Identification of G protein-coupled receptors by RNase H-mediated hybrid depletion using *Xenopus laevis* oocytes as expression system

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A method has been developed for rapidly identifying putative G protein-coupled receptors isolated initially as small cDNA fragments, following reverse transcription and polymerase chain reaction (PCR) amplification of mRNA. The method is based upon the use of synthetic oligonucleotides deduced from the sequence of the amplified receptor fragments, to direct a RNase H-mediated specific degradation of hybrids formed between the oligonucleotides and the corresponding receptor-encoding mRNA. Loss of an agonist-dependent receptor response in the *Xenopus laevis* oocyte expression system identifies the amplified receptor fragment. Taking in vitro synthesised serotonin HT_2-receptor (SR)-encoding mRNA as a model, it was shown that following incubation with RNase H and SR antisense oligonucleotides, injection of this message no longer caused the acquisition of agonist-dependent membrane currents in voltage-clamped oocytes. In contrast, when corresponding sense oligonucleotides were used, the serotonin-evoked membrane responses in oocytes were acquired as normal. The method should allow the identification of receptors which can functionally be expressed and measured in *Xenopus* oocytes.

RNase H; DNA/mRNA hybrid; G protein-coupled receptor; Voltage-clamp; *Xenopus laevis* oocytes

1. INTRODUCTION

The molecular cloning of various G protein-coupled receptors has revealed that they all contain 7 segments of hydrophobic amino acids which are generally believed to constitute transmembrane domains (reviewed in [1,2]). In addition to their structural similarity, there is a notable degree of amino acid sequence conservation, especially within the putative transmembrane domains. Based upon this observation, degenerate oligonucleotide pools deduced from the third and sixth transmembrane domains have been used as primers in the polymerase chain reaction [3]. Using this approach, several putative members of this receptor superfamily have been successfully amplified from either genomic DNA or cDNA [3] (and unpublished data).

Having identified new receptor candidates by sequence and structural homology to known members of the G protein-coupled receptor family, one is faced with the problem of identifying their ligands. To date, this has usually entailed the isolation of a full-size clone from an appropriate cDNA library, recloning of the cDNA in an expression vector, its expression in *Xenopus* oocytes or a suitable host cell line and the subsequent analysis of ligand binding profiles or the measurement of agonist-induced second messenger synthesis. As an alternative to this approach, a method is presented which allows the rapid identification of PCR amplified fragments of receptors that can be functionally expressed in mRNA-injected oocytes. It bears the advantage that large numbers of putative receptors can be rapidly screened and that a cDNA fragment is sufficient for receptor identification. The method is based on the RNAse H-mediated selective depletion of hybrids formed between antisense oligonucleotides deduced from PCR amplified receptor cDNA fragments and the corresponding receptor-encoding mRNA, leading to an absence of acquired ligand-dependent membrane responses in voltage-clamped oocytes.

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Abbreviations: SR, serotonin HT_2-receptor; TRH, thyrotropin releasing hormone

2. MATERIALS AND METHODS

Isolation of poly(A)$^+$ RNA from GH3 cells and rat brains, injection of frog oocytes and their electrophysiological recording were carried out as previously described [4]. In vitro synthesized RNA encoding functional SR was transcribed from a HT2 cDNA clone in a bluescript vector (provided by P.H. Seeburg, Heidelberg [5]), using T3 RNA polymerase and standard protocols.

18mer oligonucleotides synthesized by the phosphoamidite method, purified by reversed-phase HPLC (C_{18} column), and desalted on NAP 5 columns, were used in sense (ATGGTGATCACCTACTTC; positions 1350–1367) and antisense (GAAGTAGGTGATCACCAT) orientation derived from the SR cDNA sequence [5]. This nucleotide sequence is also conserved in the serotonin HT_{1c} receptor in a homologous position [6].

2 μg of poly(A)$^+$ RNA or 3 ng of in vitro transcribed RNA were annealed to 120 ng of SR-derived 18mer oligonucleotide (sense or antisense orientation) in 100 mM KCl in a vol. of 5 μl at 65°C for 20 min.

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The annealing mixture was allowed to cool to room temperature over a period of 5 min, placed on ice and diluted with 15 μl of a solution containing: 72 U RNase inhibitor (Pharmacia, Freiburg), 1 U RNase H (Gibco/BRL, Eggenstein), 5 μg tRNA, 15 mM $MgCl_2$, 150 mM KCl, 30 mM Tris-HCl (pH 7.5), 1.5 mM DTT, and 7.5% (w/v) sucrose. The mixture was incubated for 1 or 5 min at 37°C, quenched on ice and extracted once with phenol and once with chloroform. Nucleic acids were precipitated with ethanol and the precipitates washed twice with 70% ethanol. The pellets were dissolved in 2 μl H_2O and 50 nl were injected into oocytes.

3. RESULTS AND DISCUSSION

Injection of either rat brain poly(A)$^+$ RNA or in vitro synthesized RNA encoding the SR results in the acquisition of serotonin-dependent membrane currents in voltage-clamped oocytes [5] (Fig. 1, left). However, when the RNA preparations are first annealed to antisense oligonucleotides deduced from the fifth membrane-spanning domain of the SR (for sequence information see section 2) and treated with RNAse H prior to injection, oocytes do not demonstrate membrane currents after application of serotonin (Fig. 1, middle). In contrast, oocytes injected with RNA preparations which were previously annealed to the corresponding sense oligonucleotide and treated with RNAse H, still acquire the ability to respond to serotonin (Fig. 1, right). This indicates that RNase H in combination with short specific antisense oligonucleotides can be used to deplete a target mRNA and that this can be electrophysiogically assayed in oocytes.

To demonstrate the specificity of the method, the receptor for the tripeptide thyrotropin releasing hormone (TRH) was assayed in a control experiment. Poly(A)$^+$ RNA from GH3 cells used as source for the TRH receptor-encoding mRNA [4] was incubated with either the SR sense or antisense oligonucleotides and digested with RNase H. Fig. 2 shows that acquired TRH-mediated membrane current responses are essentially unaffected in oocytes injected with this RNA preparation. In addition, similar analysis of a mixture of in vitro synthesized SR-encoding RNA and poly(A)$^+$ RNA from GH3 cells shows that oocytes injected with such a mixture previously incubated either with the SR sense or antisense oligonucleotides but not treated with RNase H, respond with almost equal intensity to TRH and serotonin challenges (Fig. 3). However, serotonin but not TRH-induced responses, are almost completely abolished when the incubation mixtures additionally contained RNase H.

A variety of molecular approaches have been adopted to address the cloning of members of the G protein-coupled receptor family. These include functional expression of cloned receptor cDNA in frog oocytes, hybrid selection of receptor-encoding mRNAs with cloned cDNA and expression of transfected receptor genes in cell culture followed by identification using a cell sorter (reviewed in [2]). Especially powerful has been the exploitation of known similarities between the

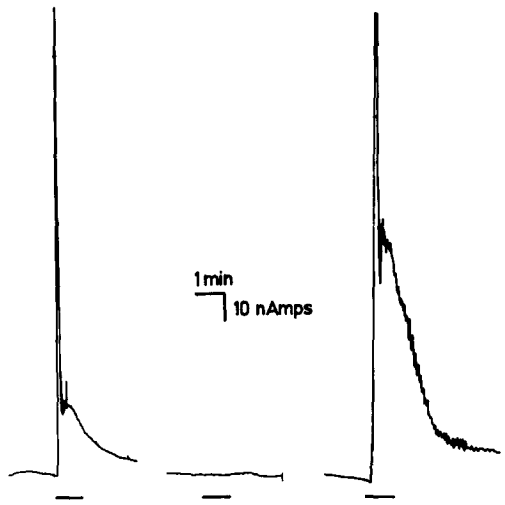

Fig. 1 RNase H/antisense oligonucleotide-mediated degradation of serotonin HT_2 receptor-encoding mRNA. Membrane current profiles of oocytes injected with 100 ng of rat brain mRNA (left), with 100 ng rat brain mRNA previously annealed to antisense oligonucleotide and treated with RNase H (middle) or with 100 ng of rat brain mRNA previously annealed to sense oligonucleotide and treated with RNase H (right) are shown. The bars indicate the duration of serotonin (10 μM) application.

family of G protein-coupled-receptors to construct degenerate oligonucleotide primers for the PCR-mediated amplification of putative new family members [3]. A problem of this approach is that the amplified receptor fragments do not possess all the information required to encode a functional receptor and must thus be used to isolate a full-size clone from a suitable cDNA library. This procedure becomes very time-consuming if many clones are to be analysed.

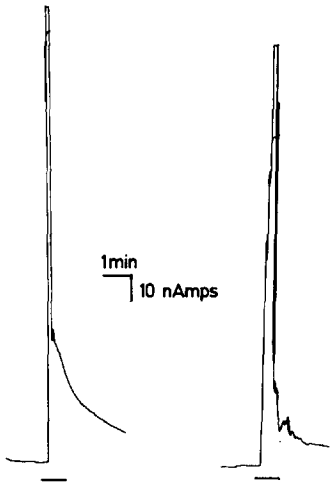

Fig. 2. RNase H/serotonin HT_2 receptor oligonucleotide treatment does not affect TRH-receptor mRNA. Membrane current responses of oocytes injected with 100 ng GH3 cell poly(A)$^+$ RNA annealed to SR sense oligonucleotide (left) or antisense oligonucleotide (right) and digested with RNase H. The bars indicate TRH application.

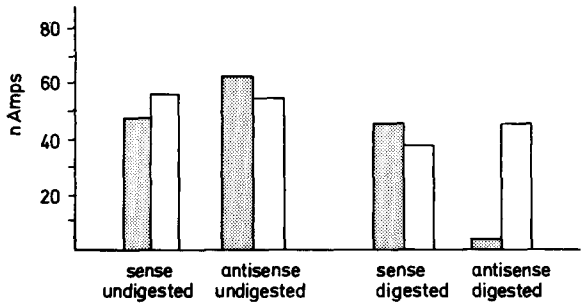

Fig. 3. Specific degradation of serotonin HT_2 receptor mRNA. A mixture of GH3 cell poly(A)$^+$ RNA (50 ng) and in vitro SR-encoding RNA (175 pg) was injected into oocytes after annealing to SR sense or antisense oligonucleotide and after additional digestion with RNase H. Groups of injected oocytes were consecutively exposed to TRH (open bars) and serotonin (closed bars). Oocytes were alternatively treated first with serotonin or first with TRH. The histogram shows the mean values of peak currents obtained after ligand application.

Some way of utilising the sequence information inherent in such PCR amplified fragments for direct receptor identification would greatly expedite these procedures.

It has several times been reported that oligonucleotide-mediated translation arrest occurs almost quantitatively in *Xenopus* oocytes [10–12]. However, initial attempts to inject hybrids preformed between rat brain poly(A)$^+$ RNA and the SR antisense oligonucleotide failed to prevent the acquisition of membrane responses induced by serotonin (not shown). This was apparently not due to the relatively downstream location of the oligonucleotide in relation to the initiation codon (oligonucleotides correspond to a conserved region of the 5th membrane-spanning domain of the serotonin receptor family), since formation of hybrids with an oligonucleotide spanning the initiator ATG codon of the serotonin receptor still failed to prevent the acquisition of serotonin responsiveness in hybrid injected oocytes (unpublished data). Such a result might, of course, be explained by the presence in rat brain poly(A)$^+$ RNA of mRNAs encoding other serotonin receptor subtypes with poor homology to the oligonucleotides used.

It has recently been demonstrated that RNase mediated predigestion of hybrids formed between mRNA and antisense oligonucleotides deduced from a particular mRNA sequence can be used to specifically prevent translation of the corresponding protein in the rabbit reticulocyte lysate system [9]. It thus seemed reasonable to suppose that a similar approach utilising antisense oligonucleotides deduced from putative PCR amplified receptor fragments and predigestion with RNase H could be used to deplete specific receptor mRNAs prior to expression in *Xenopus* oocytes. The present report shows that RNase H in combination with SR antisense oligonucleotides, can specifically and selectively degrade the corresponding message, the consequence being an absence of normally acquired agonist-dependent membrane currents in voltage-clamped *Xenopus* oocytes. Sense oligonucleotides deduced from the same sequence fail to affect the integrity of the SR message. The specificity of the method is further illustrated by the fact that functional mRNA for the TRH receptor, another G protein-coupled receptor family member, is left intact during the digestion of hybrids with RNase H presumably because of its inability to form stable RNA/DNA hybrids with the SR antisense oligonucleotide.

In summary, the method described here should be applicable to identifying PCR-amplified clones for receptors which have been functionally detected in poly(A)$^+$ RNA-injected oocytes [7,8]. Additionally, it seems probable that new subtypes of already known receptors might be identified by this approach.

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