Kostka, *ibid.* **165**, 96 (1987).
29. R_0 was estimated (*15*) with a value of 0.67 for the orientation factor, which is valid when donor and acceptor dipoles undergo a dynamic randomization of relative orientations during the excited state lifetime of the donor. This assumption is reasonable because of the considerable flexibility of the DABCYL and EDANS linkages to the peptide, as well as the flexibility of the peptide itself. A value for the donor quantum yield (Q) of 0.13 was used on the basis of the yield measured for the substrate fragment PIVQ-EDANS, made by comparison to quinine sulfate in 1*N* sulfuric acid ($Q = 0.546$) [J. N. Demas and G. A. Crosby, *J. Phys. Chem.* **75**, 991 (1971)].
30. Assuming a relative intramolecular diffusion coefficient of the ends of substrate **1** to be 5×10^{-7} cm^2 s^{-1} [E. Haas, E. Katchalski-Katzir, I. Z. Steinberg, *Biopolymers* **17**, 11 (1978)], the donor and acceptor could move approximately 13 Å relative to one another during the excited state lifetime of EDANS.
31. In principle, it should be possible to use an acceptor that is also fluorescent and to obtain reaction rates by monitoring the time-dependent decrease in sensitized acceptor fluorescence. In practice, however, obtaining comparable sensitivity by use of this method is more difficult because it requires finding a D/A pair in which (i) the donor can be excited without significant direct excitationof the fluorescent acceptor, and (ii) the acceptor and donor fluorescence spectra are well-separated from one another.
32. G. T. Wang, E. D. Matayoshi, G. A. Krafft, manuscript in preparation.
33. Steady-state fluorescence data were obtained on a Spex DM-1B spectrofluorometer equipped with photon counting and double-grating excitation and emission monochromators. Samples were measured in 3-mm-square microcells at the desired temperature. Intensity data was acquired continuously and recorded directly on an interfaced microcomputer. The rate of fluorescent substrate hydrolysis was computed from a linear regression analysis.
34. Assays were routinely run in the presence of 10% dimethyl sulfoxide (DMSO) to aid the solubilization of HIV-1 inhibitors, most of which are highly insoluble in purely aqueous solutions. Although DMSO at this concentration reduces the activity of HIV-1 PR by about 25%, we consider it a necessary experimental compromise in order to ensure maximal dissolution of the inhibitors in a monomeric state. The addition of bovine serum albumin (BSA) at 1 mg/ml was also found to greatly reduce losses of HIV-1 PR, substrate, and some inhibitors by nonspecific absorption to the walls of cuvettes, test tubes, and pipette tips. The BSA does not appear to interact with the fluorophore, as judged from its lack of effect on the fluorescence lifetime of the substrate fragment PIVQ-EDANS. In the DMSO- and BSA-containing buffer, the rate of hydrolysis of **1** was confirmed to be directly proportional to the concentrations of HIV-1 PR (*17*).
35. Recombinant HIV-1 PR was obtained from an *Escherichia coli* expression system and purified to approximately 70% (J. Rittenhouse and R. L. Simmer, manuscript in preparation). The concentration of HIV-1 PR in our dilute stock solutions was estimated by the method of M. Bradford [*Anal. Biochem.* **72**, 248 (1976)], as well as by fluorometric determination of the Trp concentration (2 mol of Trp per mole of HIV-1 PR) in samples of denatured protease in 6*M* guanidinium HCl, with *N*-acetyl tryptophanamide in the same solvent as a reference. Estimates obtained with the former method (40% higher than the latter) were used in the calculations reported here. AMV PR was purchased from Molecular Genetic Resources (Tampa, FL).
36. Other fluorogenic substrates are being investigated in which additional charged residues are included beyond the P4 or P4-min positions, in order to improve substrate solubility (*17, 20*).
37. The inner filter artifact [J. B. F. Lloyd, in *Standards in Fluorescence Spectrometry*, J. N. Miller, Ed. (Chapman & Hall, London, 1981), p. 27] is especially severe for this application because of the presence of two chromophores. Although it can be reduced by the use of fluorescence microcells, the artifact becomes significant at concentrations of **1** greater than about 10 μ*M* even with 3 × 3 mm cells. No inner filter correction is necessary if only the relative rates are to be compared in a series of measurements where the substrate concentration remains fixed (and where the varying component is optically transparent at the excitation and emission wavelengths used), since the multiplicative correction factor is the same for each measurement. However, if one wishes to convert hydrolysis rates to concentration units or to compare rates observed at different substrate concentrations (where the absorbance exceeds about 0.02 unit at either excitation or emission wavelengths), the inner filter correction is required. The correction is constant throughout the actual hydrolysis measurement, since the extinction coefficients as well as the absorption and fluorescence spectra of the donor and acceptor are not altered by hydrolysis of the substrate.
38. Free EDANS is suggested as a reference for the inner filter correction, since it is commercially available and spectrally similar to the PIVQ-EDANS fragment that is liberated by proteolysis. For the procedure discussed in the legend to Fig. 4, the added EDANS must be of sufficiently low concentrations so as to not create an inner filter artifact.
39. A. Cornish-Bowden, *Biochem. J.* **137**, 143 (1974).
40. We thank S. Cepa for carrying out HPLC–mass spectrometry, S. Dorwin for the amino acid analyses, and J. Rittenhouse, R. L. Simmer, and coworkers for providing the recombinant HIV-1 PR used in our studies. We are indebted to J. Huffaker for her skilled technical assistance in many of the experiments. This research was supported in part by NIH AI27720.

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Molecular Characterization of a Functional cDNA Encoding the Rat Substance P Receptor

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Substance P is a member of the tachykinin peptide family and participates in the regulation of diverse biological processes. The polymerase chain reaction and conventional library screening were used to isolate a complementary DNA (cDNA) encoding the rat substance P receptor from brain and submandibular gland. By homology analysis, this receptor belongs to the G protein–coupled receptor superfamily. The receptor cDNA was expressed in a mammalian cell line and the ligand binding properties of the encoded receptor were pharmacologically defined by Scatchard analysis and tachykinin peptide displacement as those of a substance P receptor. The distribution of the messenger RNA for this receptor is highest in urinary bladder, submandibular gland, striatum, and spinal cord, which is consistent with the known distribution of substance P receptor binding sites. Thus, this receptor appears to mediate the primary actions of substance P in various brain regions and peripheral tissues.

SUBSTANCE P (SP) IS A NEUROPEPtide considered to function as a neurotransmitter or modulator in the central and peripheral nervous system. Of all neuropeptides, SP is perhaps the best characterized in terms of distribution, sites of release, and biological actions (*1*). SP is a member of a family of structurally related peptides called the tachykinins (*2*). The mammalian tachykinin peptide family currently includes SP, neurokinin A (NKA), neurokinin B (NKB), neuropeptide K, and neuropeptide γ (*3, 4*). SP is produced from any of three differentially spliced mRNAs, and the mature undecapeptide is stored in secretory vesicles and secreted upon cellular stimulation (*5*). SP has excitatory effects on both peripheral and central neurons. It also elicits a variety of biological responses in nonneuronal tissues, including stimulation of smooth muscle contraction, exocrine and endocrine gland secretion, and plasma extravasation and regulation of immune and inflammatory responses (*1, 6*). The stimulation of SP receptors (SPRs) with ligand is followed by an increase in the hydrolysis of phosphatidylinositol 4,5-bisphosphate; this is a result of the G protein–mediated activation of a phosphoinositide-specific phospholipase C (*3, 7*).

We have used molecular cloning techniques in conjunction with expression analysis to determine the primary structure of the rat SPR. The initial isolation of a cDNA fragment encoding part of the SPR was obtained by the use of the polymerase chain reaction (PCR) technique (*8*). Because receptors of the G protein–coupled superfamily display homology in the putative transmembrane domains (*9*), we localized two such areas suspected to be unique for tachykinin receptors on the basis of the deduced sequence of a bovine substance K receptor (SKR) (*10*) and other members of this receptor superfamily. We designed two degenerate oligonucleotides for use as PCR primers (primer PCR II, 5′ TG$^{T}_{G}$ATGGCIGCITT$^{T}_{C}$AA$^{T}_{C}$GC 3′; and primer PCR VII, 5′ ATIGG$^{A}_{G}$TT$^{A}_{G}$TAGATNGT 3′).

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These oligonucleotides corresponded to the putative membrane-spanning domains M-II and M-VII and are mixtures of 20 and 17 bases containing two and one inosines, respectively. With the use of these oligonucleotides as primers and rat small intestine cDNA as a template, a PCR was performed. A 671-bp cDNA fragment was generated, cloned, and sequenced (*11*). This cDNA encoded an open reading frame, which, on the basis of hydrophilicity plotting (*12*), contained a hydrophobic region at either end and four interspersed hydrophobic regions. These hydrophobic regions aligned with the putative membrane-spanning regions of the SKR and thereby allowed analysis of protein homology. This rat cDNA encoded a protein fragment that displayed 73% similarity to the bovine SKR, but showed less that 30% similarity to any other G protein–coupled receptor.

The cDNA fragment was used to isolate the entire coding region of the putative tachykinin receptor by initially screening a rat genomic library (*13, 14*). The 671-bp cDNA fragment was found to be encoded by four exons. The upstream exon contained the initiator methionine on the basis of the consensus translation start sequence (*15*), and the 3′ exon extended through the end of M-VII. With the use of oligonucleotide primers based on these two ends (*16*) and PCR, mRNAs were detected in the rat submandibular gland, small intestine, cerebral cortex, and urinary bladder (but not vas deferens or kidney) that generated cDNA fragments with sequences identical to that of the four exons combined. The 671-bp cDNA was also used to screen a rat hippocampal cDNA library (*17*). A cDNA clone was isolated that extended 610 bp past the 3′ end of the M-VII sequence and encoded an open reading frame extending from nucleotide position +552 to a stop codon located 99 codons (nucleotide + 1224) after the COOH end of M-VII. In addition, this cDNA clone extended 310 bp into the 3′ noncoding region. We used PCR to generate a cDNA containing the entire coding region of this putative receptor. Oligonucleotides were synthesized corresponding to the 5′ end, as determined from the genomic analysis, and to the 3′ end as determined from the cDNA analysis. A cDNA fragment of 1248 bp was generated from rat cortex, duodenum, submandibular gland, urinary bladder cDNA (but not from vas deferens cDNA), and from the rat hippocampal cDNA library. The resulting cDNA fragments from hippocampus and submandibular gland were subcloned, and the nucleotide sequence was determined (Fig. 1). The putative SPR polypeptide consists of 407 amino acid residues with a molecular mass of 46,385 daltons and an average isoelectric point (*p*I) of 6.1. The hydrophilicity profile

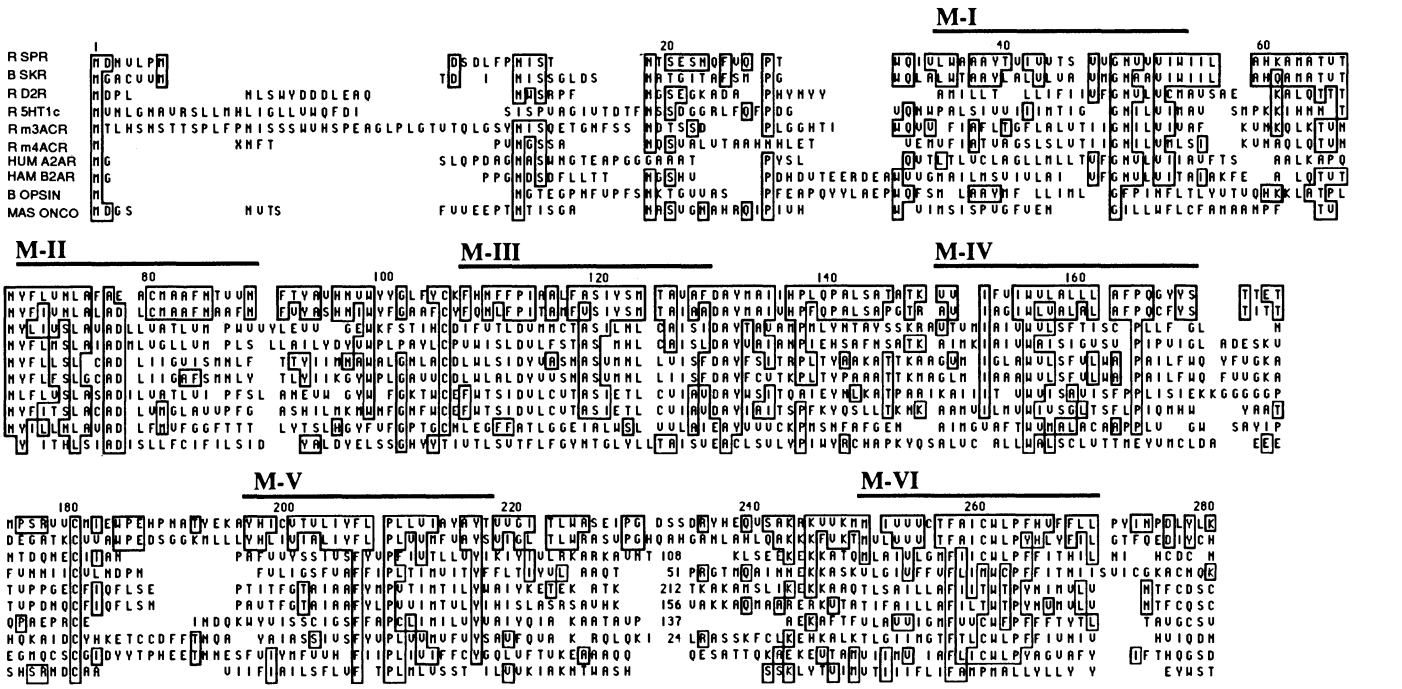

Fig. 1. Primary structure of the rat substance P receptor (R SPR) and alignment of its primary structure with the bovine substance K receptor (B SKR) (*10*), rat dopamine D_2 receptor (R D2R) (*22*), rat $5HT1_c$ serotonin receptor (R 5HT1c) (*23*), rat m3 and m4 muscarinic receptors (R m3ACR and R m4ACR) (*21*), human α_2- and hamster β_2-adrenergic receptors (HUM A2AR and HAM B2AR) (*9*), bovine opsin (B OPSIN) (*20*), and the product of the oncogene c-*mas* (MAS ONCO) (*25*). Amino acid abbreviations are indicated (*33*). The SPR nucleotide sequence has been obtained from full-coding region cDNA clones, genomics exons, and PCR-generated fragments. Full-coding region–containing clones were isolated from rat submandibular gland and hippocampal cDNAs. The nucleotide sequence (*11*) has been deposited into GenBank, accession number M31477. The putative membrane-spanning domains of the receptors are indicated by solid bars, with M-I to M-VII indicated above each. The numbers above the receptor sequence denote the numbering of the SPR primary sequence. The amino acid residues conserved in the SP receptor with other members of this superfamily are enclosed in boxes. The complete third cytoplasmic domain is not included for all sequences shown, and the numbers present in these regions represent the number of amino acid residues not presented within each gap.

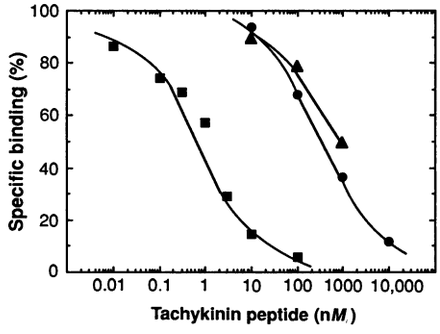

Fig. 2. Pharmacology of tachykinin peptide ligand binding to the SPR in transfected COS-7 cells. An expression vector containing the SPR cDNA coding region was created by directionally ligating the SPR cDNA (nucleotides −8 to +1224) at Hind III–Bam HI sites into pBC12BI (*18*). The expression of the SPR cDNA is under the control of the Rous sarcoma virus LTR. COS-7 cells were transfected by the DEAE-dextran method (*18*) and whole cells were used for ligand binding studies. Cells were incubated at 4°C in PBS containing ^{125}I-labeled Tyr^{8}-SP (0.1 n*M*) and the indicated concentration of unlabeled SP (■), NKA (●), or NKB (▲). Nonspecific binding of ^{125}I-labeled Tyr^{8}-SP was determined by binding of ligand to nontransfected COS-7 cells, or in cells incubated with 1000-fold excess unlabeled SP. These results were compiled from four separate transfection experiments, and the range of each data point was less than 15% of the mean value shown. Nonspecific binding represented 10 to 15% of the total ^{125}I-labeled Tyr^{8}-SP bound.

of the deduced amino acid sequence and an alignment with the bovine SKR revealed seven hydrophobic domains, consistent with this receptor being a member of the G protein–coupled receptor superfamily.

To determine the functional nature of this protein, we subcloned the full-coding region cDNA fragment into the mammalian expression vector pBC12BI and expressed it in the COS-7 cell line (*18*). These cells were then examined for expression of the SPR by ligand displacement and Scatchard analysis with ^{125}I-labeled Tyr^{8}-SP as ligand (*19*). On the basis of Scatchard analysis, the SPR has a dissociation constant (K_d) of 3.5 n*M*. The transfected cells had 5.4 fmol of binding sites per 200,000 cells, or 16,200 binding sites per cell, whereas no binding was seen in nontransfected cells. Displacement studies of ^{125}I-labeled Tyr^{8}-SP (Fig. 2) with SP, NKA, or NKB indicated that SP was the most potent competitor with a 50% inhibitory concentration (IC_{50}) of $\sim 1 \times 10^{-9} M$. The corresponding IC_{50} values for NKA and NKB were more than 100-fold as great (NKA, $3 \times 10^{-7} M$; NKB, $1 \times 10^{-6} M$).

The SPR shares many amino acid sequence features common to the G protein–coupled receptor superfamily. This superfamily includes rhodopsin and related opsins (*20*), adrenergic receptors (*9*), muscarinic cholinergic receptors (*21*), dopamine receptor (*22*), serotonin receptors (*23*), yeast peptide mating factor receptors (*24*), the product of the oncogene c-*mas* (*25*), and SKR (*10*). A comparison of some of the members of this superfamily with the SPR is shown in Fig. 1. The sequences are divided into distinct domains based on the NH_2-terminus facing the extracellular milieu, seven α-helical transmembrane domains, and the COOH-terminus oriented intracellularly. The SPR shows the greatest homology to the SKR. In general, the most homologous portion of these two receptors are the membrane-spanning regions, which show 83% similarity including conservative changes (*26*), whereas the extracellular and cytoplasmic domains are much less homologous (57 and 51% similarity, respectively). The most related portions of these two receptors are the second cytoplasmic loop domain and the sixth membrane-spanning domain, with the protein sequence similarity being 95% for both. The least related portions are the NH_2- and the COOH-terminal domains, the second extracellular domain, and the third cytoplasmic domain, with protein sequence similarity of 52%, 33%, 38%, and 59%, respectively. These divergent sequences may be important in ligand binding specificity, in the case of the extracellular differences, and G protein interactions, in the case of the cytoplasmic differences. On the basis of the relative homology of this receptor with a bovine SKR, it can be concluded that this receptor is a related tachykinin receptor, and the ligand displacement and Scatchard analysis demonstrate that it is a SPR.

One interesting structural difference between the SPR and the SKR is the greater number of potential phosphorylation sites in the third cytoplasmic loop domain and the COOH-terminal domain of the former. The SPR contains 31 serine or threonine residues in these regions, and 26 out of 99 residues in the COOH-terminal domain are either serine or threonine. The SKR contains only 18 serine or threonine residues within these regions, and 14 out of 65 residues in the COOH-terminal domain are serine or threonine. Phosphorylation of residues in these regions of the β_2-adrenergic receptor is related to desensitization (*27*). The SPR is much more rapidly desensitized than the SKR (*28*). Another interesting area of comparison is the second and third extracellular domains. Although the aspartate residue in M-II (conservatively changed to a glutamate residue in the SPR) and M-VII have been postulated to be important in ligand binding of the adrenergic receptors (*29*), the ligands for the tachykinin receptors are peptide and, therefore, because of their larger size, presumably require more sites of

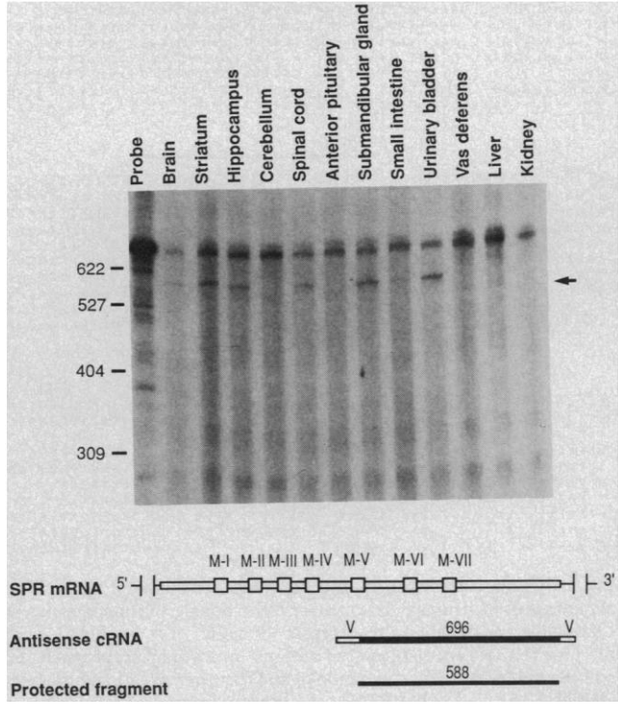

Fig. 3. Nuclease protection analysis of SPR mRNA distribution. Nuclease protection assays were performed as described (*31*). The lower portion depicts schematically the SPR mRNA, the antisense cRNA probe, and the protected SPR mRNA fragment. ^{32}P-labeled antisense cRNA of 696 bases (588 bases of SPR sequence, 108 bases of vector sequence; V, vector) was annealed with tissue RNA. After nuclease digestion, the protected species (arrow) were analyzed by polyacrylamide gel electrophoresis and autoradiography. Densitometric analysis of autoradiographic exposures from two experiments allowed quantitation of SPR mRNA relative to synthetic SPR message sense cRNA. The relative amount of SPR mRNA per 25 μg of total RNA was urinary bladder, 154 pg; striatum, 115 pg; submandibular gland, 114 pg; spinal cord, 49 pg; brain, 43 pg; hippocampus, 38 pg; and small intestine, 23 pg, with the remaining tissues containing undetectable amounts of SPR mRNA. The probe lane contains 2.5% of the total probe used in each protection assay. Size marker was ^{32}P-labeled pBR322 digested with Hpa I, with values being shown in bases.

interaction. These interactions may involve extracellular regions. The second and third extracellular loop domains are very different between the SPR and SKR, except for the COOH-terminal half of the third extracellular loop domain, where eight amino acid residues and their corresponding nucleotide sequence are exactly conserved. The latter area may provide sites for interaction with the COOH-terminal region of the tachykinin peptides, because this region of SP and NKA are highly conserved (Phe-X-Gly-Leu-Met-NH_2) and is required for high-affinity interaction (*6, 7*), whereas the rest of these two loops may provide for specific tachykinin ligand selectivity.

The expression of the SPR mRNA was examined by Northern (RNA) blot analysis and nuclease protection assays. The Northern blot analysis (*30*) with polyadenylated RNA indicated an mRNA species of ~3000 bp; however, the expression was detectable only in tissues where there was apparently the greatest abundance of mRNA. Therefore, the more sensitive nuclease protection assay (*31*) was used to examine the SPR mRNA abundance. A cRNA probe corresponding to 588 bases of the SPR was used to hybridize with RNA isolated from various tissues (Fig. 3). One mRNA species was protected that is the same size (and therefore identical sequence) in both the central nervous system and in peripheral tissues. In addition, this species is of greatest abundance in tissues known to contain relatively high numbers of SP binding sites (that is, urinary bladder, striatum, submandibular gland, and spinal cord). The levels of detectable expression represent 0.0006 to 0.003% of the total RNA, based on comparisons with message sense RNA produced from the cloned cDNA. These results further demonstrate that the mRNA encoding this SPR, which preferentially binds SP, is expressed in the tissues containing relatively high amounts of SPR ligand binding activity (*1, 6, 7*). Consequently, these studies demonstrate that the described SPR mediates the primary actions of SP in the central nervous system and in peripheral tissues.

Note added in proof: After submission of this report, a communication appeared (*32*) that provided a similar SPR sequence and a similar ligand displacement profile as that described herein.

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16. 5′ end: 5′ CCAAGCTTGCGCCACCATGGATAACGTCCTTCC 3′. M-VII 3′ end: 5′ CGCGGATCCTGTCGTTGAGGCAACAGTAT 3′.
17. The rat hippocampal cDNA library (Clontech Laboratories, Palo Alto, CA; catalog number RL 1026b, lot 3521) was generated by inserting cDNA (that was randomly primed from adult male Sprague-Dawley rat hippocampal mRNA) into the Eco RI site of λGT11. The library consisted of 2.3×10^6 independent clones. Approximately 1.25×10^6 pfu were screened with the ^{32}P-labeled, random-primed 671-bp cDNA fragment. Eleven cDNA clones were isolated and subcloned into pBluescript, and the nucleotide sequence of selected cDNAs was determined.
18. The 1248-bp fragments generated by PCR (from submandibular gland cDNA and the hippocampus cDNA library) contained a Hind III restriction site at the 5′ end and a Bam HI restriction site at the 3′ end (as engineered in the PCR primers). These fragments were digested with these two restriction enzymes in order to create overhanging 5′ ends that allowed the fragments to be directionally subcloned into Hind III–Bam HI sites of the mammalian expression vector, pBC12BI [B. R. Cullen, *Methods Enzymol.* **152**, 684 (1987)]. This plasmid is oriented so that the inserted fragment is under the control of the Rous sarcoma virus long terminal repeat (LTR). The plasmid containing the submandibular gland cDNA fragment was then used to transfect COS-7 cells by a modification of the DEAE-dextran–mediated transfection procedure [B. R. Cullen, above). This consisted of seeding a 150-mm culture dish with COS-7 cells and allowing them to reach 10 to 25% confluency. For transfection, the medium was removed, the plates were washed with phosphate-buffered saline (PBS) at 37°C, and 10 ml of medium, containing Dulbeccos's modified Eagle's medium containing glucose (4.5 g/liter) [DME(HG/HB)], 10% Nu-serum IV (Collaborative Research, Bedford, MA), 1 m*M* pyruvate, penicillin (100 U/ml), streptomycin (100 μg/ml), 25 μg of plasmid, 4 mg of DEAE-dextran, and 100 μ*M* chloroquine was added. After 3 hours at 37°C, the medium was removed, and 10 ml of 10% dimethylsulfoxide (DMSO) in PBS was added for 2 min at 22°C. The DMSO solution was then removed, the cells were washed with 30 ml of PBS, and 30 ml of DME(HG/HB) containing 10% fetal bovine serum, 1 m*M* pyruvate, penicillin (100 U/ml), and streptomycin (100 μg/ml) was added. Cells were harvested 48 to 72 hours after transfection.
19. Pharmacological analysis of the expressed receptor included ligand displacement and Scatchard analysis [G. Scatchard, J. S. Coleman, A. L. Shen, *J. Am. Chem. Soc.* **79**, 12 (1957)] with ^{125}I-labeled Tyr^8-SP (2200 Ci/mmol), prepared as described [J. E. Krause, A. J. Reiner, J. P. Advis, J. F. McKelvy, *J. Neurosci.* **4**, 775 (1984)]. Transfected cells were scraped from the tissue culture dish, washed with PBS, and resuspended at a concentration of 10^6 cells per milliliter. For each assay point, either 200,000 or 400,000 cells in a 0.5-ml volume were first incubated at 4°C for 30 min. In ligand displacement experiments, ^{125}I-labeled Tyr^8-SP (0.1 n*M*) and the appropriate amount of unlabeled SP in PBS containing 40 μg of bacitracin, 4 μg of leupeptin, 4 μg of chymostatin, and 0.2 mg of bovine serum albumin (BSA) (final amounts per assay) were added, and the cells were incubated for 1 hour at 4°C. For Scatchard analysis, ligand concentration ranged from 0.01 to 10 n*M*. Bound ligand was then separated from free ligand by rapid filtration onto glass fiber filters. The filters were washed with PBS, and the bound radioactivity was determined in a gamma counting system (TM Analytic, Elk Grove, IL). A similar peptide displacement profile was observed when ^{3}H-labeled SP was used as radiolabeled ligand.
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30. For Northern blot analysis, 10 μg of polyadenylated RNA [resuspended in 20 m*M* 3-[*N*-morpholino] propanesulfonic acid (MOPS), *p*H 7.0), 5 m*M* sodium acetate, 0.5 m*M* EDTA, 6% formaldehyde, and 50% formamide] was subjected to electrophoresis in a 1.0% agarose gel and 40 m*M* MOPS, 10 m*M* sodium acetate, 1 m*M* EDTA, and 6% formaldehyde. RNA was transfered to nitrocellulose (Schleicher & Schuell, Keene, NH) and hybridized with the ^{32}P-labeled, random-primed, 671-bp cDNA fragment.
31. For nuclease protection analysis, the general procedures previously described were used as a guide [J. Favaloro, J. Treisman, R. Kamen, *Methods Enzymol.* **65**, 718 (1980); J. E. Krause, J. D. Cremins, M. S. Carter, E. R. Brown, M. R. MacDonald, *ibid.* **168**, 634 (1989)]. A cDNA fragment encoding nucleotides +637 to +1224 of the SPR cDNA was inserted into the Hinc II site of pBluescript, and a ^{32}P-labeled antisense cRNA was transcribed from the T7 polymerase promoter. A[^{32}P]uridine triphosphate (UTP)–labeled cRNA fragment of 696 bases (200,000 cpm; specific activity, 2.37×10^8 cpm/μg) was produced, which was annealed with 25 μg of total RNA (prepared by the guanidine thiocyanate method [J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]) from various tissues for 12 hours in 80% formamide containing 1 m*M* EDTA, 400 m*M* NaCl, and 40 m*M* 1,4-piperazinediethanesulfonic acid (*p*H 6.4). Nonannealed nucleic acids were digested with 300 U of S1 nuclease in a buffer containing 280 m*M* NaCl, 4.5 m*M* $ZnSO_4$, and 30 m*M* sodium acetate (*p*H 4.4). Products were analyzed by electrophoresis in a 6% polyacrylamide gel containing 7*M* urea, followed by autoradiography. The amount of specifically hybridized SPR mRNA (the 588-base species) was quantitated by densitometry with a model 620 Bio-Rad video densitometer (Richmand, CA).
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33. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E. Glu; F, Phe; G, Gly; H, His; I. Ile; K. Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
34. We thank Y. Takeda for preparation of the ^{125}I-labeled Tyr^8-SP and helpful discussions, P. Dykema for genomic library screening, R. Hynes for the rat genomic library, and B. Cullen for the plasmid expression vector pBC12BI. Supported in part by the Division of Biology and Biomedical Sciences, Washington University; NIH grant NS21937; and the Pew Memorial Trust. J.E.K. is a Pew Scholar in the Biomedical Sciences.

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Ligand-Induced Transformation by a Noninternalizing Epidermal Growth Factor Receptor

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Identification of a mutant epidermal growth factor (EGF) receptor that does not undergo downregulation has provided a genetic probe to investigate the role of internalization in ligand-induced mitogenesis. Contact-inhibited cells expressing this internalization-defective receptor exhibited a normal mitogenic response at significantly lower ligand concentrations than did cells expressing wild-type receptors. A transformed phenotype and anchorage-independent growth were observed at ligand concentrations that failed to elicit these responses in cells expressing wild-type receptors. These findings imply that activation of the protein tyrosine kinase activity at the cell membrane is sufficient for the growth-enhancing effects of EGF. Thus, downregulation can serve as an attenuation mechanism, without which transformation ensues.

ACTIVATION OF THE EGF RECEPTOR initiates a cascade of cellular events (*1, 2*). On binding ligand, the intrinsic tyrosine kinase is triggered, and this is immediately followed by a rise in cytosolic-free calcium concentration and receptor internalization, which results in receptor degradation. Specific gene transcription is stimulated within minutes. Hours later, DNA synthesis and cell division occur. Mutational analysis has shown that the tyrosine kinase activity is necessary for all subsequent receptor actions, including internalization (*3*). The relation between these early events and the mitogenic response has been unclear. This has led to suggestions that receptor or ligand internalization, or both, may be required for DNA synthesis to occur (*4*). Cells expressing a membrane-bound EGF receptor ligand, the precursor of transforming growth factor α (pro–TGF-α), were capable of causing some of the immediate responses in neighboring cells (*5*), but effects on DNA synthesis are still unknown.

An EGF receptor that failed to undergo ligand-dependent endocytosis would allow determination of the function of internalization in initiating cell growth. Serial truncations of the COOH-terminus of the EGF receptor identified a region that couples ligand binding to receptor internalization and degradation (*6*). The COOH-terminal 213 amino acids of the 1186–amino acid EGF receptor contain the inhibitory domain (*7*) and the calpain hinge (*8*), as well as the newly defined internalization region (amino acids 973 to 1022). A receptor mutant truncated at amino acid 973 (c'973) failed to downregulate but had a competent kinase, with binding of ligand leading to increased transcription (*6*). Because this mutant was expressed in a transformed cell and at supraphysiologic levels, the effects on cell growth and morphology could not be evaluated.

Expression of the internalization-defective mutant on the surface of a nontransformed cell line that does not express endogenous EGF receptors, the NIH 3T3–derived NR6 cells (*9*), allowed delineation of the link between endocytosis and cell growth. NR6 cell lines that presented either the wild-type (WT) or the truncated (c'973) receptor were derived. Cells were transfect-

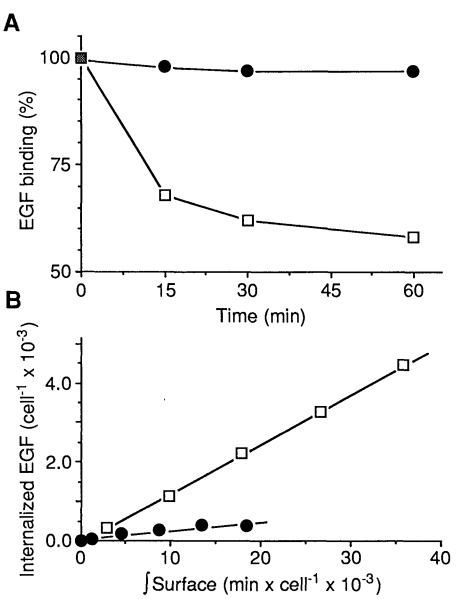

Fig. 1. Ligand-induced downregulation (**A**) and internalization (**B**) of EGF receptors. (A) NR6 cells expressing either the WT (□) or truncated c'973 (●) EGF receptors were treated with 50 n*M* EGF for the indicated times at 37°C. Cell surface ligand was removed by the acid-stripping procedure, and ^{125}I-labeled EGF binding was measured (*21*). Results are the means of triplicates, which differed by <5%. (B) Endocytic rates for NR6 cells expressing the WT (□) or c'973(●) EGF receptors. The plot was derived according to previously published methods (*22*). The internalization incubations were performed at 37°C for 5 min. The failure of the c'973 receptor to internalize was confirmed by immunofluorescent studies (*23*).

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