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16. Experimental constructs, cloned into the pBluescript (Stratagene) plasmids, were transcribed with T3 RNA polymerase under standard reaction conditions in the presence of $m^7(5')Gppp(5')G$. The mRNAs were translated in the presence of [^{35}S]methionine (Trans-^{35}S label, 1066 Ci/mmol, ICN Radiochemicals) in a wheat germ extract (Promega) treated for 30 min at 4°C with *N*-acetyl-D-glucosamine attached to agarose beads (Sigma). Protein synthesis was stopped by the addition of 30 μM cycloheximide, and the translation products were frozen in liquid nitrogen and stored at −80°C until use. Mice (DBA/2) were cervically dislocated and exsanguinated, and mitochondria were isolated from the livers (*24*). Transport assays were done as described (*25*) with the following modifications. Transport reactions (25 μl final volume) contained freshly isolated mitochondria (100 μg of total protein) in 5 μl of isolation buffer (*24*), 10 μl of untreated rabbit reticulocyte lysate (Promega) (*26*), and 5 μl of [^{35}S]methionine-labeled proteins in buffer containing 12 mM Hepes (pH 7.6), 0.5 mM spermidine, creatine phosphokinase (50 μg/ml), 10 μM creatine phosphate, 25 mM potassium acetate, 1 mM magnesium acetate, and 5 mM dithiothreitol. Hemin (Porphyrin Products, Logan, UT) prepared as described (*27*) was added in a final volume of 5 μl at the indicated concentrations. Hemin concentrations were determined as described (*28*). Animal care was in accordance with institutional guidelines. Experiments were repeated a minimum of three times with preALAS-E major, preALAS-E minor, and preOTC as controls, and all results were reproducible under all conditions tested.
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19. For the construction of the preALAS-E–OTC fusion proteins, plasmid encoding the preOTC was digested with Bam HI, Xho I, and Pvu II. The 1044-bp fragment encoding the mature OTC was ligated into the preALAS-E vectors prepared by digestion with Nco I, blunted with Klenow fragment of DNA polymerase I, and digested with Bam HI. The resulting fusion proteins contained the 92 NH_2-terminal residues of preALAS-E linked to the COOH-terminal 318 amino acids of mature OTC. For the construction of the mutant proteins 6327, 6328, and 6329, plasmid encoding preOTC was digested with Bam HI, blunted, and digested with Pst I. The 1350-bp fragment was ligated into preALAS-E vectors. The ALAS-E HRMs were mutagenized with the oligonucleotides shown in Fig. 4 according to the Altered Sites technical manual (Promega). The mutagenized plasmids were prepared by digestion with Afl II, blunted, and digested with Bam HI. The resulting fusion proteins contain the 53 NH_2-terminal residues of preALAS-E linked to the 402 COOH-terminal amino acids of preOTC.
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29. 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.
30. Protein import into mitochondria was linear under the described experimental conditions (*16*) for 90 min. The amount of transport at 1 hour was determined by densitometry of autoradiographs with the use of the Adobe Photoshop (Adobe Systems, Mountfairview, CA) 2.0 and Image 1.40b3 computer programs (W. Rashand, National Institute of Mental Health). Densitometry was standardized, and measurements were taken in the linear range as determined by standard curves. Graphs present mean values for replicated experiments. Representative autoradiographs are presented.
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Identification of the SH3 Domain of GAP as an Essential Sequence for Ras-GAP–Mediated Signaling

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Guanosine triphosphatase activating protein (GAP) is an essential component of Ras signaling pathways. GAP functions in different cell types as a deactivator and a transmitter of cellular Ras signals. A domain (amino acids 275 to 351) encompassing the Src homology region 3 (SH3) of GAP was found to be essential for GAP signaling. A monoclonal antibody was used to block germinal vesicle breakdown (GVBD) induced by the oncogenic protein Ha-ras Lys^{12} in *Xenopus* oocytes. The monoclonal antibody, which was found to recognize the peptide containing amino acids 275 to 351 within the amino-terminal domain of GAP, did not modify the stimulation of the Ha-Ras–GTPase by GAP. Injection of peptides corresponding to amino acids 275 to 351 and 317 to 326 blocked GVBD induced by insulin or by Ha-Ras Lys^{12} but not that induced by progesterone. These findings confirm that GAP is an effector for Ras in *Xenopus* oocytes and that the SH3 domain is essential for signal transduction.

Ras proteins are required for the functioning of the signal transduction pathways that regulate cell growth and differentiation (*1*, *2*). The function of Ras in mammalian cells depends on its interaction with GAP, a protein that can stimulate the intrinsic GTPase activity of Ras (*3*). A complex between Ras and GAP is responsible for interrupting muscarinic atrial K^+ channel currents (*4*, *5*). In Chinese hamster ovary cells Ras induces transcription from the polyoma virus enhancer only in the presence of full-length GAP (*6*). Mutations in Ras (in the so-called effector domain between amino acids 30 and 40) block biological activity and GAP-stimulated GTP hydrolysis (*7*, *8*). GAP binds to a number of proteins in vitro. The COOH-terminal domain is sufficient for binding to cellular Ras proteins (*9*). The NH_2-terminal domain contains SH2 and SH3 domains that allow

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tight binding to platelet-derived growth factor receptors, epidermal growth factor receptors, insulin receptors, v-Src, and phosphoproteins (*10–15*). The SH2 domains are similar to regulatory sequences of other nonreceptor tyrosine kinases, phospholipase C-γ, the p85 subunit of Pi-3 kinase, and other molecules that have been referred to as adaptors, such as v-Crk, Nck, and Sem-5 (*14, 15*). SH3 is a small protein domain containing about 60 amino acid residues that are similar yet distinct in various proteins. The presence of SH3 domains in a wide variety of otherwise unrelated proteins suggests that these domains may be important for coordinating protein-protein interactions necessary for the function or cellular localization of these proteins (*15*). In the *Xenopus* oocyte Ras is required for GVBD induced by insulin-like growth factor (IGF) or insulin but not that induced by progesterone (*16*). GAP enhances the action of transforming v-Ha-Ras and insulin in *Xenopus* oocytes (*17*). We used a monoclonal antibody (MAb200) to one isoform of GAP, the p100 GAP protein (*18*), to test the function of the domain encompassing the SH3 sequence in Ras-dependent GVBD in *Xenopus* oocytes.

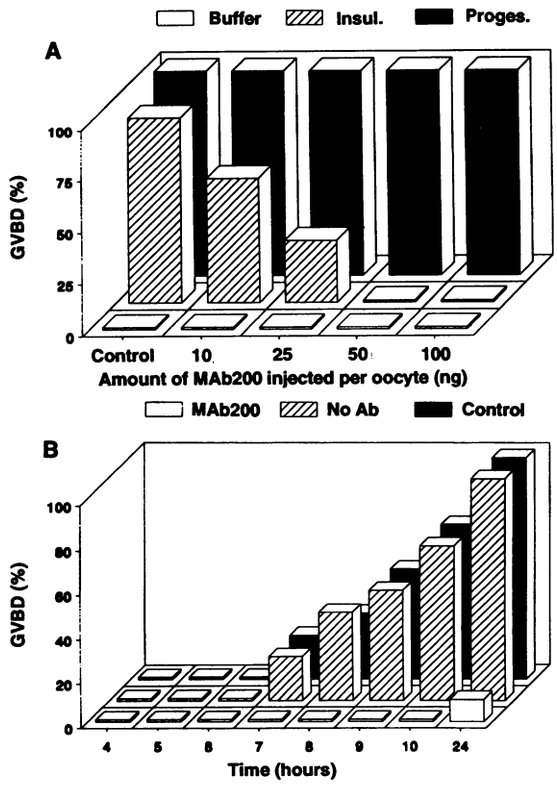

Fig. 1. Effect of microinjected MAb200 on GVBD induced by hormones (**A**) or by Ha-Ras Lys^{12} (**B**). (A) Groups of ten stage VI oocytes, recovered by stripping of the oocyte clumps for 2 hours at room temperature in collagenase (2 mg/ml) (Sigma), were microinjected with approximately 40 nl of control mouse immunoglobulin G (IgG) (2.5 mg/ml) (Control) or with 40 nl of solution containing the indicated amounts of MAb200 per oocyte. Oocytes were placed into Barth buffer (2 ml) (*24*) containing bovine serum albumin (1 mg/ml) (Buffer) and 1 μM insulin (Insul.) or 1 μM progesterone (Proges.). GVBD was scored after 18 hours of incubation by the dissection of oocytes that had been fixed in 5% trichloroacetic acid. (B) Oocytes were microinjected with 40 nl of a 1 to 1 (v/v) mixture of MAb200 (5 mg/ml) and of Ha-Ras Lys^{12} (0.5 mg/ml) (MAb200), 40 nl of Ha-Ras Lys^{12} (0.25 mg/ml) (No Ab), or 40 nl of a 1 to 1 (v/v) mixture of mouse IgG (5 mg/ml) and Ha-Ras Lys^{12} (0.5 mg/ml) (Control). The results are expressed as the percentage of each group of oocytes that underwent GVBD. Each experiment was reproduced at least four times.

Different amounts of MAb200 were microinjected in stage VI oocytes. The oocytes were then incubated with insulin or progesterone, and GVBD was evaluated by scoring of the oocytes every hour after injection. The MAb200 inhibited insulin-induced GVBD with an inhibition concentration (IC_{50}) of 20 ng per oocyte but did not modify progesterone-induced GVBD (Fig. 1A). The MAb200 also blocked GVBD that was induced in oocytes microinjected with Ha-Ras Lys^{12} (10 ng per oocyte) (Fig. 1B). The GAP domain recognized by the MAb200 was evaluated with a series of GAP constructs encoding different portions of the GAP protein (Fig. 2). The constructs were expressed as glutathione-S-transferase (GST) fusion proteins in *Escherichia coli* strain TG1. The

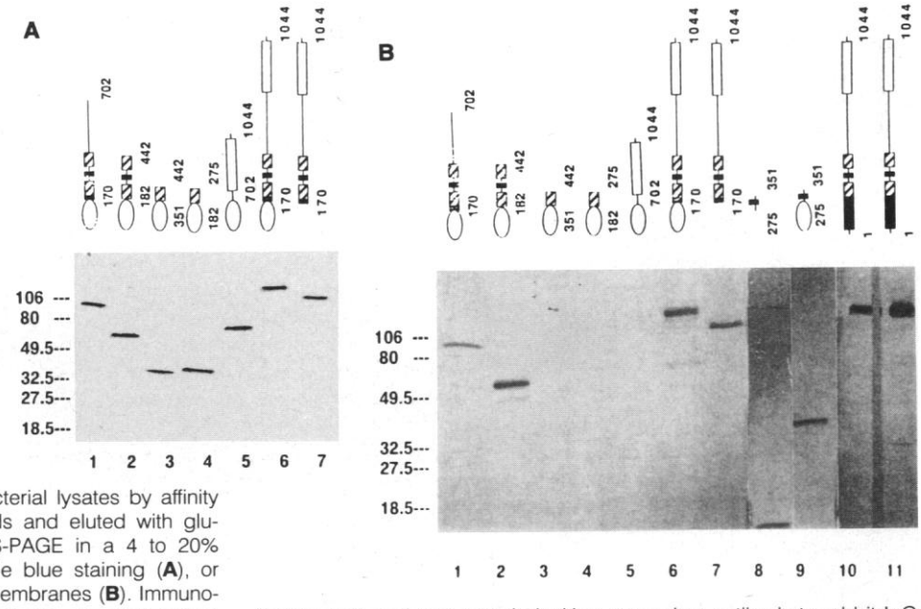

Fig. 2. Mapping of the MAb 200 epitope on GAP. The p100 GAP sequence (a.a. 170 to 1044), the catalytic domain (a.a. 702 to 1044), and the NH_2-terminal domain of p100 GAP (a.a. 170 to 702) have already been described (*6, 18*). Ras-GAP (a.a. 1 to 1044) was partially purified from bovine brain (*7*). The SH2-SH3 region (a.a. 182 to 442), the two SH2 domains (a.a. 182 to 286 and 337 to 442), and the SH3 sequence (a.a. 275 to 351) were obtained by polymerase chain reaction (PCR). Oligonucleotides corresponding to the boundaries of the SH3 domain of human PLC-γ cDNA (*25*) were used to amplify the desired stretches on human brain cDNA by PCR to obtain PLC-γ (787 to 860). Complementary DNAs were ligated to the Bam HI and Eco RI restriction sites of the pGEX 2T vector. Expression of the GST fusion proteins was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (1 μM), and the fusion proteins were isolated from TG1 bacterial lysates by affinity chromatography with glutathione-agarose beads and eluted with glutathione. The proteins were subjected to SDS-PAGE in a 4 to 20% polyacrylamide minigel, revealed by Coomassie blue staining (**A**), or transferred to polyvinylidene difluoride (PVDF) membranes (**B**). Immunoblotting was done with MAb200 (0.5 μg/ml) and was revealed with a secondary antibody to mouse IgG linked to horseradish peroxidase detected by 4-chloro-1 naphthol (lanes 1 to 10). Bovine GAP (a.a. 1 to 1044) was detected with a rabbit polyclonal antibody raised against GAP (146 to 159) and was revealed with a secondary antibody to rabbit IgG linked to horseradish peroxidase (lane 11). Molecular size markers are shown at the left (in kilodaltons). ○, GST; ▨, SH2 domain; ■, SH3 domain; □, catalytic domain.

proteins were purified on glutathione-agarose columns, separated by SDS–polyacrylamide gel electrophoresis (PAGE), and blotted with MAb200. The MAb200 recognized the NH_2-terminal domain of GAP and, within this domain, a 76–amino acid stretch [amino acids (a.a.) 275 to 351] encompassing the SH3 region (a.a. 286 to 340). The epitopes recognized by MAb200 were two hydrophilic regions of GAP (a.a. 277 to 287 and 313 to 317) (*19*).

To further evaluate whether a.a. 275 to 351 of GAP [GAP (275 to 351)] are critical for Ras signaling, we injected oocytes with this peptide. GAP (275 to 351) (100 ng per oocyte) blocked GVBD induced by either Ras or insulin. Dose-response studies showed that the minimum amount of the peptide required to block GVBD was about 10 ng per oocyte. Peptide containing a.a. 786 to 860 from PLC-γ [PLC-γ (786 to 860)], which encompasses the SH3 region of PLC-γ and is similar but not identical to the SH3 domain of GAP, did not inhibit GVBD activity when injected at this concentration (Fig. 3A). We determined the amount of GAP present in uninjected oocytes to be 1 ng per oocyte with a two-site immunometric assay (*18*). Therefore, the concentration of GAP (275 to 351) was 1400 times greater than the concentration of GAP in the oocytes. We tried to determine the involvement of the SH3 domain in Ras signaling and tested peptides within the SH3 domain or encompassing one of the MAb200 epitopes, GAP (273 to 305), for Ras-dependent GVBD. Among these peptides, GAP (317 to 326) inhibited GVBD induced by Ha-Ras Lys^{12} but not that induced by progesterone (Fig. 3B). We tested whether GAP (275 to 351) could antagonize Ras-GAP signaling by preventing GAP from associating with cellular components through its SH2 domains or by interfering with the Ras-GAP interaction. The effect of GAP (275 to 351) on the in vitro interaction of peptides containing either the two SH2 and the SH3 domains of GAP [GAP (182 to 442)] or one SH2 domain [GAP (182 to 275)] with phosphorylated epidermal growth factor receptors (EGF-R) was evaluated. EGF-R from EGF-activated cells were partially purified in the presence of phosphatase inhibitors and mixed with GAP (182 to 442) or GAP (182 to 275) fused in frame with the GST, with or without GAP (275 to 351). Phosphorylated EGF-R bound equally well to GAP (182 to 442) and GAP (182 to 275) in the presence of GAP (275 to 351) (Fig. 4), suggesting that the epitope-binding domain of MAb200 on GAP did not participate in the interaction between EGF-R and GAP. However, it cannot be excluded that GAP (275 to 351) may prevent the binding of cellular GAP to phosphoproteins in *Xenopus* oocytes via SH2 domains.

The GTPase activity of Ha-Ras stimulated by p100 GAP was also analyzed. In the presence or absence of MAb200 or GAP (275 to 351), there was no difference in the rate of the GTPase reaction (*20*), whereas the neutralizing antibody to Ras, Y13-259, or antibodies directed to the so-called "effector domain" of Ha-Ras (*21*) (a.a. 30 to 40) inhibited the ability of p100 GAP to enhance the GTPase activity of Ras. The peptide GAP (317 to 326) did not modify either the association of GAP (182 to 442) to phosphorylated EGF-R or the enhancement of the GTPase activity of Ras by GAP (*20*). MAb200 or GAP (273 to 351) block Ras-mediated maturation of *Xenopus* oocytes by interacting with a domain of GAP different from the catalytic domain. GAP (275 to 351) probably acts by competing with intact GAP for binding to downstream components. Although there are a number of similarities between SH3 domains from different proteins, identification of a putative SH3 is not so obvious. The same domain derived from PLC-γ was not effective in blocking Ras-induced GVBD. This specificity is not surprising considering that single point mutations within the hydrophilic regions of the NH_2-

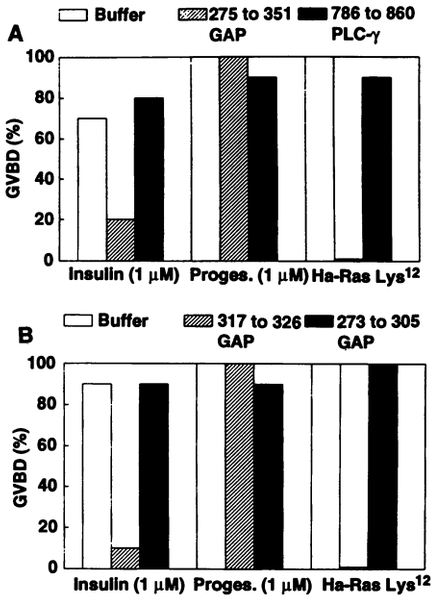

Fig. 3. Effect of GAP (275 to 351) and GAP (317 to 326) on GVBD induced by hormones or Ha-Ras Lys^{12}. (**A**) Recombinant GAP (275 to 351) and PLC-γ (786 to 860) were separated from the GST moiety by site-specific proteolysis in which human thrombin [0.2% (w/w) of fusion protein] was applied directly to the glutathione-agarose column. Thrombin was eliminated by adding *p*-aminobenzamidine–agarose beads. The polypeptides were concentrated in Centricon devices (Amicon). Oocytes were microinjected as described in Fig. 1 with 40 nl of hydrolysis buffer [tris-HCl (pH 8), 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and GAP (275 to 351) (2.5 mg/ml) or PLC-γ (786 to 860) (2.5 mg/ml)] and subsequently incubated in Barth buffer (2 ml) containing insulin or progesterone as described in Fig. 1A or microinjected with 40 nl of a mixture of Ha-Ras Lys^{12} in hydrolysis buffer with GAP (275 to 351) or with PLC-γ (786 to 860) (Ha-Ras Lys^{12}). (**B**) The peptides GAP (273 to 305) and GAP (317 to 326) were synthesized by the solid-phase procedure of Merrifield (*26*) with the use of 9-fluorenyl metoxy carbonyl chemistry. The products were characterized by mass spectroscopy and sequencing, and their purity (>95%) was determined by reversed-phase high-pressure liquid chromatography. Results are expressed as the percentage of each group of ten oocytes that underwent GVBD. GAP peptides were dissolved in 5% dimethyl sulfoxide (DMSO) at 2.5 mg/ml, vortexed, and centrifuged for 5 min at room temperature. Oocytes were microinjected as described in Fig. 3A with 40 nl of 5% DMSO (control) or with cleared solutions of peptides. Each experiment was repeated at least four times.

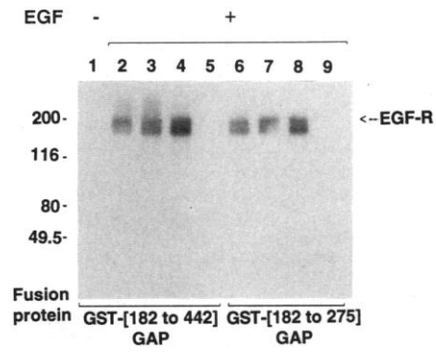

Fig. 4. Lack of effect of GAP (275 to 351) on binding of GAP (182 to 442) to the phosphorylated EGF-R. Quiescent Chinese hamster lung fibroblasts overexpressing human EGF-R were stimulated or not (lane 1, no EGF) with 80 nM EGF (Collaborative Research) (lanes 2 to 9) for 10 min in Dulbecco's modified Eagle's medium containing sodium orthovanadate. Cells were lysed in lysis buffer (HNTG) [50 mM Hepes (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, aprotinin (10 μg/ml), 1 mM Na_3VO_4, 10 mM tetrasodium pyrophosphate, and 100 mM sodium fluoride]. After centrifugation (15,000*g* for 15 min) the supernatants were incubated with 10 μg of either GST-GAP (182 to 442) (lanes 1 to 5) or GST-GAP (182 to 275) (lanes 6 to 9) that had been coated on 50 μl of glutathione-agarose beads and buffer (lanes 2 and 6), GAP (275 to 351) (50 μg) (lanes 3 and 7), GAP (275 to 351) (100 μg) (lanes 4 and 8), or GAP (182 to 275) (100 μg) (lanes 5 and 9). After being gently mixed for 90 min, the complexes were washed three times with HNTG buffer. Complexed proteins were separated by SDS-PAGE (7.5% gels) and immunoblotted with antibodies to phosphotyrosine. Molecular size markers are shown at the left (in kilodaltons).

terminal and COOH-terminal SH3 regions alter the activity of the SEM-5 protein from *Caenorhabditis elegans* (*22*). GAP (275 to 351) may thus in itself constitute a domain allowing binding to one or several downstream molecules. These data are consistent with evidence suggesting that the SH2-SH3 domains of GAP are sufficient to inhibit muscarinic atrial K^+ channel currents (*5*).

Our results provide evidence that SH3 domains can regulate cellular signal transduction. Ras might be regulated both downstream and upstream by SH2-SH3–containing proteins. GAP (275 to 351) should prove to be useful for the identification of downstream components that participate in Ras-GAP signaling. Recently a complementary DNA was isolated that encoded a protein (3BP-1) similar to proteins with GAP activity that bound the SH3 domain of Abl (*23*). The Ras-GAP–associated protein p190 (*10*) has sequence similarities with 3BP-1 and may participate in Ras signaling by binding to the SH3 domain of Ras-GAP. Direct links have yet to be made between Ras and the growth factor receptors that increase the GTP-bound active form of Ras and between Ras and activation of the mitogen-actived protein kinases. The SH3 domain of GAP may constitute a direct link in the control of Ras signaling, and within this SH3 domain the GAP (317 to 326) peptide may represent a binding site for an effector protein.

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19. C. Pernelle (Rhone Poulenc Rorer), unpublished data. Overlapping decapeptides derived from GAP (273 to 351) were synthesized according to the technique reported by Cambridge Research Biochemicals. Peptides were reacted with MAb200 (10 nM), and the enzyme-linked immunosorbent assay was developed with the 2,2′-azino-di-(3-ethyl benzthiazoline sulfonate)-hydrogen peroxide substrate.
20. M. N. Thang and M. Duchesne, unpublished data. MAb 200, when added to the GAP assay solution, did not modify the rate of GTP hydrolysis, even after immunoprecipitation. The effect of an increase in the amount of GAP (275 to 351) or GAP (317 to 326) on the rate of p100-GAP–stimulated GTPase activity was measured according to (*9*).
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Modulation of Anxiety and Neuropeptide Y-Y1 Receptors by Antisense Oligodeoxynucleotides

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The function of neuropeptide Y, one of the most abundant peptide transmitters of the mammalian brain, remains unclear because of a lack of specific receptor antagonists. An antisense oligodeoxynucleotide corresponding to the NH_2-terminus of the rat Y1 receptor was constructed and added to cultures of rat cortical neurons. This treatment resulted in a reduced density of Y1 (but not Y2) receptors and diminished the decrease in adenosine 3′,5′-monophosphate (cAMP) usually seen after Y1 receptor activation. Repeated injection of the same oligodeoxynucleotide into the lateral cerebral ventricle of rats was followed by a similar reduction of cortical Y1 (but not Y2) receptors. Such antisense-treated animals displayed behavioral signs of anxiety. Thus, specific inhibition of neurotransmitter receptor expression can be accomplished in the living brain and demonstrates that altered central neuropeptide Y transmission produces an anxiety-like state.

Neuropeptide Y (NPY) (*1*) is present in high concentrations in the hypothalamus, the limbic system, and the cortex of mammals (*2*). Because NPY is abundantly expressed, exhibits bioactivity in numerous systems at nanomolar concentrations, and shows a remarkable degree of conservation throughout phylogenesis (*3*, *4*), it is likely to be an important brain transmitter. On the basis of NPY administration to the central nervous system, the peptide has been proposed to participate in the central regulation of endocrine and autonomic function, circadian rhythms, and food intake. In addition, disturbed NPY transmission may contribute to clinical symptoms of anxiety and depression (*5*). In rats, central administration of NPY produces effects indistinguishable from clinically effective anxiolytics (*6*). A direct demonstration of the role played by endogenous NPY, however, has been hampered by the lack of specific NPY receptor antagonists.

Two subclasses of receptors for NPY have been described both in the periphery and in the brain (*7*–*12*). NPY-Y1 receptors require the intact sequence of amino acids 1 to 36 of NPY for activation, whereas Y2 receptors can also be activated by shorter COOH-terminal fragments such as $NPY_{13–36}$ (*7*). The anxiolytic action of NPY in animal models of anxiety has been linked to the Y1 subclass of NPY receptors (*6*). The rat NPY-Y1 receptor and the highly homologous human NPY-Y1 receptor have been cloned (*8*), and their nucleotide sequences indicate that the Y1 receptor belongs to the superfamily of G protein–coupled receptors. On the basis of the sequence of the rat Y1 receptor, we designed and synthesized an antisense 18-base oligodeoxynucleotide (D-oligo) that corresponded to the receptor NH_2-terminus immediately downstream from the initiation codon; the corresponding sense and missense D-oligos were used as controls.

Rat cortical neurons obtained at embryonic day 14 were cultured for 7 days. RNA was then isolated, and the presence of Y1 receptor mRNA was determined by reverse transcriptase–polymerase chain reaction (RT-PCR) with the use of the sense D-oligo and a reverse primer (Fig. 1B) (*13*). Intact cultured cortical neurons displayed specific binding sites for ^{125}I-labeled peptide YY (PYY), which binds to multiple NPY receptors (*7*–*12*). Heterogeneity among the ^{125}I-

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