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Stable association between the bovine papillomavirus E5 transforming protein and activated platelet-derived growth factor receptor in transformed mouse cells

LISA PETTI AND DANIEL DIMAIO*

Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06510

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ABSTRACT **The 44-amino acid E5 transforming protein of bovine papillomavirus is the shortest protein known to induce tumorigenic transformation of fibroblasts. We showed previously that expression of the E5 protein activates the cellular β receptor for platelet-derived growth factor (PDGF) and proposed that the activated receptor transmits the transforming signal to the cell. Here we use coimmunoprecipitation analysis to show that the E5 protein and the activated PDGF receptor exist in a stable complex in transformed mouse C127 cells. These results suggest a distinct mechanism of growth factor receptor activation and provide further evidence that the PDGF receptor is an important target of the E5 protein.**

DNA tumor viruses frequently encode proteins that stimulate cell proliferation and cause tumorigenic transformation. These viral proteins are thought to act by modulating the activity of the products of cellular oncogenes and tumor suppressor genes, but the biochemical mechanisms through which these putative cellular targets control normal cell growth are in general not well-defined. Bovine papillomavirus type 1 (BPV) induces the formation of fibropapillomas and other mesenchymal tumors in infected animals. The ability of BPV to transform fibroblasts *in vivo* is thought to reside in the 44-amino acid E5 protein that can cause tumorigenic transformation of rodent fibroblast cell lines and can stimulate cellular DNA synthesis in quiescent cells (1–7). The BPV E5 gene can also stimulate the transforming activity of transfected genes encoding cell-surface receptors (8). The E5 transforming protein is localized largely to intracytoplasmic membranes in transformed cells and consists of an extremely hydrophobic domain that can be functionally replaced by many random sequences of hydrophobic amino acids and a hydrophilic C terminus that contains 7 of the 8 specific amino acids identified as being important for transformation (9–12). The small number of specific amino acids required for transformation suggests that the E5 protein may transform cells by influencing the activity of a cellular membrane protein involved in growth control (10). Although the E5 protein binds to a 16-kDa subunit of the vacuolar H^+-ATPase, the biochemical and physiological consequences of this interaction are not known (12–15). We recently showed that expression of the E5 protein results in activation of precursor and mature intracellular forms of the endogenous platelet-derived growth factor (PDGF) β receptor in transformed fibroblasts, and we proposed that the activated receptor transmits a proliferative signal to the cell (ref. 16; unpublished data). Here we use coimmunoprecipitation analysis to show that the E5 protein and the activated PDGF receptor form a stable complex in transformed mouse C127 cells.

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MATERIALS AND METHODS

Cells, DNA, and Antisera. Normal C127 mouse fibroblasts and the BPV E5-transformed derivative L1D-1 have been described (16). Synthetic oligonucleotides encoding an 11-amino acid sequence from influenza virus hemagglutinin (a gift of D. Goldstein, Georgetown University) were inserted into the E5 gene at the *Bst*XI site of pBPV-142.6, a plasmid containing the entire BPV genome (17), to generate p142.6-HA-12. Calcium phosphate-mediated DNA transfection was performed on C127 cells by using 500 ng of p142.6-HA-12 DNA digested with *Bam*HI to liberate the viral DNA. Cell line CBHA-8 was established from a transformed focus 17–20 days after DNA transfer. Untransformed cell line CCT was established from cells transfected with calf thymus DNA, a treatment that did not induce foci. C127 cells transformed by transfection with a cloned v-*sis* gene were established from a transformed focus by L. Nilson (Yale University, New Haven, CT). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (DMEM-10) supplemented with antibiotics and were passaged serially before reaching confluence.

The E5 protein was immunoprecipitated with αE5, a rabbit antiserum raised to the 16 C-terminal amino acids of the BPV E5 protein (18). PDGF β receptor was detected with αPR4, a rabbit antiserum recognizing the 13 C-terminal amino acids of the human PDGF β receptor (19) and a gift of S. Courtneidge. Anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY).

Analysis of Metabolically Labeled Cells. Confluent cells in 10-cm dishes were washed twice with phosphate-buffered saline (PBS), incubated for 45 min in serum- and methionine-free DMEM, and then incubated in fresh serum-, methionine-, and cysteine-free medium containing 0.33 mCi of Tran^{35}S-label per ml (1 Ci = 37 GBq) (70% [^{35}S]methionine; ICN) for 6 hr at 37°C. Cells were then washed twice with PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), scraped off the dish, pelleted, and frozen at −70°C overnight. Cell pellets were thawed and then lysed in 50 μl (normal C127 cells) or 200 μl (L1D-1 E5-transformed cells) of cold RIPA buffer (20 mM Mops/150 mM NaCl/1 mM EDTA/1% Nonidet P-40/1% sodium deoxycholate/0.1% SDS/1 mM PMSF/0.1 mM sodium orthovanadate). Identical lysates were pooled and then cleared of cell debris by spinning in a microcentrifuge for 5–10 min. Fifteen microliters of αPR4 or nonimmune rabbit serum was added to 235 μl of extract, or 10 μl of αE5 was added to 115 μl of L1D-1 extract. After rotation at 4°C for 2 hr, 90 μl of a 1:1 suspension of protein A-Sepharose beads (Pharmacia) in TBS-BSA [10 mM Tris·HCl, pH 7.4/165 mM NaCl/10% (wt/vol) bovine serum albumin] was added and the mixture was rotated for 45 min at 4°C. The beads were pelleted and washed five times with

Abbreviations: BPV, bovine papillomavirus; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride.

*To whom reprint requests should be addressed.

cold RIPA buffer. Immune complexes were eluted from the beads by boiling for 5 min in 200 μl of RIPA buffer containing 0.5% SDS, and 0.8 ml of RIPA buffer was added to the eluate. After preclearing with 20 μl of normal rabbit serum, immunoprecipitation with 19 μl of the E5 antiserum was performed as described above and the washed immune complexes were resuspended in 70 μl of Laemmli sample buffer, boiled 5 min, and then electrophoresed on a SDS/12–20% polyacrylamide gel. Standard procedures were used to process the gel for fluorography using Amplify (Amersham).

Analysis of Unlabeled Cells by Immunoblotting. Confluent cells in 10-cm dishes were either untreated or treated with recombinant PDGF BB for 10 min at 37°C. Cell extracts were prepared by rinsing cells twice with PBS, lysing in 1 ml of RIPA buffer for 20 min on ice, and spinning out cell debris. Anti-E5 or nonimmune rabbit serum was added to extracts, which were then incubated for 2 hr at 4°C. For denaturation before immunoprecipitation, SDS and dithiothreitol (final concentrations, 0.5% and 1 mM, respectively) were added to the extract, which was then boiled for 5 min and chilled on ice. (A control experiment showed that the E5 protein is efficiently precipitated by the E5 antiserum under these conditions.) Immunoprecipitates were collected, washed five times with cold NET-N buffer (20 mM Tris·HCl, pH 8/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40/1 mM PMSF), resuspended in protein sample buffer, heated, and electrophoresed on a 7.5% acrylamide/0.17% bisacrylamide gel. Gels were subjected to immunoblot analysis as described (16) with a 1:600 dilution of αPR4 or a 1:500 dilution of anti-phosphotyrosine monoclonal antibody 4G10. To document the total amount of PDGF receptor present in each extract, αPR4 immunoprecipitates were subjected to immunoblot analysis with a 1:600 dilution of αPR4.

RESULTS

To determine whether the PDGF receptor was in a molecular complex with the E5 protein, the ability of a PDGF receptor antiserum to immunoprecipitate the E5 protein was assessed. Extracts of metabolically labeled normal C127 cells and cells transformed by the E5 protein were prepared in RIPA buffer and immunoprecipitated with either a control antiserum or an antiserum raised to a PDGF receptor peptide. The immunoprecipitates were denatured and subjected to a second round of immunoprecipitation with an antiserum raised against an E5 peptide, and E5 protein was visualized after gel electrophoresis (Fig. 1). As expected, no E5 protein is detectable in extracts prepared from untransformed cells (lane a). In contrast, when extracts of E5-transformed cells are analyzed, the E5 protein is readily detectable in the immunoprecipitate generated with the PDGF receptor antiserum (lane b), but it is absent from the immunoprecipitate generated with nonimmune serum (lane c). Approximately 10% of the E5 protein is present in PDGF receptor immune complexes under our relatively stringent conditions of cell lysis and immunoprecipitation. Immunoprecipitation of the E5 protein by PDGF receptor antiserum is blocked by incubating the antiserum with the receptor peptide used for immunization (lane d). Since there is no sequence similarity between this peptide and any portion of the E5 protein, these results suggest that the E5 protein and the PDGF receptor are present in a stable complex in transformed cells.

The existence of an E5 protein–PDGF receptor complex was confirmed in the reciprocal experiment in which immunoprecipitation was performed with the E5 antiserum and the immunoprecipitates were analyzed for the presence of PDGF receptor by immunoblotting with the PDGF receptor antiserum. As shown in Fig. 2 (*Left*), mature and precursor forms of the PDGF receptor were present in E5 immune complexes prepared from extracts of E5-transformed cells (lane f) but not from extracts of normal cells (lane a). This result was obtained with several independent lines of normal and transformed cells (data not shown). Moreover, PDGF receptor is not immunoprecipitated by the E5 antiserum from extracts of normal cells treated with PDGF (lane c) or from extracts of cells transformed by the v-*sis* gene, a transforming version of the gene encoding the PDGF B chain (data not shown). The amounts of PDGF receptor in these various extracts are similar (Fig. 2 *Right*). The fact that the E5 antiserum immu-

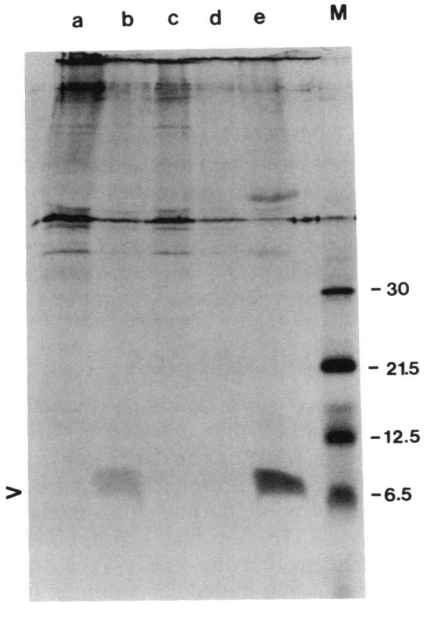

FIG. 1. Coimmunoprecipitation of the E5 protein with PDGF receptor antiserum. Labeled extracts were prepared and analyzed as described. Lanes: M, radiolabeled molecular size markers (sizes indicated in kDa); a, normal C127 cells; b–e, transformed L1D-1 cells. Antibody used in the first round of immunoprecipitation was nonimmune serum (lane c), PDGF receptor antiserum (lanes a and b), PDGF receptor antiserum preincubated with an excess of PDGF receptor peptide (lane d), or E5 antiserum (lane e). Arrowhead indicates position of the BPV E5 protein.

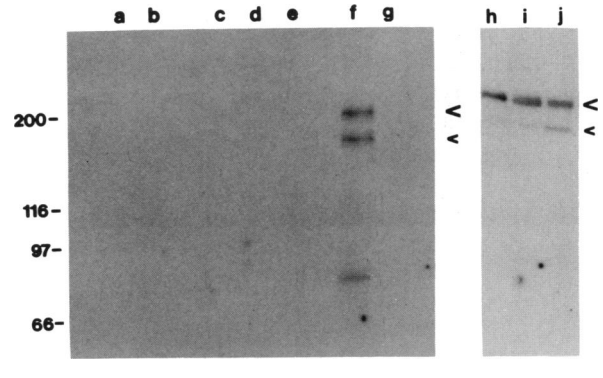

FIG. 2. Coimmunoprecipitation of the PDGF receptor with E5 antiserum. Unlabeled extracts were analyzed for the presence of PDGF receptor by immunoblotting with αPR4. Sources of extract: lanes a, b, and h, normal C127 cells; lanes c–e and i, C127 cells treated with PDGF; lanes f, g, and j, transformed L1D-1 cells. Lanes b, d, e, and g show immunoprecipitation after denaturation of the extract. (*Left*) Each lane represents 630 μg of extracted protein immunoprecipitated with 10 μl of αE5 (except for lane e, which was precipitated by preimmune serum), and a 6-day exposure is shown. (*Right*) Each lane represents 240 μg of extracted protein immunoprecipitated with 2 μl of αPR4, and a 20-hr exposure is shown. Large arrowheads show position of mature PDGF receptor; small arrowheads show position of PDGF receptor precursor. Smaller molecular mass band (kDa) detected by the PDGF receptor antiserum is presumably a degradation product of the receptor.

noprecipitates the PDGF receptor only from cells expressing the E5 protein indicates that this antiserum does not directly recognize the PDGF receptor. In accordance with this interpretation, coimmunoprecipitation of the PDGF receptor by the E5 antiserum is blocked by denaturation of protein complexes before immunoprecipitation (lane g). In addition, PDGF receptor is not precipitated from transformed cells by preimmune serum, and precipitation of the receptor by the E5 antiserum is blocked by the E5 peptide used for immunization (data not shown).

Activation of the PDGF receptor by PDGF treatment or by E5 expression results in increased tyrosine phosphorylation of the receptor, a transmembrane tyrosine kinase (20–22). Immunoblotting with anti-phosphotyrosine antibody was used to determine whether the E5 antiserum immunoprecipitates activated receptor. As shown in Fig. 3, the E5 antiserum immunoprecipitates substantial amounts of tyrosine-phosphorylated PDGF receptor and receptor precursor from transformed cells (lane d) but not from PDGF-treated normal cells (lane b) or from cells transformed by v-*sis* (lane c) [both of which contain abundant tyrosine-phosphorylated PDGF β receptor (data not shown; see Fig. 4, lane b)]. Identification of tyrosine-phosphorylated proteins as PDGF receptor was confirmed by sequential immunoprecipitation with E5 and PDGF receptor antisera, followed by phosphotyrosine immunoblotting (data not shown). No other tyrosine-phosphorylated proteins are readily detectable in E5 immunoprecipitates (Fig. 3, lane d). An E5 antiserum raised to the same peptide in a different rabbit and an antiserum raised to an overlapping E5 peptide also immunoprecipitates abundant tyrosine-phosphorylated PDGF receptor from E5-transformed C127 cells but not from PDGF-treated untransformed cells (data not shown).

A small amount of tyrosine-phosphorylated PDGF receptor can occasionally be detected in immunoprecipitates generated from PDGF-treated normal cells by the E5 antiserum or by preimmune serum (e.g., Fig. 4, lane f), but it is invariably a minute fraction of the total tyrosine-phosphorylated receptor in the cells (lane b). Importantly, the ability of the E5 antiserum to precipitate this small amount of receptor from PDGF-treated normal cells is not inhibited by the E5 peptide, whereas precipitation of E5-activated receptor is blocked by this peptide or by denaturation of protein complexes (data not shown). Thus, the variable recovery of low amounts of PDGF-activated receptor from normal cells appears nonspecific.

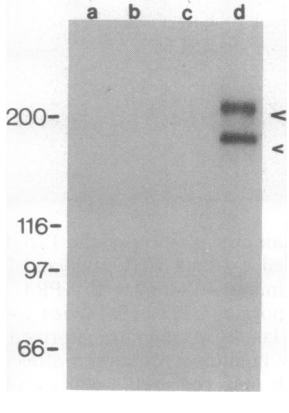

FIG. 3. Association of E5 protein with activated PDGF receptor. Each lane represents 850 μg of extracted protein immunoprecipitated with the E5 antiserum, followed by immunoblotting with anti-phosphotyrosine antibody. Lanes: a, normal C127 cells; b, C127 cells treated with 10 ng of PDGF (PDGF, Boston) per ml; c, C127 cells transformed by v-*sis*; d, E5-transformed L1D-1 cells. Sizes shown on left are kDa.

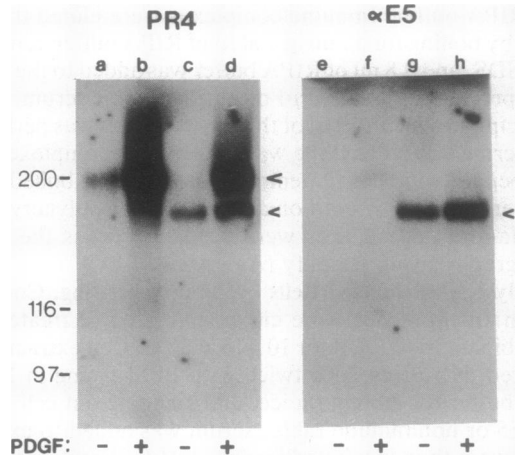

FIG. 4. Specific association of the E5 protein with E5-activated PDGF receptor. C127 cells transformed by the E5-HA protein (CBHA-8) and control CCT cells were either untreated (lanes −) or treated with 7.5 ng of PDGF per ml (lanes +). Extracts were immunoprecipitated by using 30 μl of E5 antiserum and 2 mg of protein (*Right*) or 5 μl of PR4 PDGF receptor antiserum and 400 μg of protein (*Left*). Immunoblots were probed with anti-phosphotyrosine antibody 4G10. Sources of extracts: lanes a, b, e, and f, CCT cells; lanes c, d, g, and h, CBHA-8-transformed cells. Sizes shown on left are kDa.

The specificity of the interaction between the E5 protein and the PDGF receptor was examined further in C127 cells transformed by an E5 mutant containing an in-frame 11-amino acid insertion after codon 4 of the E5 gene (Fig. 4). This mutant causes marked tyrosine phosphorylation of the precursor form of the PDGF receptor but has little effect on the mature form (lane c; data not shown). Accordingly, as assessed by phosphotyrosine blotting, the E5 antiserum immunoprecipitates only the receptor precursor from extracts of cells transformed by this mutant (lane g). When cells transformed by this mutant are treated with PDGF, both forms of the receptor are tyrosine phosphorylated, the mature form in response to the growth factor and the precursor form in response to the E5 protein (lane d). Strikingly, the E5 antiserum immunoprecipitates only the E5-activated precursor from extracts of PDGF-treated transformed cells (lane h). Thus, although there are two classes of activated, tyrosine-phosphorylated PDGF receptors in these cells, there appears to be a specific interaction between the viral protein and those receptor molecules it activates. This result also indicated that the E5 protein did not nonspecifically associate with all PDGF receptor molecules upon cell lysis.

DISCUSSION

The results reported here establish that the BPV E5-transforming protein and the activated PDGF receptor are stably associated in transformed C127 cells. Complex formation between these two proteins has also been detected in BPV-transformed bovine dermal fibroblasts and in E5-transformed rat FR3T3 fibroblasts (unpublished results). Several facts indicate that the PDGF receptor and the E5 protein form a bona fide molecular complex in living cells. First, the complex is detected in transformed cells expressing both proteins at physiologic levels from their natural promoters. Second, the interaction is stable enough to withstand extraction with RIPA buffer and to be detected without the necessity of using chemical crosslinkers. Third, association is detected between the E5 protein and only the specific form of the PDGF receptor that it activates. Fourth, the E5 protein and the activated PDGF receptor precursor apparently colocalize in endoplasmic reticulum and Golgi compartments of

transformed cells. Finally, this physical interaction is mirrored by the physiological interaction described previously—namely, the rapid and specific activation of the endogenous PDGF β receptor induced by expression of the E5 protein.

The complex between the PDGF receptor and the E5 protein may be a consequence of direct physical contact between the two proteins, or both may be present in a larger multiprotein complex. We can envision two potential sites of direct contact between the PDGF receptor and the E5 protein. One is between the transmembrane domain of the PDGF receptor and the very hydrophobic central portion of the E5 protein. The other potential site of contact is the ligand binding domain of the receptor and the hydrophilic C terminus of the E5 protein, both of which are localized on the same face of the membrane (9). The amino acid sequence of this hydrophilic active site of the E5 protein resembles a small segment of PDGF (16), an observation consistent with the suggestion that the E5 protein may bind directly to the PDGF receptor. Like PDGF, the E5 protein is a disulfide bond-linked dimer, which may mediate receptor activation by simultaneously binding two receptor molecules, thereby inducing receptor dimerization, which is thought to be an early and obligatory step in receptor activation (23–25). Binding and activation of immature and intracellular PDGF receptor forms by the E5 protein has a precedent in the activation of these forms by the v-sis protein, a known ligand of the PDGF receptor (26–28). On the other hand, the E5 protein and PDGF may activate the receptor by using distinct mechanisms. For example, receptor activation by the E5 protein may require additional proteins such as the 16-kDa subunit of the vacuolar H^+-ATPase, as has been recently suggested (14, 15). The experiments reported here were not designed to determine whether the E5 protein, the PDGF receptor, and the ATPase subunit coexist in the same complex, and experiments to examine this issue remain to be done. Coimmunoprecipitation analysis of cells expressing wild-type and mutant versions of the E5 protein and the PDGF receptor should identify the domains of the two proteins that are required for their interaction, and this information may shed light on the nature of the interaction and the mechanism of activation.

The PDGF receptor is the only tyrosine-phosphorylated protein detectable in E5 immune complexes, indicating that it is the only activated receptor tyrosine kinase associated with the E5 protein in transformed fibroblasts. This apparent specificity may result from the inability of the E5 protein to interact with other growth factor receptors. Alternatively, the E5 protein could bind to a variety of growth factor receptors but preferentially activate the PDGF receptor, or the E5 protein could activate promiscuously, with specificity conferred by the spectrum of receptors expressed in the host cell. The reported ability of the E5 gene to cooperate with a variety of transfected cell-surface receptors to transform NIH 3T3 cells implies that in this cotransfection assay the E5 protein does not exclusively affect the PDGF receptor (8). In some cell types expressing low levels of PDGF receptors or in cells overexpressing foreign receptors, the E5 protein may interact productively with other targets. This latter interpretation is consistent with our recent observation that the BPV E5 gene can transform keratinocytes, which express little if any PDGF receptor (29).

We proposed previously that activation of the PDGF receptor by the E5 protein initiates the signal transduction cascade responsible for the proliferative effects of the E5 protein in fibroblasts. Our finding that the E5 protein and the activated PDGF receptor exist in a stable complex in transformed fibroblasts indicates that receptor activation is a direct consequence of E5 expression and not a secondary response to the transformed state and provides biochemical evidence that the PDGF receptor is an important target of the viral protein. Furthermore, cells that lack PDGF receptor and respond poorly to the E5 protein can be rendered highly susceptible to E5-mediated tumorigenic transformation by introduction of the gene encoding the PDGF β receptor (L. Nilson and D.D., unpublished results). These results imply that DNA tumor virus-transforming proteins can induce transformation by binding a component of a normal signal transduction pathway and stimulating its activity. The ability of a mutant E5 protein to transform cells with little or no activation of the mature PDGF receptor implies that signals emanating from intracellular receptor forms may be sufficient to induce E5-mediated transformation. However, it is difficult to rule out the possibility that this mutant activates a low level of cell-surface receptor.

A number of viruses encode proteins that closely resemble the ligands of growth factor receptors. Examples include v-*sis*, a transduced version of the cellular PDGF B gene, and the vaccinia virus growth factor, a close homologue of epidermal growth factor. However, it is now clear that intrinsic viral proteins that do not appear to be derived from cellular genes encoding the normal ligand can also activate growth stimulatory pathways to induce transformation. The first example was polyomavirus middle T antigen and $pp60^{c\text{-}src}$ (30, 31), but the role played by *src* family tyrosine kinases in normal fibroblast proliferation is not known. In the case of RNA viruses, intracellular erythropoietin receptor appears to be the important cellular target of a retrovirus envelope protein, Friend leukemia virus gp55, which confers erythropoietin-independent growth on infected cells (32–34). However, unlike the PDGF receptor, activation of the erythropoietin receptor and its effects on downstream substrates are poorly understood and not readily demonstrable by biochemical criteria. Further analysis of transformation by the E5 protein and of the interaction between the E5 protein and the PDGF receptor promises to yield mechanistic insights into tumorigenesis, cell cycle control, and activation and function of growth factor receptors.

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