Human Growth Hormone-stimulated Growth of Human Cultured Lymphocytes (IM-9) and Its Inhibition by Phorbol Diesters through Down-regulation of the Hormone Receptors

POSSIBLE INVOLVEMENT OF PHOSPHORYLATION OF A 55,000 MOLECULAR WEIGHT PROTEIN ASSOCIATED WITH THE RECEPTOR IN THE DOWN-REGULATION*

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We report in this paper that human growth hormone (hGH) stimulates the growth of human cultured IM-9 lymphocytes in a low concentration (3%) of serum. The hormone-stimulated growth was inhibited with the phorbol diesters phorbol 12-myristate 13-acetate and phorbol 12,13-didecanoate, but not with the phorbol monoesters phorbol 12-myristate and phorbol 12,13-dibutyrate (PDBu). The binding experiments of 125I-hGH to the phorbol diester-treated cells and to their detergent-solubilized receptors revealed that the phorbol diesters caused internalization of the hGH receptors from the cell surfaces but did not significantly affect their affinity (Kd = 8.5 × 10⁻⁸ M⁻¹). About half of the receptors (1.4 × 10⁶/cell) were internalized in 30 min at 37 °C, and the half-effective doses of phorbol 12-myristate 13-acetate and PDBu were 5 and 35 nM, respectively. When culture was continued after washing with the culture medium, the phorbol diester-treated cells recovered their hGH-responsive growth, and the number of the surface hGH receptors was restored. The down-regulation of the hormone receptor was also induced with another phorbol diester, phorbol 12,13-didecanoate, but not with the phorbol or phorbol monoesters phorbol 12-myristate and phorbol 12-acetate. The synthetic activators of protein kinase C 1-oleoyl-2-acetyl-glycerol and N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide had an effect similar to that of the phorbol diesters. Staurosporine and sphingosine, inhibitors of protein kinase C, inhibited the phorbol diester-caused down-regulation with a half-inhibitory dose (IC₅₀) of 8 nM and 130 μM, respectively. This suggests that protein kinase C was involved in the reaction. When ³²P⁺-loaded IM-9 cells were stimulated with PDBu at 37 °C, the phosphorylation of M, 55,000, 88,000, and 114,000 proteins increased rapidly. The PDBu-stimulated phosphorylation of 55,000 protein was also inhibited by staurosporine at 10 nM, which was a comparable concentration to inhibit the phorbol diester-induced down-regulation of hGH receptors. Furthermore, among these proteins, the 55,000 protein was specifically coisolated with the hGH receptors by three different experiments: 1) immunoprecipitation by anti-hGH antibody; 2) immunosolubilization using protein A-cellulose column; and 3) affinity purification by hGH-fixed agarose gel.

These results suggest that phorbol diesters reduce the hGH-stimulated growth of cultured IM-9 lymphocytes by the down-regulation of hGH receptors and that the receptor-associated 55,000 protein may be involved in this regulation through phosphorylation by protein kinase C.

Human growth hormone (hGH), a peptide hormone (molecular weight, 22,000) required for normal growth in man, has been synthesized by biotechnological methods and is used widely as a therapeutic drug for growth-deficient syndromes. However, the mechanisms of the hormone action have not been established mainly because it has different activities, both direct and indirect (via insulin-like growth factor 1), and shows various activities in different tissues and cells, including liver, cartilage, lymphocytes, and adipocytes (1-3). The amino acid sequences of the growth hormone receptors of rabbit and human livers have been deduced from sequences of cloned DNAs, and the structures have been found to be distinct from the receptors of other growth factors (4). Recently, it has been demonstrated that growth hormone has an important role in regulating many immunological phenomena via specific receptors on lymphocytes or phagocytes (for review, see 5). Human IM-9 lymphocytes, which have high affinity receptors for growth hormone (6), have been used for the radioreceptor assay of growth hormones (7) or for biochemical studies of the hormone receptors (8-10). The biological functions and dynamics of the receptors have, however, been obscure, partly because hGH does not promote growth in cultured lymphocytes under common culture conditions.

In this paper, we present lines of evidence that hGH is involved in controlling the growth of IM-9 lymphocytes. We also describe the mechanisms by which phorbol diesters regulate the hGH receptors in these cells.

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‡ The abbreviations used are: hGH, human growth hormone; G1H, growth hormone; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; HBSS, Hank's balanced salt solution; TBS, Tris-HCl-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
MATERIALS AND METHODS

Reagents—hGH was provided by Sumitomo Pharmaceutical Co. (Osaka, Japan). The radioiodination of hGH was carried out according to the method of the Amer sham Corp. Phorbol esters were obtained from LC Services Co. (Woburn, MA). Sphingosine was purchased from Du Pont—New England Nuclear. Rabbit anti-hGH antibody was isolated from the rabbit immunized with hGH four times in 3 months by affinity chromatography on Protein A-Cellulofine (Seikagaku-Kogyo, Tokyo). Anti-Gel 15 (Bio-Rad) was used for preparing hGH-fixed agarose gel.

All other chemicals were commercial preparations of the highest purity.

Cell Culture and Measurement of Cell Growth—Human IM-9 lymphocytes were cultured at 37°C in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (Flow Laboratories, Australia), 10 mM HEPES (pH 7.5), and kanamycin (60 µg/ml). No contamination by mycoplasma was detected. Cell growth was measured as incorporation of [3H]thymidine into the cells, treated as follows. IM-9 lymphocytes growing at log phase and collected by centrifugation (100 x g, 10 min) at 4°C were suspended in the culture medium containing 3% fetal bovine serum and the indicated concentration of hGH (1 x 10⁵ cells/ml). The cells were cultured at 37°C for 24 h. The cells were then treated with PMA (100 nM), washed as described later, and incubated for 24 h in culture medium containing 3% fetal bovine serum and the indicated concentration of hGH (1 x 10⁵ cells/ml) and cultured at 37°C for 24 h. The cells were then treated with PMA and washed as described later, and incubated for 24 h in culture medium containing 3% fetal bovine serum and the indicated concentration of hGH (1 x 10⁵ cells/ml) and cultured at 37°C for 24 h.

Phorbol Diester Treatment—IM-9 lymphocytes (4 x 10⁵) were washed with HBSS containing 5 mM HEPES (pH 7.2) and resuspended in 4 ml of HBSS containing 0.1% Nonidet P-40, 0.3 mM benzamidine, 20 mM NaF, 2 mM potassium pyrophosphate, and 1 mM MgCl₂. Two microliters of hGH-fixed agarose gel (250 µg of hGH/ml of gel) which had been equilibrated previously with TBS containing 0.1% Nonidet P-40, 0.3 mM benzamidine, 20 mM NaF, and 2 mM potassium pyrophosphate was used as a label for the photoaffinity labeling. The column was thoroughly washed with the buffer, and the elution was carried out with 50 mM acetic acid buffer (pH 3.0) containing 0.1% Nonidet P-40 and 0.3 mM benzamidine. The eluate was concentrated to 1 ml by a Microcon Millipore (10,000), and 30 µl of sample was analyzed by SDS-PAGE and autoradiography. When ¹²⁵I-hGH (final concentration, 0.5 nM) was used as a ligand for the photoaffinity labeling, all experimental procedures were as described previously except for the P₂O₅-loaded cells. The percent of cross-linking was about 1.7.

Affinity Isolation of hGH Receptors by hGH Agarose Gel—The cell extract, prepared as above, was applied to a column (0.5 x 2 cm) of hGH-fixed agarose gel (250 µg of hGH/ml of gel) which had been equilibrated previously with TBS containing 0.1% Nonidet P-40, 0.3 mM phenylmethylsulfonyl fluoride, and the columns were washed with TBS until no radioactive material was detected in the NaCl-eluted fraction. hGH-bound receptors were eluted with 2.8 ml of the buffer containing 0.5 M NaCl, and specifically bound hGH receptors were eluted with 2.8 ml of the buffer containing 0.5 M NaCl and 6 M urea. The recovery of the activity in the urea-eluted fraction was calculated to be 21% after the removal of urea by dialysis. The samples were concentrated by Molcut II and analyzed on SDS-PAGE by autoradiography.

RESULTS

Human Growth Hormone-stimulated Growth of IM-9 Lymphocytes and Inhibitory Effect of Phorbol Diesters on the Growth—The growth of IM-9 lymphocytes is dependent on the...
presence of fetal bovine serum in the culture medium, and usually 10% of the serum is required for normal growth. When the concentration of the serum was lowered to 3%, cell growth was reduced to less than 30% 48 h after the culture had begun (data not shown). However, cultivating IM-9 lymphocytes in RPMI 1640 containing 3% fetal bovine serum for 24 h caused the cells to respond dose dependently to hGH in the culture medium (Fig. 1). In the presence of 10 nM hGH, growth was restored to more than 80% of that in 10% fetal bovine serum in RPMI 1640, as measured by [3H]thymidine incorporation into DNA. Since other human cell lines such as K-562, HL-60, and CCRF-CEM, which do not have detectable hGH receptors on their surfaces, showed no response to hGH under the same culture conditions (data not shown), the effect of hGH on the growth stimulation of IM-9 cells under the fetal bovine serum-lowered conditions appears to be a receptor-mediated phenomenon.

Serum concentration in the culture medium seemed to be critical to elicit the hGH dependence of the growth. IM-9 cells kept their ability to grow in RPMI 1640 containing 5% fetal bovine serum and were able to respond only weakly to hGH. When the cells were cultured in RPMI 1640 containing 1% of fetal bovine serum, more than half of the cells died within 24 h.

It has been shown that phorbol diesters can modulate cell growth positively (15-17) or negatively (18); therefore, we investigated the effect of PMA on the hGH-dependent growth of IM-9 cells in RPMI 1640 containing 3% fetal bovine serum. As shown in Fig. 1, the addition of PMA in the culture medium (100 nM) completely abolished the responsiveness of the cells to hGH, although PMA did not show any cytotoxicity at this concentration (the viability of the cells tested by the dye exclusion test was 98%). Washing the cells to remove unbound PMA restored the cell responsiveness to hGH under the same culture conditions.

**Phorbol Diester-induced Decrease of [125I]-hGH Binding**

To elucidate the mechanisms of PMA-caused cell unresponsiveness to hGH, we studied the effects of phorbol diesters on [125I]-hGH binding to IM-9 cells. The cells were preincubated with a phorbol diester in HBSS at 37 °C for 30 min and harvested. [125I]-hGH was bound at 4 °C for 2 h in 10 mM Tris-HCl (pH 7.2)-buffered saline. Fig. 2 shows that PMA and PDBu dose dependently decreased the specific binding of [125I]-hGH to the receptors on the cell surfaces. Half-effective doses of PMA and PDBu were 5 and 35 nM, respectively. These effects of phorbol diesters did not occur when the cells were treated at 4 °C, although the specific binding of the [125I]-hGH to the IM-9 cells was not affected at this temperature (data not shown). It is possible that the mobility of the hGH receptors on the cell surfaces is involved in the phorbol diester-induced decrease of [125I]-hGH binding.

**Time Course of Phorbol Diester-induced Change of hGH Receptors on IM-9 Cell Surfaces**

To investigate the biochemical effects on the hGH receptors which occur after the phorbol diester treatment, we studied the time course of the effect of PDBu (1 μM) on the [125I]-hGH binding to the IM-9 cells. Fig. 3A shows that the specific binding of [125I]-hGH to IM-9 cells decreased rapidly depending on the time of pretreatment with PDBu. After 30 min, the lowest level of about one-half the original binding value was reached. In the presence of PDBu in the culture medium, the decreased level of hGH receptors on the cell surfaces was continued for at least 20 h without a significant decline of cell viability as indicated by the trypan blue dye exclusion test.
Phorbol Diester-induced Down-regulation of GH Receptor

In another experiment, the cells were treated with PDBu for 30 min to reduce the cell surface binding sites, washed with culture medium to remove the unbound drug, and cultured for 20 h in RPMI 1640 containing 10% fetal bovine serum to study the change in the number of receptors on the cell surfaces and cellular morphology. A typical result is shown in Fig. 3B. \( {\text{^{125}I}\text{-hGH}} \) binding capacity remained at a low level (about 50% of the original capacity) for about 10 h after the PDBu treatment and then gradually recovered to the same level as the phorbol diester-un-treated cells 20 h after the removal of the drug from the medium. These kinetic profiles of the change of the number of hGH receptors after the phorbol diester treatment (Fig. 3, A and B), together with the result shown in Fig. 2 appear to explain the drug-induced change in the responsiveness to the hormone (Fig. 1).

The phorbol diester treatment caused the IM-9 cell membranes to become ruffled or damaged, and washing followed by incubation for 20 h allowed the membranes to return to sharply outlined forms similar to those in untreated cells.

Internalization of hGH Receptors Caused by Phorbol Diesters—Phorbol diesters have been shown to lead to the enhanced internalization of a variety of cell surface receptors (19-24). Therefore, we investigated whether the phorbol diester-induced change of hGH binding was due to a decrease in the number of the hGH receptors, a decrease in their affinity, or both. Scatchard plot analysis (25) of PDBu-treated and -untreated IM-9 cells revealed that the number of the receptors of hGH was decreased from 2.7 x 10^5 to 1.3 x 10^5 sites/cell by PDBu, but there was no significant change in their affinity (\( K_d = 8.5 x 10^{-10} \)M^-1) (Fig. 4). These results are fully consistent with the observation shown in Fig. 3A which indicates that the binding of \( {\text{^{125}I}\text{-hGH}} \) was decreased by PDBu treatment to about 50% of that of untreated cells. When all the receptors were extracted from the whole cells by sonication in the presence of Nonidet P-40, which solubilized the active receptors with the highest recovery (about 84%) among the detergents tested, almost the same hGH-binding activity appeared in the extracts from either PDBu-treated or -untreated cells (Table I).

These results indicate that the decrease in the binding of \( {\text{^{125}I}\text{-hGH}} \) by PDBu treatment of IM-9 cells was not due to a decrease in the affinity of the receptors to this ligand but was induced by the internalization of the receptors. The phorbol diester-induced loss of the proliferative responsiveness of the cells to hGH (Fig. 1) can also be explained by the drug-induced internalization of the hGH receptors.

Effects of Various Phorbol Ester Derivatives and Activators of Protein Kinase C on \( {\text{^{125}I}\text{-hGH}} \) Binding—Since protein kinase C is a receptor of phorbol diesters that also activate the enzyme directly (26), we examined whether or not various phorbol derivatives and activators of protein kinase C could down-regulate the hGH receptors. Phorbol diesters including PDBu, PMA, phorbol 12,13-dibenzoate, and phorbol 12,13-didecanoate down-regulated the hGH receptors, but phorbol monoesters phorbol E-myristate and phorbol 12,13-acetate did not (Fig. 5). The spectrum of the activity of phorbol ester derivatives was highly correlated with their ability to activate protein kinase C (26). 1-Oleoyl-2-acetyl-glycerol and \( N^-6(\text{phenylhexyl})^-5\text{-chloro}-1\text{-naphthalenesulfonamide} \), synthetic activators of protein kinase C (27, 28), also moderately down-regulated the hGH receptors. PDBu, PMA, and phorbol 12,13-didecanoate had the greatest effect.
Phorbol Diester-induced Down-regulation of GH Receptor

Bound [125I]hGH (X10^4 cpm)

|      | 0   | 1.5 | 2.0 | 2.5 | 3.0 | 3.5 |
|------|-----|-----|-----|-----|-----|-----|
| None |     |     |     |     |     |     |
| PDBu|     |     |     |     |     |     |
| PMA |     |     |     |     |     |     |
| PDBz|     |     |     |     |     |     |
| PDD |     |     |     |     |     |     |
| PHR |     |     |     |     |     |     |
| PM  |     |     |     |     |     |     |
| PA  |     |     |     |     |     |     |
| OAG |     |     |     |     |     |     |
| SC-9|     |     |     |     |     |     |

Fig. 5. Effects of various phorbol derivatives and activators of protein kinase C on [125I]-hGH binding to IM-9 cells. The cells were treated with the drugs at 37°C for 30 min, and the specific binding of [125I]-hGH to 1.3 x 10^6 cells was determined as in Fig. 2. PDBz, phorbol 12,13-dibenoate; PDD, phorbol 12,13-didecanoate; PHR, phorbol; PM, phorbol 12-myristate; PA, phorbol 13-acetate. The concentrations of all of the phorbol derivatives, 1-oleyl-2-acetyl-glycerol (OAG) and N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), were 100 nM, 30 μM, and 50 μM, respectively.

and these drugs decreased the hGH receptors on the cell surfaces up to about 50%.

These results indicate the involvement of protein kinase C in the down-regulation of the hGH receptors on IM-9 cells.

Effects of Staurosporine and Sphingosine on the Down-regulation of hGH Receptors—If protein kinase C is involved in the down-regulation of hGH receptors on IM-9 cells, an inhibitor of this enzyme should prevent the down-regulation. We therefore investigated the effect of staurosporine, a strong inhibitor of protein kinase C (29), on the PDBu-induced decrease of hGH receptors on IM-9 lymphocyte surfaces. A typical result is shown in Fig. 6A. Staurosporine strongly inhibited the phorbol diester-induced down-regulation of the hGH receptors at low concentrations (IC_{50} = 7 nM). It is reported that the IC_{50} of staurosporine against isolated protein kinase C was 2.7 nM (29). Therefore it appears that the inhibition of the down-regulation by staurosporine was caused by the suppression of protein kinase C activity. Sphingosine, which was used as an inhibitor of protein kinase C (30, 31), also showed an inhibitory activity against the down-regulation (Fig. 6B). These results suggest that protein phosphorylation catalyzed by protein kinase C is essential to the down-regulation of the receptors.

Phorbol Diester-stimulated Protein Phosphorylation and Its Inhibition by Staurosporine—We studied the effect of phorbol diester on protein phosphorylation of IM-9 lymphocytes preincubated with ^32P_i. As shown in Fig. 7, PDBu stimulated the phosphorylation of M, 55,000, 88,000, and 114,000 proteins. The reaction was rapid and reached a maximum within 2 min. This clearly preceded the down-regulation of the hGH receptors. The PDBu-stimulated phosphorylation was inhibited by staurosporine, dose dependently. The effective concentration of staurosporine for the phosphorylation of the 55,000 protein was comparable with that in the inhibition of PDBu-induced down-regulation (Fig. 6A). To inhibit the phosphorylation of M, 88,000 and 114,000 proteins, higher concentrations of staurosporine were required.

Immunoprecipitation of hGH Receptors—It has been reported that some receptors for peptide ligands are phosphorylated by phorbol diester-activated protein kinase C (19-22, 32). We investigated whether the PDBu-stimulated phosphoproteins were hGH receptors or not. After PDBu-stimulated phosphorylation was performed as in Fig. 7, hGH receptors were solubilized with Nonidet P-40, and ligand-receptor complexes were made by addition of hGH. The complexes were then precipitated by anti-hGH antibody. The recovery of hGH receptors in the precipitate was estimated to be more than 88% from a similar cold experiment.

As shown in Fig. 8, the PDBu-stimulated 55,000 phosphoprotein was specifically precipitated with hGH and anti-hGH antibody. Several other phosphoproteins were also observed in the precipitates; however, the 55,000 phosphoprotein seemed to be a constituent of hGH receptors or a receptor-associated protein. To clarify this point, we tried to purify the hGH receptors by the following two different techniques.
Phorbol Diester-induced Down-regulation of GH Receptor

FIG. 7. PDBu-stimulated protein phosphorylation of IM-9 lymphocytes and its inhibition by staurosporine. After preincubation with PDBu at 37°C for 60 min, the cells were incubated with staurosporine or vehicle (dimethyl sulfoxide) for 10 min and then treated with 1 μM PDBu for 5 min at 37°C. The phosphoproteins were analyzed on 7.5% gel by SDS-PAGE followed by autoradiography. Each lane represents phosphoprotein derived from 1.1 × 10⁶ cells. Lane 1, no PDBu (control); lane 2, with PDBu; lane 3, 1 nM staurosporine and PDBu; lane 4, 10 nM staurosporine and PDBu; lane 5, 100 nM staurosporine and PDBu.

Immunoisolation of hGH Receptor by Protein A-Cellulose—We cross-linked hGH with its receptors on the ³²P-loaded cells using ultraviolet irradiation (14). The hGH-receptor conjugates were isolated from the solubilized cells as an anti-hGH antibody-hGH-receptor ternary complex by affinity chromatography on a protein A-cellulose column. The direct affinity labeling by UV irradiation was mild and easy to control with much less overcross-linking. To determine the molecular weight of the hGH-receptor conjugates, we first conducted a similar experiment with ¹²⁵I-hGH and ³²P-unloaded cells. The hGH-receptor conjugates appeared as a rather broad band with a molecular weight of 135,000 (Fig. 9A, lane 1). Since this band disappeared in the presence of 100 times excess of unlabeled hGH (Fig. 9A, lane 2), this 135,000 protein band seems to be the cross-linked hGH-receptor conjugates. These observations are consistent with the results reported previously (8, 9). In a similar experiment, when the cells were prelabeled with ³²P, treated with PDBu for 3 min, and the hGH and receptors were cross-linked by UV irradiation, no radioactive protein band was detected with a molecular weight of 135,000 (Fig. 9B). In this case, two radioactive bands with lower molecular weight (50,000 and 55,000) were observed. They were not observed when normal rabbit IgG was used in place of the anti-hGH antibody. This 55,000 band appeared only with the cells treated with PDBu and seems to be identical to the 55,000 band shown in Figs. 7 and 8. Identity of the M, 50,000 protein is unclear at present. Since the phosphoprotein was observed only when protein A column was used, it may not be related with hGH receptors. However, we cannot rule out the possibility that it is a receptor-associated protein.

Isolation of hGH Receptor by hGH-Agarose Gel—We isolated the hGH receptors directly using hGH-fixed agarose gel without anti-hGH antibody. More than 99% of hGH-binding activity was absorbed to the affinity gel, and after extensive washing, a purified hGH receptor fraction was obtained by elution with 6 M urea. Although the PDBu treatment caused no significant change in the amount of 55,000 protein, in the purified receptor fraction, the autoradiograms show clearly that the 55,000 protein phosphorylated with PDBu was copurified with the hGH receptor (Fig. 10B). As ³²P was not detected at the position of hGH receptors (110,000), the receptor itself does not seem to be phosphorylated. The 55,000 protein, a portion of which seemed to associate with the hGH receptors, may play an important role in the phorbol diester-induced down-regulation of the receptor through its phosphorylation.

DISCUSSION

In this paper we present simple culture conditions for observing hGH-stimulated growth of IM-9 lymphocytes. When IM-9 cells were cultured in a medium with low serum content (3%) for 24 h, the cells acquired the hGH dependence for growth. However, with a serum content less than 1%, the
cells did not proliferate even in the presence of hGH. Above
5% serum, the cells readily proliferated, but the dependence
was much lower than it was in 3% serum. When the serum
concentration in the medium was lowered from 10 to 3% and
the cells were immediately stimulated by adding hGH, no
clear growth response to the hormone was observed (data not
shown). Therefore, it seems necessary to culture the cells in
a medium containing 3% serum for more than several hours
to obtain substantial responsiveness to the hormone.

The hGH-stimulated growth of IM-9 cells appears to be a
receptor-mediated event for the following reasons. 1) Other
human cell lines such as K-562, HL-60, CCRF-CEM (32) and
WI-38, which have no detectable hGH receptors on their
surfaces, did not respond to hGH in a medium with low serum
content (3%) after the same treatment of the cells. 2) When
the cells were treated with a phorbol diester, the number of
hGH receptors expressed on the cell surfaces was decreased
by half of those on the untreated cells, and the hormone
responsiveness was completely abolished (Figs. 1 and 3). 3)
After washing and incubation, both the hGH responsiveness
(Fig. 1) and the number of receptors were restored in the
phorbol diester-treated IM-9 cells (Fig. 3). Since the number
of the hGH receptors and their affinity for the ligand re-
mained unchanged during the cultivation of the cells in me-
dium containing 3% serum, the acquired dependence of the
growth on hGH was not able to be explained simply by the
changes in the receptor number and/or affinity for the ligand.

We examined the changes of the hGH receptors and the
effects of the phorbol diesters, activators, and inhibitors of
protein kinase C on the receptor internalization. The results
shown in this paper indicate that hGH receptors of IM-9 cells
are linked to a system that modulates the growth of the cells.
However, whether or not the hGH receptors are functioning
in a medium with high serum concentration (10%) remains
undetermined. One possibility is that the hGH receptors are
always working, and when the serum concentration is high,
endogenous growth hormone is utilized. By lowering the
concentration of endogenous growth hormone by decreasing
the serum concentration in the medium, the cell may become
responsive to the exogenous growth hormone. However, this
possibility is unlikely since the cells in low serum concentra-
tion must be cultivated for more than several hours to acquire
responsiveness. An alternative explanation is that when the
cells are exposed to the low serum condition, which is appar-
ently unfavorable to growth, the cells may use a system linked
to the hGH receptors for growth, and more than several hours
is necessary for the cells to switch to this system.

The present studies demonstrated that tumor-promoting
phorbol diesters down-regulated without ligand binding both
the hGH-stimulated growth and the hGH receptors of IM-9
lymphocytes through an internalization of hGH receptors
without any change of their affinity to the hormone. It has
been reported that hGH receptors were down-regulated by a
ligand-induced process (9, 33, 34). The differences in the
mechanisms of the phorbol diester-induced and ligand-in-
duced down-regulation of hGH receptors are not clear at
present.

Receptor internalization has received much attention in
relation to the possible role of specific phosphorylation. In
particular, studies have been concentrated on the role of
protein kinase C in the internalization of various receptors.
(19–24). The fact that activators of protein kinase C decrease the number of specific binding sites of hGH on IM-9 cells (Fig. 4) and that staurosporine and sphingosine, inhibitors of protein kinase C, inhibit the PDBu-induced decrease of the number of hGH receptors (Fig. 6) strongly suggest that protein kinase C is involved in the phorbol diester-induced down-regulation of the hGH receptors. In general, phosphorylation machineries on cell membranes have been shown to be involved in signal transduction and in the dynamic movement or interaction of proteins (35–37). Recently, it has been reported that phosphorylation of certain receptors for peptide ligands induced down-regulation either through internalization (such as transferrin receptors (19, 20), T-cell receptors (21, 22), and insulin receptors (38, 39)) or by decreasing their affinity (such as epidermal growth factor receptors (18) and tumor necrosis factor receptors (24)).

As with other receptor systems (19–24), the internalization of hGH receptors reached a plateau at 50% reduction of surface receptors. Two different explanations are possible for this. 1) As in the case of transferrin receptors (19), all the receptors are recycled under a rate constant such that half of the receptors are exposed to the cell surfaces. 2) There are two distinct sets of hGH receptor systems on IM-9 cell surfaces, only one of which responds to growth control, and its expression is modulated on the cell surface by phorbol diesters. The other set of hGH receptors is not coupled to growth control and always remains on the cell surfaces.

When IM-9 cells were treated with PDBu, the M₅5,000 protein and some other proteins were rapidly phosphorylated. Although the 55,000 phosphoprotein was partly copurified with hGH receptors, it does not seem to be a hGH-binding molecule itself, since the molecular weight of the hGH receptor of IM-9 cells has been estimated to be 110,000 (8, 9). We confirmed this by the ultraviolet-cross-linked cross-linking of hGH to its receptors (Fig. 9). By this technique, covalently bound hGH-receptor conjugates migrated by SDS-PAGE as a rather broad band with a molecular weight of 135,000. When the cross-linking was carried out using the cells preincubated with ³²P, according to the conditions in the experiment shown in Fig. 9A, ³²P was not incorporated into the 135,000 protein (Fig. 9B). We therefore suggest that the hGH receptors of IM-9 cells are not phosphorylated in response to PDBu.

Although the function of the 55,000 protein remains to be determined, we assume that at least a portion of it is associated with hGH receptors to form a functional complex, since the internalization is modulated on the cell surface by phorbol diesters (40).

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