●, 10 percent serum-containing medium; ○, serum-free medium containing $10^{-9}M$ insulin.

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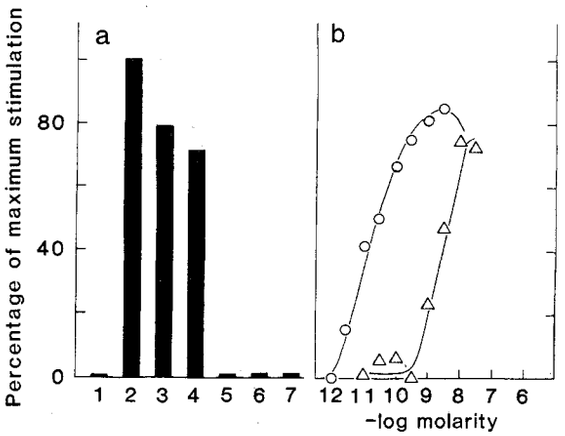

Fig. 2. Specificity of the insulin-induced growth response. (a) Cells were arrested as described in the legend to Fig. 1. The medium was changed from serum-free to: 1, fresh serum-free medium; 2, fresh 10 percent serum-containing medium; 3 to 7, fresh serum-free medium plus, in 3, 10 n*M* insulin; 4, 10 n*M* proinsulin; 5, 10 n*M* insulin A chain; 6, 10 n*M* insulin B chain; and 7, 10 n*M* autoclaved insulin. At 15.5 hours the cells were exposed to [^{3}H]thymidine as described in Fig. 1; and at 16 hours the cells were harvested and prepared for autoradiography. (b) After they were arrested, the cells were stimulated with the indicated concentrations of insulin (○) or proinsulin (△) for 16 hours. They were labeled continuously during this period and then harvested and processed for autoradiography. In both (a) and (b), between 900 and 1100 cells were counted to determine the percentage of labeled nuclei, and the value for the percentage of labeled nuclei in serum-stimulated cells was normalized to 100 percent. The actual values for the percentage of labeled nuclei with serum were 91 percent in (a) and 89 percent in (b).

clone demonstrates a variety of functions observed with the uncloned population. It possesses tyrosine aminotransferase that is inducible with glucocorticoids, insulin, and analogs of adenosine 3′,5′-monophosphate (*8*). It has an arylhydrocarbon hydroxylase that is inducible with methylcholanthrene (*9*), and an aldehyde dehydrogenase that is inducible with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD (*10*)] and, as reported by Knutson and Poland (*11*) for an uncloned H35 line, this clone is not killed by prolonged exposure to TCDD (*10*).

This clone stops growing under conditions of limited serum and our studies indicate that the point of arrest is in early G_1. While examining the ability of serum to initiate the growth of these arrested cells, we found that insulin was also capable of stimulating the arrested cells to reenter the cell cycle. By measuring the percentage of labeled nuclei after serum stimulation of arrested H35 cells, we observed that the cells enter the S phase in a synchronous fashion (Fig. 1a). There is a lag period of about 8 or 9 hours before any increase in the percentage of labeled nuclei is observed, and the mitotic index begins to increase at about 17 or 18 hours. The maximum value for the mitotic index occurs at 24 hours (seen in other experiments carried out for longer periods of time). This coincides with an average generation time of 22 to 24 hours for H35 cells growing randomly in culture. Ten percent serum is the optimum concentration for growth of the cell as well as for reinitiation of growth in the serum-starved cells (data not presented).

When H35 cells are stimulated by 1 n*M* insulin they show a very similar pattern of response (Fig. 1b). Thus, the lag period before entry into S phase that occurs with insulin is essentially identical to that observed with optimum concentrations of serum. The maximum percentage of labeled nuclei attained with 1 n*M* insulin is also the same as that obtained with 10 percent serum. However, a difference between the response to insulin and to serum was detected later in the cell cycle. The decline in the percentage of labeled nuclei and the increase in index of mitosis were more rapid in the presence of 10 percent serum than with 1 n*M* insulin. With insulin, the index of mitosis increased slightly more at 25 to 26 hours but never reached the value seen with medium containing 10 percent serum (data not presented). These results are similar to those reported for insulin stimulation of chick embryo fibroblast growth (*12*). It was suggested that the S and G_2+M phases were longer in the presence of insulin than in serum, which would account for the less rapid increase in mitotic cells and the slower rate of decline in labeled nuclei.

It should also be noted that the differences in the rate of increase in mitotic index, the magnitude of the peak, and the time point at which the peak was observed were reflected in the changes in cell number. Treatment with serum led to a doubling in cell number that was complete within approximately 30 hours. However, with insulin, there was not a complete doubling of the cells, although cell number increased from 30 to 40 percent depending on the experiment. Sustained growth in the presence of serum-free, insulin-containing medium was not observed.

The significance of the data in Fig. 1b is further illustrated by comparing the rate of entry of cells into S phase when they are stimulated with 10 percent serum-containing medium or with serum-free medium containing 1 n*M* insulin. Plotted as the log of the percentage of cells remaining in G_1 (*13*), the straight lines generated indicate that the two treatments cause similar rates of cell entry into S phase (Fig. 1c). The apparent rate constants that can be calculated from these data are 24 percent per hour for serum and 21 percent per hour for insulin. Thus, insulin in the absence of any serum factors is capable of triggering these arrested cells to traverse G_1 and enter S phase in a response virtually indistinguishable from that observed with 10 percent serum-containing medium. This concentration of insulin is well within the physiological range (*14*), and is the optimum concentration for provoking cell cycle reentry. Also, the concentration of insulin in the serum-containing medium was well below the levels required to give the maximum response with serum-free, insulin-containing medium. Thus it is not solely the insulin in serum that stimulates growth in the arrested cells.

To demonstrate the biological specificity of this response, we tested isolated insulin A and B chains, proinsulin, and chemically inactivated insulin for their ability to stimulate the arrested cells (Fig. 2a). The isolated A and B chains and inactivated insulin provoked no response at concentrations as high as 10 n*M*. In contrast, 10 n*M* proinsulin provoked a response similar in magnitude to that of 10 n*M* insulin. To determine whether this was an indication of a lack of specificity, the concentration dependencies for insulin and proinsulin were determined (Fig. 2b). These data show that at 10 n*M*, insulin and proinsulin stimulate equally the entry of cells into S phase. However, the potencies of the two peptides are different. The 50 percent effective concentration (EC_{50}) for insulin ranges from 30 to 70 p*M*, whereas the EC_{50} for proinsulin is approximately 3 n*M*. That the potency of proinsulin is about 1 percent of that of insulin is compatible with its biological potency in other systems (*15*).

The half-maximum response obtained with 30 to 70 p*M* insulin is indicative of an extremely sensitive response. This value corresponds closely to that found for induction of tyrosine aminotransferase in these cells (*16*) and also for stimu-

lation of aminoisobutyrate uptake in uncloned H35 cells (*17*). Such sensitivity suggests mediation of the response through the insulin receptor. This in contrast to other reports in which the ability of insulin to act as a growth factor has been attributed to its acting through a receptor for an insulin-like growth factor, MSA (multiplication-stimulating activity) from the BRL-3A cell line (*18-20*). Both of these reports were substantiated by the observation that the F(ab) fragment of immunoglobulin G antibodies to the insulin receptor would not block insulin mitogenic activity in spite of its ability to displace iodinated insulin from its receptor (*18-20*) and to block insulin-stimulated glucose oxidation.

The significance of the ability of insulin to act as a potent growth factor is reinforced by early observations that insulin provokes an increase in hepatic DNA content, an effect that is attributable to increased DNA synthesis and not to a change in the ploidy of the cells (*21*). Also, investigators have reported the concomitant occurrence of insulin resistance in patients with growth disorders suggesting the possibility that the lesion responsible for the insulin resistance is a contributing factor in the associated growth defect (*22, 23*).

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24. This research was supported by grant AM 24047 from NIH to J.W.K. We especially thank W. D. Wicks in whose laboratory this work was conducted.

28 April 1980; revised 21 November 1980

Rapid Induction of Cellular Strain Specificity by Newly Acquired Cytoplasmic Components in Amoebas

Abstract. *A new strain-specific character was induced in amoebas by bacterial endosymbionts after only 10 to 15 cell generations of symbiosis. The nuclei of changed amoebas not only became incompatible with the cytoplasm of the original strain, but also exerted a strong lethal effect on intact amoebas of the same original strain.*

We previously reported bacterial endosymbionts of *Amoeba proteus* that changed from parasites (*1*) to required cytoplasmic components within a few years (*2*). These symbiotic bacteria are capable of infecting other amoebas and of causing the latter to become dependent on the symbionts in 200 cell generations (18 months) (*3*). We now report that within 4 weeks (10 to 15 generations) of infection with the symbiotic bacteria, host amoebas may become genetically distinct from the original strain.

Amoebas do not reproduce sexually, and the only way to test genetic compatibility between two strains is through nuclear transplantation. Exchange of nuclei between amoebas of the same strain (homotransplants) results in the formation of viable cells, whereas heterotransplants (that is, between amoebas of different strains) are nonviable because of nuclear-cytoplasmic incompatibility (*4*). Heterologous nuclei also exert a strong lethal effect when implanted into amoebas of different strains (*5*) or genera (*6*). Thus, when a heterokaryon is produced from different strains, both donor and host nuclei become nonviable within minutes, in contrast to homokaryons which produce viable clones. The lethal effect of heterologous nuclei has been attributed to a strain-specific lethal factor (*5*), which consists of high-molecular-weight proteins (*7*). Thus an internuclear lethal effect is one criterion for determining cell variation and strain specificity.

Taking advantage of the specificity of this phenomenon, we studied the role of newly acquired cell components, namely, endosymbionts of the xD strain of *A. proteus* and their ability to effect permanent cell changes in their hosts. The xD strain arose in 1966 after spontaneous infection of the D strain with rod-shaped X bacteria (*1*). The xD strain now requires these endosymbionts and its nuclei are not viable in their absence (*2*). Our studies were designed to determine (i) if the D and xD strains are sufficiently different to display nuclear incompatibility, (ii) how many cell generations are required before such incompatibility is expressed, and (iii) whether the lethal effect is dependent on the continued presence of the endosymbiotic bacteria. Changes in compatibility between D and xD amoebas are easily studied, since symbiosis between D amoebas and X bacteria can be established at will (*8*). We report here that xD amoeba nuclei are unilaterally lethal to D amoebas (that is, D nuclei are nonlethal to xD amoebas). Furthermore, the introduction of endosymbionts into a D amoeba induces irreversible changes in the host progeny in 10 to 15 generations (about 4 weeks), and these changes render the nuclei of new xD amoebas lethal to D amoebas when they are transplanted into these cells.

First, to test mutual lethal effects, we inserted nuclei of one strain into amoebas of the other by means of a de Fonbrune micromanipulator on agar-coated slides (*9*). After 5 minutes we removed the nuclei micrurgically. In previous studies, this period was sufficient for heterologous nuclei to exert lethal effects (*5*). Homokaryons were studied concurrently. The amoebas were cultured singly in watch glasses (U.S. Bureau of Plant Industry; Thomas, Philadelphia) containing modified Chalkley's medium (*2*), and we observed the amoebas until they either formed viable clones (five or more divisions) or died. The amoebas were fed three times a week with *Tetrahymena* (*10*) and kept at 20°C.

As shown in Table 1, 84.4 percent of the D hosts that had contained xD nuclei for 5 minutes died, whereas only 14.5 percent of the corresponding homokaryons died. The lethal effect exerted

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