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Possible involvement of GTP-binding proteins in growth regulation of human epidermoid carcinoma cell line A431

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A431 cells, a human epidermoid carcinoma cell line, express an unusually large number of cell surface receptors for the epidermal growth factor (EGF). The growth rate of A431 cells was estimated by measuring [^{3}H]thymidine incorporation at the logarithmic growth phase. The growth of the cells in protein-free medium was partially inhibited by exposure of the cells to pertussis toxin, islet-activating protein (IAP). The growth in both serum-containing and protein-free medium was inhibited by high concentrations of EGF, and these inhibitions were partially reversed by treatment of the cells with IAP. The effects of IAP were well correlated with the degree of ADP-ribosylation of a membrane 40-kDa protein. Thus, IAP sensitive G-proteins appear to be involved in the signal transduction of both positive and negative regulation of A431 cell growth. The possibility is also discussed that phosphatidylinositol turnover may participate in growth regulation.

Cell growth; GTP-binding protein; Pertussis toxin; Islet-activating protein; Epidermal growth factor; A431 cell

1. INTRODUCTION

The human epidermoid carcinoma cell line A431 expresses an extraordinarily high number of epidermal growth factor (EGF) receptors (2–3 × 10^6 receptors per cell) [1], yet its growth is inhibited by concentrations of EGF that are mitogenic with other cell lines [2,3]. While it is believed that this inhibition results from surplus transduction of intracellular signals through EGF receptors, the signals that play a critical role in this inhibition are unclear.

Islet-activating protein (IAP), pertussis toxin, is known to ADP-ribosylate and inactivate GTP-binding proteins (G-proteins) of intact cells and hence is useful for the identification of processes mediated by IAP-sensitive G-proteins [4]. It has been shown that IAP-sensitive G-proteins are also involved in the signal transduction systems of some growth factors which induce cell growth [5–11]. In this study, we aimed at elucidating the possible role of G-proteins in growth regulation of A431 cells. The results indicate that IAP-sensitive G- proteins are involved in the signal transduction leading to both positve and negative regulations of A431 cell growth. A possible role of phosphatidylinositol turnover in the growth regulation is also suggested.

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2. MATERIALS AND METHODS

2.1. *Materials*

[*methyl*-^{3}H]Thymidine (1.11 TBq/mmol) was purchased from ICN Biomedicals Inc., and [α-^{32}P]NAD (1.44 TBq/mmol) and *myo*-[2-^{3}H]inositol (740 GBq/mmol) were from Du Pont-New England Nuclear. EGF (from mouse submaxillary glands) was purchased from Toyobo Co., Ltd. (Osaka, Japan). The sources of all other materials were those described in [7,12,13].

2.2. *Cell culture*

A431 cells were obtained from the Japanese Cancer Research Resources Bank and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10 U/ml of mycostatin, and a high concentration of glucose (4.5 g/l). The cells were maintained at 37°C in a humidified atmosphere of 5%CO_2–95%air. The medium was changed every 2 days.

For protein-free culture, exponentially growing cells were suspended in serum-containing medium, and plated in multi-well culture plates or culture dishes at a density of 1 × 10^4 cells/cm^2, and cultured in the same medium for an additional 24 h. The cell monolayers were then washed twice with DMEM and further cultured in the mixture of 3 parts of DMEM and 1 part of Ham's F12. After 24 h, the cells were submitted to DNA synthesis assay, measurement of generation of [^{3}H]inositol phosphates, and preparation of membranes to be studied for IAP-induced ADP-ribosylation of membrane G-proteins.

For DNA synthesis assay in serum-containing medium, the cells were grown as described above except for the omission of the medium change to a protein-free one.

2.3. *DNA synthesis assay*

The cells were grown on 12-well culture plates as described in section 2.2 and the incubation medium was replaced by the fresh medium containing EGF. After 24 h, [^{3}H]thymidine (7.4–18.5 kBq/ml, 1 mM) was added to the cultures, which were further maintained for 2 h. The cell monolayers were washed twice with cold phosphate-buffered saline, three times with cold 10% trichloroacetic acid and were dissolved in 0.1 N NaOH for the determination of radioactivity content in a liquid scintillation counter.

2.4. *ADP-ribosylation of membrane proteins*

The cells were grown in protein-free medium as described in section 2.2. Preparation of crude plasma membranes and ADP-ribosylation of membrane proteins by IAP were carried out as in [13].

2.5. *Measurement of generation of [^{3}H]inositol phosphates*

The cells were grown in protein-free medium as described in section 2.2. except that [^{3}H]inositol (370 kBq/ml) was added at 24 h before the end of the culture. The cell monolayers were incubated at 37°C for 10 min in fresh, protein-free, medium fortified with 10 mM LiCl and 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) prior to the addition of EGF. The 30-min incubation with EGF was terminated by quick aspiration of the medium followed by the addition of 0.4 M perchloric acid. The supernatant was titrated to pH 7.5 with 0.7 N KOH/0.6 N $KHCO_3$ and kept at 4°C overnight. After removal of the precipitate by centrifugation, the supernatant was subjected to Dowex column chromatography as described by Berridge et al. [14].

3. RESULTS AND DISCUSSION

3.1. *IAP-sensitive inhibition by EGF of DNA synthesis in A431 cells cultured in serum-containing medium*

The inhibition of A431 cell growth by EGF is influenced by cell density [2]. In this study, we estimated the growth rate of A431 cells by measuring [^{3}H]thymidine incorporation at the logarithmic growth phase in order to minimize the influence of time-dependent increases of cell density. When A431 cells were cultured in serum-containing medium with various concentrations of EGF, there was a progressive inhibition of cell growth as the concentration of EGF was increased (Fig. 1). When the growth was studied in A431 cells that had been cultured with 100 ng/ml of IAP for 24 h, this inhibition was partially reversed. In the absence of EGF, IAP had no effect on the growth of A431 cells. These results suggest that IAP-sensitive G-proteins are involved in the inhibition of A431 cell growth by EGF.

3.2. *Biphasic effects of EGF on DNA synthesis in A431 cells cultured in protein-free medium*

A431 cells grew in the mixture of 3 parts of DMEM and 1 part of Ham's F12 as fast as in serum-containing medium (data not shown). Because the biological effects of growth factors have been often identified better in serum-free media than in serum-containing media, we next examined the effect of EGF on the DNA synthesis of A431 cells in protein-free medium (Fig. 2). Unlike the observation in serum-containing medium in Fig. 1, more DNA was synthesized in A431 cells in the presence of 2–3 nM EGF than in its absence in protein-free medium. Further increase in the concentration of EGF up to 4–10 nM caused progressive inhibition of DNA synthesis. In contrast to failure of IAP to inhibit DNA synthesis in serum-containing medium, the exposure of cells to IAP resulted in marked inhibition of DNA synthesis without or with low (2–3 nM) concentrations of EGF in the serum-free medium. The inhibition of DNA synthesis by higher (4–10 nM) concentrations of EGF was much less marked in IAP-treated cells than in non-

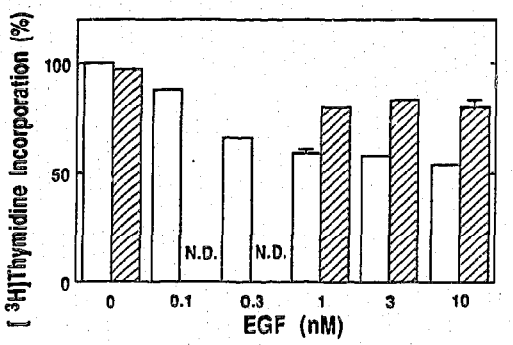

Fig. 1. Reversal by IAP of EGF-induced inhibition of DNA synthesis in serum-containing medium. A431 cells that had been cultured for 24 h with (hatched columns) or without (open columns) 100 ng/ml of IAP were further incubated for 24 h in fresh medium containing various concentrations of EGF. DNA synthesis was measured as described in section 2.3. Each value is the mean ± SEM from 3 observations. The value corresponding to 100% was 8747 dpm/well.

treated cells in protein-free medium. Thus, EGF-induced inhibition of DNA synthesis in A431 cells was mostly abolished by the IAP treatment of cells in protein-free medium as well as in serum-containing medium, excluding possible involvement of any factor(s) in serum. In any event, EGF at concentrations higher than 4 nM elicited more striking stimulation of DNA synthesis in IAP-treated cells than in non-treated cells in protein-free medium (Fig. 2). It is likely that IAP-sensitive G-proteins are responsible for EGF-induced growth inhibition.

Failure of IAP to inhibit spontaneous DNA synthesis in A431 cells cultured in the serum-containing medium (Fig. 1), despite the marked inhibition observable in the protein-free medium (Fig. 2), would be accounted for by contamination of the serum with EGF. The lack of stimulatory phase at lower EGF concentrations in the serum-containing medium (Fig. 1) would be explained likewise. The effective concentrations of the growth factor to inhibit DNA synthesis were much lower in the serum-containing medium (above 0.1 nM in Fig. 1) than

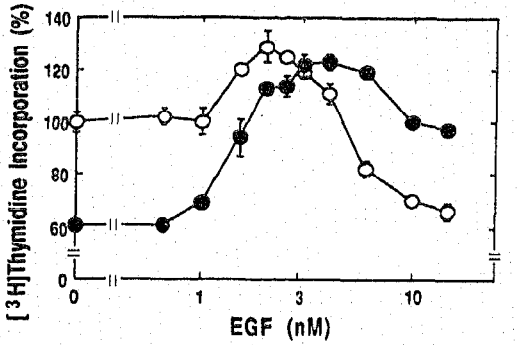

Fig. 2. Biphasic effects of EGF on DNA synthesis in A431 cells in protein-free medium. A431 cells that had be cultured for 3 h with (●) or without (○) 10 ng/ml of IAP were further incubated for 24 h in fresh medium containing various concentrations of EGF. DNA synthesis was measured as described in section 2.3 and plotted as mean ± SEM from 3 observations. The value corresponding to 100% was 13853 dpm/well.

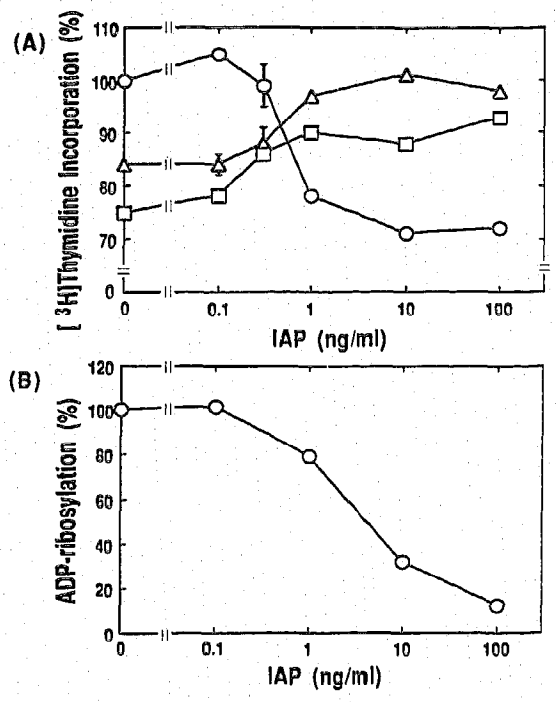

Fig. 3. Dual effects of IAP on DNA synthesis as correlated with ADP-ribosylation of the 40 kDa membrane protein. A431 cells were cultured in the presence of increasing concentrations of IAP for 3 (A) or 6 (B) h. In (A), cells were further cultured for 24 h with 4 nM (△) or 10 nM (□) EGF or without (○) EGF before DNA synthesis was measured. The value corresponding to 100% was 10730 dpm/well. In (B), membranes were prepared from cells to be analyzed for IAP-induced [^{32}P]ADP-ribosylation by the method described in section 2.4. The ADP-ribosylation is expressed as percentage of the control value obtained with cells cultured in the absence of IAP.

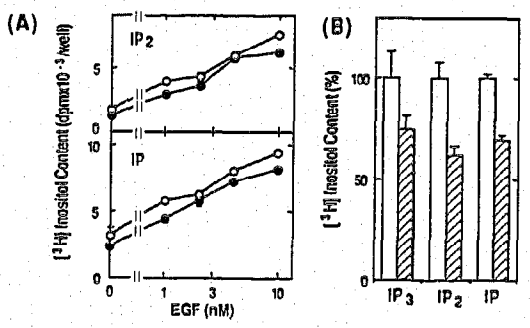

Fig. 4. Generation of inositol phosphates by A431 cells in the presence or absence of EGF. (A) [^{3}H]inositol-labeled A431 cells were first cultured for 6 h with (●) or without (○) 100 ng/ml of IAP and then incubated for 40 min in fresh medium fortified with 10 mM LiCl. Various concentrations of EGF were added to the medium at 10 min of incubation. The ^{3}H-contents of the inositol bisphosphate (IP_2) and inositol monophosphate (IP) fractions were measured as described in section 2.5. (B) The ^{3}H contents of inositol phosphates after incubation of A431 cells without addition of EGF are shown. Hatched and open columns (mean ± SEM from 5 observations) show the data in the presence and absence of IAP, respectively. Other experimental conditions are the same as those in (A). The values corresponding to 100% were: IP_3, 1559; IP_2, 1153; IP,1669 dpm/dish. The effect of IAP treatment was significant ($P < 0.01$).

in the protein-free medium (above 3 nM in Fig. 2), probably because omission of serum protein from the culture medium would afford less favorable conditions for growth regulation of the cultured cells. Alternatively, the action of EGF would be stabilized by the association with serum protein. Two plots drawn without and with the IAP treatment crossed each other at the EGF concentration of 3 nM in protein-free medium in Fig. 2. This critical concentration of the growth factor for susceptibility to IAP would be much lower in the medium fortified with serum, possibly reaching to the concentration anticipated to occur in the serum-containing culture medium. In fact, a significant amount of EGF could be liberated from platelets during preparation of serum [15]. EGF at low concentrations is thus likely to stimulate growth, and spontaneous DNA synthesis without EGF appears to be susceptible to IAP inhibition, under any conditions in A431 cells.

The inhibition of DNA synthesis by IAP in the absence of EGF and the reversal of the EGF-induced inhibition by IAP were equally dependent on the concentration of IAP used (Fig. 3A) with the half-maximally effective concentration of 0.2–0.5 nM for both parameters. Cell membranes were prepared from the exponentially growing A431 cells in protein-free medium containing increasing concentrations of IAP to be studied for ADP-ribosylation of the membrane 40-kDa G-proteins by sufficient amount of preactivated IAP in the presence of [α-^{32}P]NAD (Fig. 3B). A progressively smaller amount of the G-protein was ADP-ribosylated as the concentration of IAP in the medium of cell culture was increased, clearly indicating that the G-protein had in fact been ADP-ribosylated during cell culture with IAP. Disparity of the EC_{50} value of IAP between DNA synthesis inhibition and ADP-ribosylation may reflect the difference in the time of cell exposure to the toxin. Thus, these results suggest that the IAP-substrate G-proteins are involved in both spontaneous growth of A431 cells in protein-free medium and inhibition of cell growth by EGF in either protein-free or serum-added medium.

3.3. *Inositol phosphate production in A431 cells*

Exposure of A431 cells to EGF resulted in time- and concentration-dependent accumulation of the inositol phosphates, inositol trisphosphate, inositol bisphosphate, and inositol monophosphate (data not shown). Increased generation of inositol phosphates in response to EGF was unaffected by prior exposure of cells to IAP (Fig. 4A). IAP-sensitive G-proteins do not appear to mediate stimulation of phospholipase C via EGF receptors in A431 cells, in accordance with recent observations that phospholipase Cγ is activated by tyrosine phosphorylation as a result of direct association with phosphorylated EGF receptors [16–20]. The spontaneous generation of inositol phosphates, observable in the absence of EGF, was partially inhibited by IAP treatment of cells (Fig. 4B). Since inositol phospholipid turnover is involved in cellular proliferation [21,22], spon-

taneous activation of phospholipase C is likely to play a critical role in spontaneous growth of A431 cells in protein-free medium. A431 cells are known to produce prostaglandins [23]. Growth of A431 cells in protein-free medium was inhibited by cyclooxygenase inhibitors (unpublished data). Conceivably, autocrine prostaglandins may stimulate membrane receptors initiating growth-promoting signals through phosphatidylinositol turnover.

Although intracellular signals involved in EGF-induced growth inhibition remain obscure, the present data suggest that IAP-susceptible G-proteins are likely to be involved in signal transductions responsible for both positive and negative regulation of A431 cell growth.

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REFERENCES

[1] Haigler, H., Ash, J.F., Singer, S.J. and Cohen, S. (1978) Proc. Natl. Acad. Sci. USA 75, 3317–3321.
[2] Gill, G.N. and Lazar, C.S. (1981) Nature 293, 305–307.
[3] Barnes, D.W. (1982) J. Cell Biol. 93, 1–4.
[4] Ui, M. (1986) in: Phosphoinositides and Receptor Mechanisms (Putney, J.W., ed) Receptor Biochemistry and Methodology, vol. 7, pp. 163–195, Liss, New York.
[5] Letterio, J.J., Coughlin, S.R. and Williams, L.T. (1986) Science 234, 1117–1119.
[6] Nishimoto, I., Hata, E., Ogata, E. and Kojima, I. (1987) J. Biol. Chem. 262, 12120–12126.
[7] Murayama, T. and Ui, M. (1987) J. Biol. Chem. 262, 12463–12467.
[8] Paris, S., Chambard, J.-C. and Pouyssegur, J. (1987) J. Biol. Chem. 262, 1977–1983.
[9] Seuwen, K., Magnaldo, I. and Pouyssegur, J. (1988) Nature 335, 254–256.
[10] Zachary, I., Millar, J., Nanberg, E., Higgins, T. and Rozengurt, E. (1987) Biochem. Biophys. Res. Commun. 146, 456–463.
[11] Taylor, C.W., Blakeley, D.M., Corps, A.N., Berridge, M.J. and Brown, K.D. (1988) Biochem. J. 249, 917–920.
[12] Katada, T., Oinuma, M. and Ui, M. (1986) J. Biol. Chem. 261, 8182–8191.
[13] Okajima, F. and Ui, M. (1984) J. Biol. Chem. 259, 13863–13871.
[14] Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) Biochem. J. 206, 587–597.
[15] Oka, Y. and Orth, D.N. (1983) J. Clin. Invest. 72, 249–259.
[16] Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A. and Schlessinger, J. (1989) Cell 57, 1101–1107.
[17] Meisenhelder, J., Suh, P.-G., Rhee, S.G. and Hunter, T. (1989) Cell 57, 1109–1122.
[18] Nishibe, S., Wahl, M.I., Hernandez-Sotomayor, S.M.T., Tonks, N.K., Rhee, S.G. and Carpenter, G. (1990) Science 250, 1253–1256.
[19] Carpenter, G. and Cohen, S. (1990) J. Biol. Chem. 265, 7709–7712.
[20] Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203–212.
[21] Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S. and Takenawa, T. (1988) Science 239, 640–643.
[22] Smith, M.R., Ryu, S., Suh, P., Rhee, S. and Kung, H. (1989) Proc. Natl. Acad. Sci. USA 86, 3659–3663.
[23] Berchuck, A., MacDonald, P.C., Milewich, L. and Cazey, M.L. (1988) Mol. Cell. Endocrinol. 57, 87–92.