don and a stretch of 25 A's at the 3′ end of the pLaI-3 cDNA fragment. Although the region is dramatically different in length from that of mammalian insulin, the four mRNA's contain two common features: the AAUAAA sequence, 22 bases from the polyadenylated tail, which is present in most eukaryotic polyadenylated mRNA's, and a stretch of nucleotides rich in cytidine and lacking thymidine found in the early portion of the 3′ untranslated region. Although a similar cytidine-rich sequence is present in the same region of human (*15*), rat (*16*), and bovine (*17*) growth hormone and human chorionic somatomammotropin hormone (*18*), it is not evident in other hormone mRNA's that have been sequenced, such as human chorionic gonadotropin (*19*), rat prolactin (*20*), bovine ACTH (*21*), or fish somatostatin (*8*).

In addition to establishing the sequence for the preproinsulin peptide, the fish insulin cDNA provides a specific probe for the detection and isolation of the genomic DNA fragment (or fragments) containing the fish insulin gene and for analysis of the genomes of more divergent organisms for insulin-like sequences.

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Modulation of Epidermal Growth Factor Receptors on 3T3 Cells by Platelet-Derived Growth Factor

Abstract. *Platelet-derived growth factor does not compete with epidermal growth factor (EGF) for binding to EGF receptors on the murine 3T3 cell surface, but it modulates EGF receptors in two ways: (i) it induces a transient down regulation of EGF receptors and (ii) it inhibits EGF-induced down regulation of EGF receptors. These data suggest a common cellular internalization mechanism for the receptors for both hormones.*

Traditionally, cell culture media have been supplemented with serum, which contains a number of polypeptide growth factors that induce cellular proliferation (*1*). Several of these growth factors, including platelet-derived growth factor (PDGF) (*1*), fibroblast growth factor (FGF) (*2*), and epidermal growth factor (EGF) (*3*), have been isolated from their tissues of origin, and their mitogenic activity for cultured cells has been a subject of intensive investigation in several laboratories. The best studied of these hormones is EGF, which initiates its action by binding to specific receptors on the surfaces of target cells (*4*). Following this highly specific association with its surface receptors, EGF is internalized by cells and degraded by lysosomal proteases; the internalization and degradation of EGF is associated with EGF receptor down regulation, that is, the loss of EGF binding activity by cells (*5*). Studies with affinity-labeled EGF receptors have demonstrated that EGF receptors are down-regulated by internalization and degradation in lysosomes. The internalization and degradation of affini-

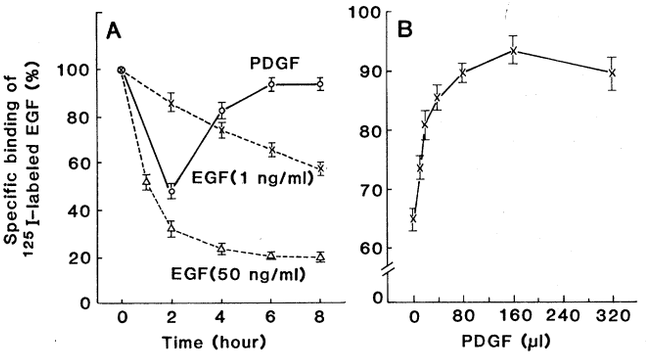

Fig. 1. (A) PDGF-induced transient down regulation of EGF receptors. The 3T3 cells (clone 42, obtained from G. Todaro) were grown to confluence on 16-mm culture dishes in medium containing 10 percent fetal calf serum (FCS) (*6*, *7*). Prior to use, the cells were incubated for another 24 hours in 0.5 ml of medium plus 0.5 percent FCS. Unlabeled EGF at concentrations of 1 ng/ml (×) or 50 ng/ml (△), or 40 μl of a stock solution of PDGF (○), were added to the dishes and the cells were incubated further at 37°C. At the times indicated, medium containing EGF or PDGF was removed by aspiration, the cells were washed twice, and binding of ^{125}I-labeled EGF was determined after 60 minutes of incubation at 23°C (*6*, *7*). Maximum binding of ^{125}I-labeled EGF indicated approximately 70,000 EGF receptors per cell. All values are corrected for nonspecific EGF binding occurring in the presence of 5 μ*M* EGF, and all values are normalized to EGF bound by cells incubated in the absence of PDGF or unlabeled EGF prior to assay of ^{125}I-labeled binding. The EGF was purified and labeled with ^{125}I as described previously (*6*, *7*). The PDGF was purified 30,000-fold from human serum (*9*). Seven microliters of the stock solution of PDGF (780 ng/ml) induced a four-fold (maximal) stimulation of DNA synthesis, determined by measuring [^{3}H]thymidine uptake into DNA (*7*) during a 1-hour exposure period occurring 24 hours after the addition of PDGF to serum-starved cells. (B) Inhibition of EGF-induced down regulation of EGF receptors by PDGF. The indicated amounts of PDGF were added to cells at 37°C and EGF (1 ng/ml) was added 1 hour later. After an additional 6 hours of incubation at 37°C, PDGF and EGF were removed by aspiration, the cells were washed twice, and the binding of ^{125}I-labeled EGF was determined (*6*, *7*).

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ty-labeled EGF receptors parallels the loss of EGF binding activity from the cell surface (*6*, *7*).

Internalization of hormone-receptor complexes is a common response to peptide hormones (*8*). We therefore have tested for interactions between peptide hormone receptors at the level of their cell surface binding and internalization, since evidence for interactions at this level would indicate a common mechanism for peptide hormone receptor down regulation. A partially purified preparation of PDGF was tested for its influence on the behavior of EGF receptors. The preparation of PDGF was purified 30,000-fold from outdated platelet-rich human plasma (*9*) and did not inhibit the binding of ^{125}I-labeled EGF to cells at the highest PDGF concentration used (Table 1; compare samples 6 and 7).

The PDGF modulated EGF receptors of cultured murine 3T3 cells in two ways (Fig. 1). First, it induced a transient loss of EGF binding activity (Fig. 1A). This down-regulatory phenomenon was most prominent 2 hours after PDGF addition and was followed by recovery of EGF binding activity within 6 to 8 hours. Transient down regulation cannot be explained by a change in EGF receptor affinity for EGF; its extent was independent of the EGF concentration in the binding assay (Table 1, compare samples 1 and 4 with 8 and 9). The rebound in EGF binding activity 4 hours after PDGF addition did not result from loss of the activity which gave rise to the initial decrease in EGF binding. When a sample of medium containing PDGF was incubated with cells for 6 hours and tested for its ability to induce transient down regulation of EGF receptors, no loss in this ability was observed (Table 1; compare samples 4 and 5). The PDGF-induced down regulation was unlike that which occurred in response to EGF, where EGF binding remained at the reduced level (Fig. 1A). The dose response to PDGF for transient down regulation of EGF receptors closely paralleled the PDGF dose response for stimulation of cellular DNA synthesis. The PDGF-induced transient down regulation of the EGF receptors was half-maximal (Table 1) at approximately twice the dose required for half-maximal stimulation of [^{3}H]thymidine incorporation into DNA during a 1-hour period of labeling 24 hours after PDGF addition (data not shown).

The second modulatory effect of PDGF on EGF receptors is an inhibition of EGF-induced down regulation of EGF receptors (Fig. 1B). Cells were first incubated with PDGF, unlabeled EGF was added 1 hour later, and the incubation was continued for an additional 6 hours—a time sufficient for recovery from PDGF-induced transient down regulation of EGF receptors (Fig. 1A). The cells were then washed thoroughly to remove unassociated PDGF, and labeled EGF was added to determine the EGF binding activity which remained. Control experiments, where labeled EGF was used during the aforementioned 6-hour incubation period, detected too little persistently bound EGF (*10*) to explain the observed reductions in EGF binding. PDGF had a long-term modulatory influence on the ability of EGF to induce down regulation of its own receptors (Fig. 1B). This blockade of EGF-induced down regulation of EGF receptors persisted for at least 12 hours, and did not appear to be exerted through a PDGF-induced displacement of EGF from EGF receptors since PDGF did not inhibit EGF binding (Table 1; compare samples 6 and 7). The inhibitory phenomenon was maximal at approximately twice the PDGF concentration which produced maximal transient down regulation of EGF receptors (Table 1).

Table 1. Modulation of EGF receptors by PDGF. The medium was removed from cells incubated for 24 hours in medium containing 0.5 percent FCS, and 60 μl of fresh medium containing 0.5 percent FCS and no PDGF or PDGF was added to each well for a 2-hour incubation period at 37°C. The cells were then processed for determination of EGF binding as described in Fig. 1 and at the EGF concentrations indicated. Nonspecific binding was determined in samples incubated with 5 n*M* or 50 n*M* ^{125}I-labeled EGF plus 5 μ*M* unlabeled EGF and did not exceed 5 percent of specific binding. Nonspecific binding is deducted from the values shown. Duplicate determinations were made for all samples and the average value is given (range in parentheses).

Sample	Additions to cell system for 2 hours (μl/0.5 ml)	^{125}I-labeled EGF	
		In binding assay (n*M*)	Specific binding (fmole/10^5 cells)
1	None	5	12.4 (11.8 to 13.0)
2	4	5	8.8 (8.3 to 9.3)
3	12	5	6.5 (6.2 to 6.8)
4	40	5	4.9 (4.6 to 5.2)
5	40*	5	5.3 (5.2 to 5.4)
6	320	5	5.0 (4.8 to 5.2)
7	320†	5	5.4 (5.1 to 5.7)
8	None	50	14.1 (13.9 to 14.3)
9	40	50	5.6 (5.2 to 6.0)

*The PDGF (40 μl/0.5 ml) was incubated with cells at 37°C for 6 hours in the standard system. This medium was then transferred to a second well and incubated with the cells for 2 hours prior to determination of EGF binding. †The ^{125}I-labeled EGF was added directly to the medium containing PDGF for the binding assay.

The results reported here for PDGF-induced modulation of EGF receptors have also been obtained with highly purified preparations of pituitary-derived FGF (*11*) and with relatively crude preparations of PDGF prepared after heating platelets at 100°C (*12*). In all of these studies, the dose responses of these hormones for modulating EGF receptors on murine 3T3 cells closely paralleled their dose responses for stimulating DNA synthesis (*13*). This indicates that the modulatory effects are mediated through events that occur after the binding of FGF or PDGF to their receptors. The transient down regulation of EGF receptors induced by either PDGF or FGF (*11*) could occur through the internalization of a common structure that responds to the ligand for any receptor which resides within it. The clustering of cell surface receptors in common regions is indicated by the internalization of a variety of fluorescent ligands within common vesicles (*14*). The existence of clustered regions of EGF receptors prior to the addition of EGF to cells is indicated by the presence of ferritin-labeled EGF clusters on fixed cells (*15*) and by the sizable fraction of EGF receptors that retain a particulate character when membranes are treated with nonionic detergent solution (*16*). Nearly half the EGF binding activity on murine 3T3 cell membranes sediments rapidly in nonionic detergent solution; this is approximately the fraction of EGF receptors that down-regulates transiently in response to PDGF (Fig. 1A). Regardless of the mechanism by which PDGF and FGF (*13*) modulate EGF receptors, the phenomena reported here show that polypeptide hormones modify the responsiveness of cells to other peptide hormones through modulation of receptor down regulation.

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Ratoon Stunting Disease of Sugarcane: Isolation of the Causal Bacterium

Abstract. *A small coryneform bacterium was consistently isolated from sugarcane with ratoon stunting disease and shown to be the causal agent. A similar bacterium was isolated from Bermuda grass. Both strains multiplied in sugarcane and Bermuda grass, but the Bermuda grass strain did not incite the symptoms of ratoon stunting disease in sugarcane. Shoot growth in Bermuda grass was retarded by both strains.*

Ratoon stunting disease (RSD) of sugarcane (*Saccharum* interspecific hybrids) occurs worldwide and causes significant yield losses, especially when sugarcane is stressed by lack of water (*1*). A virus was originally thought to cause RSD, but in 1973 a small coryneform bacterium was implicated as the causal agent (*2*, *3*). The bacterium was observed in expressed cane juices and xylem exudates by phase-contrast and dark-field microscopy and in extracted fibrovascular fluids and ultrathin sections of vascular bundles by electron microscopy. That the RSD-associated bacterium was the causal agent soon became widely accepted, even though the bacterium had not been isolated in axenic culture (*4*).

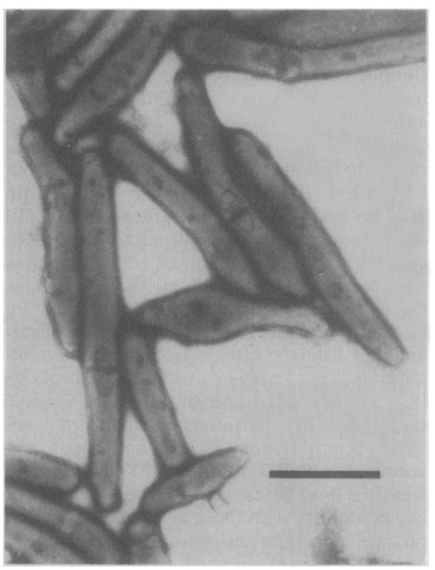

Fig. 1. Transmission electron micrograph of RSD bacteria from culture negatively stained with 1 percent phosphotungstate (*p*H 7.0). The bacteria resemble those seen in fibrovascular extracts (*3*) and expressed juice (*2*) of sugarcane with RSD. Scale bar, 1 μm.

We have now isolated the RSD-associated bacterium in axenic culture and have shown that it causes RSD. In addition, we have found that a disease which stunts Bermuda grass [*Cynodon dactylon* (L.) Pers.] is caused by a similar bacterium. The simultaneous occurrence in Bermuda grass with witches-broom symptoms of a bacterium morphologically resembling the RSD bacterium and a mycoplasma-like organism was previously described (*5*).

Diagnosis of RSD is difficult because internal symptoms do not develop in all sugarcane cultivars; the only external symptom is a nonspecific stunting associated with water stress. Thus, biological assays and serological tests have been used for diagnosis. As biological assays for RSD we used (i) the development of salmon-pink discoloration in the stem tissues just below the meristematic area in young plants of sugarcane cultivar CP 44-101 (*6*), (ii) orange-red discoloration of the vascular bundles at the nodes of mature cane (*1*), and (iii) wilting of sorghum-Sudan grass hybrid NB 280S upright (*7*). In addition, antiserum to RSD bacteria extracted from diseased sugarcane was used throughout the study in an indirect fluorescent antibody staining test to identify isolates (*8*).

The RSD bacterium was first isolated from inoculated hybrid NB 280S, which supports large populations of the bacterium (*9*). Internodes of mature plants were surface-sterilized (*10*), and fibrovascular fluid was obtained by vacuum extraction (*3*). The presence of the bacterium in the fluids was confirmed by phase-contrast microscopy (×1250), and plates containing semisolid media were inoculated with 10-μl portions of the fluid diluted two to ten times with 0.01*M* phosphate buffer (*p*H 6.8). After testing numerous formulations for their ability to support axenic cultures of RSD bacteria, we developed the SC medium (*11*). Colonies raised on the SC medium were 0.1 to 0.3 mm in diameter, circular with entire margins, convex, and nonpigmented after 2 weeks of aerobic incubation at 30°C.

We subsequently isolated the bacterium from infected sugarcane from Louisiana, Brazil, South Africa, and Japan. The bacterium was consistently isolated from sugarcane with RSD but not from healthy sugarcane (Table 1). Attempts were made to isolate the bacterium from fibrovascular fluids obtained from sugar-

Table 1. Isolation of the RSD bacterium. Plants of 20 sugarcane cultivars varying widely in susceptibility to RSD were sampled from Louisiana field plots. Although not completely effective for the elimination of RSD (*14*), heat treatment of seed pieces was used to establish a "healthy" plot with a low incidence of RSD. Two mature plants of each cultivar were sampled from the healthy plot and a "diseased" plot. A portion of the cane from each plant was bioassayed for RSD (*6*); the remainder was washed with soap and water, rinsed with water, washed with 70 percent ethanol, and flamed. An internodal section approximately 12 cm in length was aseptically excised and placed in a sterile, 50-ml conical tube for centrifugation at 1000 rev/min for 1 minute to extract fibrovascular fluid. The presence of bacteria in the extracts was determined by phase-contrast microscopy (×1250), and the SC medium was inoculated with serial tenfold dilutions of each extract to $1:10^{-8}$.

Plot	Result of RSD bioassay	Number of plants		
		Total	Bacteria observed	Bacteria isolated
Diseased	Positive	29	26	26
	Negative	10	4	5
Healthy	Positive	4	3	3
	Negative	36	2	2

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