Plant cells in culture secrete a sulfated peptide named phytosulfokine-α (PSK-α), and this peptide induces the cell division and/or cell differentiation by means of specific high and low affinity receptors. Putative receptor proteins for this autocrine type growth factor were identified by photoaffinity labeling of plasma membrane fractions derived from rice suspension cells. Incubation of membranes with a photoactivable 125I-labeled PSK-α analog, [N-^4-azidosalicyl]Lys5]PSK-(AS-PSK-α), followed by UV irradiation resulted in specific labeling of 120- and 160-kDa bands in SDS-polyacrylamide gel electrophoresis. The labeling of both bands was completely inhibited by unlabeled PSK-α and partially decreased by PSK-α analogs possessing moderate binding activities. In contrast, PSK-α analogs that have no biological activity showed no competition for 125I-AS-PSK-α binding, confirming the specificity of binding proteins. Analysis of the affinity of 125I incorporation into the protein by ligand saturation experiments gave apparent Kd values of 5.0 nM for the 120-kDa band and 5.4 nM for the 160-kDa band, suggesting that both proteins correspond to the high affinity binding site. Treatment of 125I-AS-PSK-α cross-linked proteins with peptide N-glycosidase F demonstrated that both proteins contained approximately 10 kDa of N-linked oligosaccharides. Specific cross-linking of 125I-AS-PSK-α was also observed by using plasma membranes derived from carrot and tobacco cells, indicating the widespread occurrence of the binding proteins. Together, these data suggest that the 120- and 160-kDa proteins are PSK-α receptors that mediate the biological activities of PSK-α.

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Plant cells in culture secrete a sulfated pentapeptide named phytosulfokine-α (PSK-α); Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln) in response to externally added auxin and cytokinin, and PSK-α triggers cell proliferation at nanomolar concentrations in collaboration with plant hormones (1, 2). Therefore, dispersed cells cannot proliferate under low cell density conditions in which secreted PSK-α is diluted below the critical concentration with excess culture medium. This autocrine type peptide growth factor has been found in conditioned medium derived from both monocot and dicot cell cultures (3, 4), implying that PSK-α is the general factor involved in plant cell growth. PSK-α also stimulates tracheary element differentiation of Zinnia mesophyll cells (3) and somatic embryogenesis in carrots under defined conditions (5).

A cDNA encoding a PSK-α precursor has been isolated from cDNA library constructed using poly(A)+ mRNA purified from rice cells cultured for 10 days (6). The cDNA is 725 base pairs in length, and the 89-amino acid product, preprophytosulfokine, has a 22-amino acid hydrophobic region that resembles a cleavable leader peptide at its NH2 terminus. The PSK-α sequence occurs only once within the precursor, close to the COOH terminus. The critical importance of preprophytosulfokine in cell growth was shown by transforming rice cells with sense and antisense rice PSK gene that is regulated by the constitutive rice actin promoter. The sense transgenic cells divided about two times faster than the controls, whereas the antisense transgenic cells had decreased mitogenic activity.

Evidence for the existence of high affinity binding sites for PSK-α in rice plasma membrane was provided initially by us using [35S]PSK-α (7) and later using [3H]PSK-α (8). The observed binding was saturable, reversible, and localized on the outer surface of the plasma membrane of rice cells. Because the specific binding was significantly altered by the external pH and ion strength, the ligand-receptor binding may be mainly controlled by ion interactions. Ligand saturation analysis using [3H]PSK-α revealed the existence of both high and low affinity binding sites with Kd values of 1.4 and 27 nM, respectively. Specific binding activities for [3H]PSK-α have been detected in plasma membrane fractions derived from cell lines of many plant species containing carrot, maize, asparagus, and tomato, indicating the widespread occurrence of [3H]PSK-α-binding sites (8). At present, however, the molecular structures of PSK-α-binding sites remain rather unclear. To understand the molecular mechanism of how plant cells perceive and transduce the PSK-α signal, it is critically important to identify and characterize the receptor molecules that initially perceive the PSK-α signal.

Derivatization of small peptide hormones with photoactivatable groups has been utilized for characterization and purification of hormone receptors (9). In these cases, a key factor in the application of such functional groups may be how to modify peptides without loss of binding activity and biological activity. Structure-activity studies for PSK-α have shown that the active core of PSK-α is an NH2-terminal tripeptide containing two sulfated groups (10). Thus, we focused on the COOH-terminal region of PSK-α for the functional derivatization of PSK-α.

In this study, we prepared an 125I-labeled photoactivatable PSK-α analog containing 4-azidosalicylic acid, [N^4-azidosalicyl]Lys5]PSK-α, which possesses comparable binding activity with that of PSK-α in order to specifically label mem-
brane proteins. After photoaffinity cross-linking, proteins of 120 and 160 kDa in rice plasma membranes were labeled with this ligand. These proteins fulfill the criteria expected for the high affinity PSK-α receptor(s) in terms of their affinity and specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**—[125I]NaI solution (carrier-free) was obtained from ICN Biomedicals (Costa Mesa, CA). N-Hydroxysuccinimidyl-4-azidosalicylic acid was obtained from Pierce. A reverse-phase HPLC column, Develosil ODS-5 and ODS-10, was purchased from Nomura Chemicals (Seto, Japan). Molecular weight standards were from Amersham Pharmacia Biotech. Fmoc amino acids were from Peptide Institute (Osaka, Japan). Preloaded HMP resin was from Applied Biosystems (Chiba, Japan). DMF-30 was from Sigma. Peptide N-glycosidase F was from Takara (Tokyo, Japan). Nonidet P-40 was from Nacalai Tesque (Kyoto, Japan). All the other inorganic and organic chemicals were obtained from Wako Pure Chemicals (Osaka, Japan).

**Preparation of Plasma Membranes**—Cell lines of rice Oryza sativa L. (Oc) and carrot Daucus carota L. (NC) were subcultured every 2 weeks in Murashige and Skoog medium supplemented with 1.0 mg/liter of 2,4-dichlorophenoxyacetic acid and 30 g/liter of sucrose. A cell line of tobacco Nicotiana tabacum L. (BY-2) was maintained with subculturing every 12 weeks in Murashige and Skoog medium supplemented with 30 mg/liter of 2,4-dichlorophenoxyacetic acid and 30 g/liter sucrose. Cultures were incubated at 25 °C in the dark with rotary shaking at 120 rpm as described previously (7). Cells (150 g fresh weight) were homogenized in a blender, and a microsomal fraction was obtained by ultracentrifugation as described previously (5). Plasma membranes were further enriched by the two-phase partitioning protocol (11). Pellets of plasma membrane fraction were suspended in suspension buffer containing 10 mM Tris-HCl, pH 7.0, and 250 mM sucrose using a Potter-Elvehjem type homogenizer and stored at −80 °C until use. Protein concentration was measured as described by Bradford (12) with bovine serum albumin as a reference.

**Preparation of 125I-Labeled Photoactivatable Analog of PSK-α**—The partially protected peptide resin, Fmoc-Tyr-Ile-Tyr-Thr(β-butyl)-Lys(t-butyrocarbonyl)-linker resin, was synthesized by Fastmoc chemistry with a peptide synthesizer (Applied Biosystems model 433A). This peptide resin (0.05 mmol) was suspended in DMF/pyridine mixture (4:1, 1.5 ml) and sulfated by the addition of 230 mg of DMF/PSK-α. After 16 h, peptides were cleaved and deprotected as described previously (10). The peptides were purified by HPLC on a Develosil ODS-10 column (20 × 250 mm) by an isocratic elution of 0.1% trifluoroacetic acid/60 mM ammonium acetate at a flow rate of 10.0 ml/min. After lyophilization, a precursor peptide, Fmoc-Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Lys in which NH2-terminal protected peptide eluted at 7.7 min was obtained. AS-PSK-α, a PSK-α derivative conjugated with 4-azidosalicylic acid (AS), was prepared by coupling N-hydroxysuccinimidyl-4-azidosalicylic acid and the synthesized precursor peptide. N-Hydroxysuccinimidy1-4-azidosalicylic acid (3.3 mg), Fmoc-Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Lys (32 mg) and NaHCO3 (1.0 mg) were dissolved in 1.0 ml of 50% acetone and stirred at room temperature for 3 h. The reaction mixture was then evaporated to dryness, dissolved in 1.0 ml of DMF/piperidine mixture (1:1), and further stirred for 1 h. Deprotected peptides were purified by HPLC on a Develosil ODS-5 column (10 × 250 mm) by an isocratic elution of 20% acetonitrile containing 0.1% ammonium acetate at a flow rate of 4.0 ml/min. After lyophilization, Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Lys (42% yield) was obtained. AS-PSK-α was radioiodinated using chloramine T as the oxidizing agent. Six microliters of Na125I (20 MBq), 0.6 μl of unlabeled 4 mM NaI as a carrier, 50 μl of 0.4 mM AS-PSK-α, and 10 μl of 0.5 M phosphate buffer, pH 7.5, were mixed in an Eppendorf tube. Reaction was started by adding 2.5 μl of 4 mM chloramine T to this solution, and the mixture was allowed to stand for 30 min at room temperature. Iodinated peptides were purified by HPLC on a Develosil ODS-5 column (10 × 250 mm) by an isocratic elution of 25% acetonitrile containing 0.1% ammonium acetate at a flow rate of 2.0 ml/min. Under this condition, two monoiodinated (or ortho or para position of hydroxyl group) peptides were obtained at a retention time of 6.8 and 7.7 min, respectively. From the results of binding assay, the monoiodinated AS-PSK-α eluted at 7.7 min showed higher affinity for PSK-α-binding sites in the supernatant, the radiotracer activity contained in the pellet was determined with a liquid scintillation counter.

**Photoaffinity Labeling**—Aliquots of plasma membrane fraction (125 μg of protein) was incubated for 30 min at 4 °C in a total volume of 250 μl of binding buffer containing 10 mM [3H]PSK-α and various concentrations of ligands as a competitor. Incubations were terminated by layering the reaction mixture onto 900 μl of buffer containing 0.5 M sucrose and centrifuged for 5 min at 10,000 × g at 4 °C. After discarding the supernatant, the radioactivity contained in the pellet was assessed in experiments in which increasing amounts of products, the peptide eluted at 7.7 min showed higher affinity for PSK-α receptor binding owing to the presence of sulfate ester. The competition by PSK-α and two isomers of monoiodinated peptides was assessed in experiments in which increasing amounts of unlabeled PSK-α and PSK-α derivatives competed with [3H]PSK-α for receptor binding (Fig. 2). Unlabeled PSK-α, monoiodinated AS-PSK-α eluted at 6.8 min, and monoiodinated AS-PSK-α eluted at 7.7 min, all competed for the binding sites with half-maximal inhibitory concentrations (IC50) of ~30, ~300, and ~100 nM, respectively. Although the monoiodinated positions on phenyl ring of 4-azidosalicylic acid could not be chemically determined owing to the limited amounts of products, the peptide eluted at 7.7 min showed higher affinity for PSK-α-binding sites than that eluted at 6.8 min.

The affinity of the monoiodinated AS-PSK-α eluted at 7.7 min was calculated by nonlinear regression analysis software (GraphPad PRISM®) using the equation for competitive binding to two classes of receptor. By fitting competition data from Fig. 2, the K1 and K2 values for the peptide to rice microsomes.
were determined to be 5.1 and 500 nM, respectively. The high affinity of the monoiodinated peptide eluted at 7.7 min for PSK-α receptor was also confirmed by the bioassay using asparagus mesophyll cells (Fig. 3). Half-maximal induction of cell division occurred at 3 nM, which is virtually equal concentration for half-maximal activity of unmodified PSK-α. Based on these results, we prepared the monoiodinated peptide eluted at 7.7 min using Na125I and used as a photoaffinity ligand in the following experiments. The specific radioactivity of this radioiodinated peptide designated as 125I-AS-PSK-α was 230 Ci/mmol.

Identification of High Affinity Binding Proteins by Photoaffinity Labeling—Photoaffinity labeling was performed after incubation of plasma membrane fraction with 10 nM 125I-AS-PSK-α for 30 min on ice. Cross-linked membrane proteins were solubilized by SDS sample buffer and analyzed by SDS-PAGE under the reducing and the nonreducing conditions. Autoradiographic analysis of the SDS-PAGE gels showed that 120- and 160-kDa proteins were labeled under the reducing condition (Fig. 4). The incorporation of 125I-AS-PSK-α into these bands was completely inhibited with a 320-fold excess (3.2 μM) of unlabeled PSK-α, indicating the specific binding of the ligand to the proteins. Under the nonreducing condition, relative mobility of 120-kDa protein was not altered at all, but 160-kDa protein showed a relatively smear band. The 160-kDa protein may contain intramolecular disulfide bonds that define the tertiary structure of the binding protein.

To verify the specificity of the interaction of 125I-AS-PSK-α with its binding sites, rice plasma membranes were incubated with the radioligand in the presence of increasing concentrations of unlabeled PSK-α. Photoaffinity cross-linking revealed a dose-dependent reduction in the label intensity of the 120- and 160-kDa proteins (Fig. 5). By quantifying the radioactivities of each band using an image analyzer, the ligand concentrations required to half-maximally displace 125I-AS-PSK-α from the binding sites were shown to be ~30 nM for 120-kDa...
bands. Binding specificity of the 120- and 160-kDa protein to the structurally related PSK-α analogs was further analyzed by competitive displacement of 125I-AS-PSK-α with the unlabeled analogs. A 320-fold molar excess of the analogs was added concomitantly with the radioligand to the assay mixture, and the samples were proceeded as described above. Results from a representative experiment are shown in Fig. 6. PSK-α analogs with moderate mitogenic activity such as Tyr-Ile-Tyr(SO3H)-Thr-Gln and Tyr(SO3H)-Ile-Tyr-Thr-Gln showed some competition for cross-linking of 125I-AS-PSK-α to rice plasma membrane. In contrast, PSK-α analogs that have no biological activity such as Tyr-Ile-Tyr-Thr-Gln showed no competition for 125I-AS-PSK-α binding, confirming the specificity of binding proteins.

The binding affinities of 125I-AS-PSK-α to the 120- and 160-kDa band were estimated from the concentration dependence of the ligand incorporation into the protein band (Fig. 7). The specific binding between the ligand concentration range from 0.32 to 10 nM determined by quantifying band densities using a bio-imaging analyzer showed the saturable mode of incorporation of radioactivity to the 120- and 160-kDa bands (Fig. 7A). The Scatchard analysis of the binding data showed linear profile and yielded the following parameters: $K_d = 5.0 \text{ nM}$, $B_{max} = 81 \text{ fmol/mg protein}$ (square), and $K_d = 5.2 \text{ nM}$, $B_{max} = 34 \text{ fmol/mg protein}$ (circle).

Fig. 4. Detection of PSK-α-binding proteins by photoaffinity labeling. Rice plasma membrane was incubated with 10 nM 125I-AS-PSK-α and irradiated with UV light after the removal of excess unbound ligand. Cross-linked membrane proteins were solubilized by SDS sample buffer and analyzed by SDS-PAGE and autoradiography under the reducing or the nonreducing conditions in the presence or absence of excess unlabeled PSK-α. Total protein bands in SDS-PAGE gels were visualized by the fluorescent dye Nile Red. Numbers at left indicate molecular mass markers given in kilodaltons.

Fig. 5. Competitive displacement of 125I-AS-PSK-α incorporation by unlabeled PSK-α. Rice plasma membranes were incubated with 10 nM 125I-AS-PSK-α in the presence of increasing concentrations of unlabeled PSK-α. After UV irradiation, cross-linked membrane proteins were solubilized by SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Fig. 6. Binding specificity of PSK-α-binding proteins. Rice plasma membranes were incubated with 10 nM 125I-AS-PSK-α in the presence of 320-fold excesses (3.2 μM) of various PSK-α analogs as competitors. After UV irradiation, cross-linked membrane proteins were solubilized by SDS sample buffer and analyzed by SDS-PAGE and bio-imaging analyzer. Lane 1, no competitor; lane 2, Tyr(SO3H)-Ile-Tyr(SO3H)-Thr-Gln (PSK-α); lane 3, Tyr-Ile-Tyr(SO3H)-Thr-Gln; lane 4, Tyr(SO3H)-Ile-Tyr-Thr-Gln; lane 5, Tyr-Ile-Tyr-Thr-Gln.

Fig. 7. Binding constants of PSK-α-binding proteins. A, rice plasma membranes were incubated with various concentrations of 125I-AS-PSK-α. After UV irradiation, cross-linked membrane proteins were solubilized in SDS sample buffer and analyzed by SDS-PAGE and bio-imaging analyzer. B, Scatchard analysis of the binding data showing linear profiles with the following parameters: $K_d = 5.0 \text{ nM}$, $B_{max} = 81 \text{ fmol/mg protein}$ (square), and $K_d = 5.2 \text{ nM}$, $B_{max} = 34 \text{ fmol/mg protein}$ (circle).
Peptide N-Glycosidase F Treatment of Cross-linked Membrane Proteins—Peptide N-glycosidase F (PNGase F) treatment of the photoaffinity labeled membranes demonstrated the presence of N-linked carbohydrate side chains on the receptor protein (Fig. 8). PNGase F is known to cleave asparagine-bound N-glycans to give proteins free of N-linked carbohydrate chains. After 24 h of treatment in the presence of SDS, the labeled 120- and 160-kDa proteins were reduced to apparent molecular masses of 110 and 150 kDa, respectively, suggesting the presence of approximately 10 kDa of N-linked carbohydrate chains in both proteins.

Widespread Occurrence of the PSK-α-binding Proteins—Recent studies revealed that a significant amount of PSK-α is produced by several plant cell lines including dicots as well as monocots and stimulates cell division and differentiation at a nanomolar level. Therefore, we investigated the distribution of PSK-α-binding proteins in plasma membrane fractions derived from carrot and tobacco cells. In both species, specific 125I-AS-PSK-α cross-linked proteins were detected by incubating plasma membrane fractions in the buffer containing 10 nM of the ligand followed by UV irradiation (Fig. 9). In the membrane fraction derived from D. carota L. (NC) cell line, 130- and 170-kDa proteins were specifically labeled at a relatively high efficiency. Tobacco (BY-2) membrane also possessed similar binding proteins with apparent molecular masses of 110 and 150 kDa. The differences in labeling efficiency of these proteins between rice, carrot, and tobacco membranes are probably due to the differences in the population of the binding sites.

**FIG. 8.** Peptide N-glycosidase F treatment of photoaffinity labeled membranes. 125I-AS-PSK-α labeled membranes were suspended Tris-HCl buffer containing 0.5% SDS and incubated at 95 °C for 3 min. Aliquots of this solution were incubated with Nonidet P-40 (1.0% final concentration) and peptide PNGase F (2 milliunits) at 37 °C for 24 h. After deglycosylation, samples were mixed with SDS-PAGE buffer and subjected to SDS-PAGE.

**FIG. 9.** Widespread occurrence of the PSK-α-binding proteins. Plasma membrane fractions derived from rice, carrot, and tobacco cells were incubated with 10 nM 125I-AS-PSK-α and irradiated with UV light after the removal of excess unbound ligand. Cross-linked membrane proteins were solubilized by SDS sample buffer and analyzed by SDS-PAGE and autoradiography under the reducing conditions in the presence or absence of excess unlabeled PSK-α.

Although we detected both high and low affinity binding sites in the rice plasma membrane fraction by the ligand binding assay using [3H]PSK-α (8), the photoaffinity labeling described here only revealed the presence of two binding proteins with high affinity binding constants. The absence of a low affinity protein detected by photoaffinity labeling might be explained by the following. Low affinity binding site could be produced by ligand-induced changes in receptor affinities and, therefore, not present at low ligand concentrations. A negatively cooperative model for hormone-receptor interaction has been reported for several mammal growth factor receptors (16, 17). We therefore tried to determine the binding constants of 125I-AS-PSK-α for the two proteins at high ligand concentrations, but unfortunately the high background precluded meaningful results. Alternatively, these may be two distinct PSK-α-binding sites that have different binding affinities in the rice plasma membrane fraction, but the low affinity site could not be labeled due to its conformational characteristics.

The 160-kDa protein is not a multicomponent complex resulting from covalent cross-linking of the 120-kDa protein with other membrane proteins, since 125I-AS-PSK-α possesses only one photoactivable site which usually reacts with only one target molecule. This conclusion is further supported by the fact that the relative amount of labeling of the 120- and 160-kDa proteins did not change over a wide range of 125I-AS-PSK-α concentrations. The finding of two different PSK-α-binding proteins with different molecular weights can be interpreted in several ways. (a) Only the 160-kDa protein is the biologically relevant receptor, whereas the lower molecular mass 120-kDa protein is a proteolytic breakdown product. (b) The 120-kDa protein is a translation product of a truncated form of the mRNA encoding the 160-kDa protein. (c) The high affinity PSK-α receptor is associated with both of these two proteins without covalent links such as disulfide bonds. (d) Iodine radicals generated by UV irradiation may react at a site somewhat distant from the site of nitrene insertion (18). (e) The two high affinity binding sites are structurally unrelated and are involved in different biological functions of PSK-α.

In the case of mammal growth factor receptor, limited and

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specific proteolytic processes are known to transform the native insulin receptor (19) and the epidermal growth factor factor receptor. A431 cells also possess a 95-kDa form originating from such a truncated mRNA (21). In this context, interpretation a or b shown above may be a better explanation for the presence of two different PSK-α-binding proteins with different molecular weights.

Several well characterized mammal growth factor receptors, including epidermal growth factor receptor (22), platelet-derived growth factor receptor (23), and insulin receptor (24), contain N-linked carbohydrate side chains. It has been demonstrated that core oligosaccharide addition is essential for the acquisition of epidermal growth factor binding activity (25). In this context, the oligosaccharide moieties of the insulin receptor precursor are crucial for proper processing, intracellular translocation, and formation of functionally competent insulin receptors (26). Although the function of the carbohydrate moiety in PSK-α receptors is still unclear, the presence of glycosylated side chain allows us to predict that immobilized lectins will be a useful tool in the purification of the PSK-α receptors.

Occurrence of specifically 125I-AS-PSK-α cross-linked proteins with similar size across distantly related plant species, rice, carrot, and tobacco, is in good agreement with the widespread occurrence of PSK-α. Although the presence of a number of receptor-like membrane proteins involved in plant growth and development has been predicted based on sequence similarities (27–30) and biochemical characterization (31), little is known about the receptor ligand(s) that ultimately activates the receptor function through the receptor-ligand interaction. Recent evidence implies that plants, like animals, may actually make wide use of peptide signaling (1, 32, 33), so that plant cell-to-cell communication is mediated by peptide-receptor interactions. Although further analysis of the PSK-α-binding proteins is needed to determine whether a relationship exists between the two proteins detected here, our work provides a basis for the sequence and sequence analysis of PSK-α receptor(s) that perceive the extracellular peptide signal and transduce the intracellular secondary messengers activating sets of genes involved in plant cell proliferation and differentiation.

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