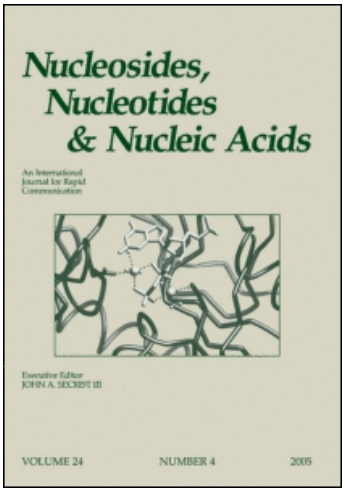

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SPECIFICITY OF THE RECEPTOR-G-PROTEIN INTERACTION: ASSIGNING FUNCTIONS TO G-PROTEIN SUBUNITS BY THE ANTISENSE TECHNIQUE

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ABSTRACT: A given heptahelical receptor uses a limited set of G proteins to interact with and to affect a given effector system. It was assumed that the specificity in receptor-G protein-effector coupling is only determined by the α subunit of the G protein, but a large body of evidences supports the idea that in addition to the α subunits the $\beta\gamma$ dimers determine G-protein specificity. We focus to the question how the antisense technology can be used to determine the identity of individual subtypes of G protein subunits involved in signal transduction cascades.

In recent years, the antisense technology has attracted much attention as it permits to inhibit selectively the expression of one particular protein and to investigate which cell function is altered (1-5). In principal, there are several ways to inhibit the expression of a particular mRNA. One is to express the sequence of this mRNA as antisense RNA in the cells. For this purpose, the target sequence is cloned in reversed orientation into an expression plasmid which will be transfected or injected into the cells. The length of such antisense RNA can vary from 40-50 to several hundred nucleotides. Antisense sequences can also be delivered as synthetic oligoribo- or oligodeoxyribonucleotides. Different chemical modifications can be introduced into the molecules to increase the stability or the cellular uptake of the oligomers as far as this does not impair the recognition of the complementary sequence (4). To inhibit the expression of G proteins in this way, either unprotected phosphodiester oligonucleotides or phosphorothioate oligonucleotides are used (6-8).

We used the antisense technique to elucidate whether G proteins of a particular subunit composition mediate hormone-induced inhibition of voltage-operated Ca^{2+} channels or stimulation of intracellular calcium release (increase in $[Ca^{2+}]_i$) (9,10). For this purpose, we microinjected antisense oligonucleotides, which are able to anneal to the mRNAs of α, β and γ subunits of G proteins, into the nucleus of GH_3 cells. Injections of oligonucleotides were performed either by using an automated (AIS, Zeiss, Oberkochen) or a manual injection system (Eppendorf, Hamburg). We used commercial pipettes (femtotips, Eppendorf) or pulled them from borosilicate glass pipettes (outer diameter 1.12 mm, inner diameter 0.96 mm, with filament, Hilgenberg). The outlet tip diameter was approximate 0.5 µm for the Eppendorf pipettes and 0.5-1.0 µm for the Hilgenberg pipettes. The injection solution routinely contained 10 µM oligonucleotides in water; use of other concentrations (5 or 20 µM) for some experiments did not influence the results. The increase in nuclear and entire cell volumes were used as a visual control for successful injection (presumably 10-20 fl were injected). To measure microinjection efficiency, cells were injected with a 10 µM solution of fluorescein isothiocyanate (FITC)-marked oligonucleotides. The fluorescence signal remained for two days in the nuclei of about 90% of injected cells, although its intensity (reflecting the amount of injected oligonucleotides) varied from cell to cell. Following injection, cells were usually cultured for 40-54 hours (GH_3 cells), for 46-76 hours (RINm5F and RBL-2H3-hm1 cells) or for 72-96 hours (PC-12 cells) before electrophysiological or fura-2 calcium measurements were performed.

For injection into different cell lines, we used antisense oligonucleotides which were designed in different ways; for injection into GH_3 cells, we used phosphodiester oligonucleotides, for injection into RINm5F cells we used partially protected oligonucleotides, i.e. in the last two nucleotides at each the 5′ and 3′ end one of the non-bridging oxygens of the phosphate group was replaced by sulfur (phosphorothioates) to protect the oligonucleotides from degradation by exonucleases. For the injection into the nuclei of PC-12 cells, we used completely phosphorothioate-protected oligonucleotides in which in each phosphate group one oxygen was replaced by a sulfur. With these completely protected oligonucleotides, we observed non-specific effects only at concentrations above 50 µM.

In GH_3 cells, we demonstrated that the G protein coupling to the somatostatin receptor to mediate inhibition of voltage-dependent Ca^{2+} channel is composed of the subunits $\alpha_{o2}\beta_1\gamma_3$ and that the G protein coupling the muscarinic M_4 receptor to the same effector system is composed of the subunits $\alpha_{o1}\beta_3\gamma_4$ (11-13). This specificity in coupling of a given receptor to a certain heterotrimeric G protein is retained in other cell models. The muscarinic M_4 receptor

in PC-12 cells and the somatostatin receptor in RINm5F cells use the identical heterotrimers as those in GH_3 cells to inhibit the voltage-dependent Ca^{2+} channel (14). By investigating the G protein coupling of the galanin receptor to the voltage-dependent Ca^{2+} channel, we found that this receptor couples *via* two different G-protein heterotrimers to the same effector system. Only one α subunit, namely α_{o1}, but two different β subunits, β_2 and β_3, and two different γ subunits, γ_2 and γ_4, participate in the inhibition of voltage-dependent Ca^{2+} channels by galanin in GH_3 and RINm5F cells. From the degree of the effects of the antisense oligonucleotides and from published data of coexpression of $\beta\gamma$ dimers we assume that the galanin receptor in GH_3 and RINm5F cells couples preferentially to the G-protein trimer consisting of $\alpha_{o1}\beta_2\gamma_2$ and less efficiently to the one consisting of $\alpha_{o1}\beta_3\gamma_4$, which is also used by the M_4 muscarinic receptor (15). By using the same technique, we were able to show that in PC-12 cells the α_2-adrenoceptor couples to the heterotrimer consisting of $\alpha_{o1}\beta_3\gamma_4$ to inhibit the voltage-operated Ca^{2+} channel, the same heterotrimer which is used by the M_4 muscarinic receptor in PC-12 and GH_3 cells. Figure 1 summaries the specific heterotrimeric G proteins which we found to interact with receptors involved in Ca^{2+}-channel inhibition.

In all these studies, the specificy of the antisense oligonucleotides has been shown by the specificy of functional effects; e.g. in the same cell, in which antisense oligonucleotides annealing to the β_1 subunit was injected, the inhibition of voltage-dependent Ca^{2+} channels by somatostatin was diminished, while the inhibition induced by carbachol or galanin was still present. *Vice versa*, the inhibition of Ca^{2+} channels by galanin was diminished in cells injected with β_2 antisense oligonucleotides, but the inhibition by somatostatin or carbachol was unchanged. We got similar results by injecting antisense oligonucleotides annealing to the mRNAs of $G\alpha$ and $G\gamma$.

We also used this technique to show that two pathways are involved in TRH-induced stimulation of voltage-gated Ca^{2+} channels in GH_3 cells. On one hand, $G\alpha_{i2}$ is required for stimulation of Ca^{2+} currents. On the other hand, $G_{q/11}$-mediated stimulation of phospholipase C with subsequent activation of protein kinase C necessary for phosphorylation of a signal transduction component (e.g. a Ca^{2+} channel or G-protein subunit), what finally results in stimulation of voltage-gated Ca^{2+} channels (16).

Recently, we extended the antisense studies to another effector systems, i.e. phospholipase C-β. As indication of PLC activity, we determined the release of intracellular Ca^{2+} by binding of calcium to fura-2 in a single cell imaging system. As a model system we used a rat basophilic leukaemia cell line that was stably transfected with a construct expressing the human muscarinic m_1 receptor (RBL-2H3-hm1). This receptor has been shown in other cell

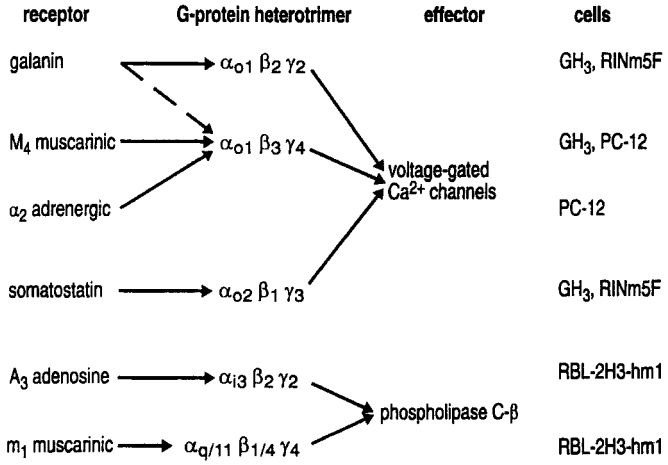

FIGURE 1: Summary of functional active G-protein-heterotrimers determined by intranuclear microinjection of antisense oligonucleotides.

systems to couple to G_q and G_{11}. To confirm this for RBL-2H3-hm1 cells, we performed carbachol-stimulated photolabeling with the stable GTP analog [α-^{32}P]GTP azidoanilide. Immunoprecipitation with a specific anti-$\alpha_{q/11}$ antiserum demonstrated an increased incorporation of [α-^{32}P]GTP azidoanilide into two proteins of 41/42 kDa, i.e. Gα_q and Gα_{11}; Gα_i proteins were not activated by the m1 receptor as shown by immunoprecipitation with an anti-α_{icom} antiserum.

Antisense oligonucleotides directed against the mRNAs coding for α_q and α_{11} subunits both suppressed the carbachol-induced increase in $[Ca^{2+}]_i$. The amounts of the corresponding immunochemically detected proteins were reduced by 85% after two days. The expression of Gα_q and Gα_{11} completely recovered after four days. In cells injected with antisense oligonucleotides directed against the β and γ subunits, anti-β_1-, anti-β_4- and anti-γ_4-injected cells showed a suppression of the carbachol-induced increase in $[Ca^{2+}]_i$. The results from RBL-2H3-hm1 cells indicate that the m1 receptor interacts with a specific G-protein complex composed of α_q, α_{11}, β_1, β_4 and γ_4 to activate phospholipase C. To increase the stability of the oligonucleotides we used peptide nucleotide acids (PNA) directed against Gα_q and Gα_{11}. Microinjected PNAs at ten time lower concentration displayed similar effects compared to completely protected phosporothioate oligonucleotides, i. e. 1 μM vs. 10 μM concentration of injection solution. The toxic effects, e.g. unspecific suppression of not targetted signal transduction pathways were also seen at ten time lower concentration.

To control the specificity of the antisense oligonucleotide approach, we compared the m1 receptor-induced increase in $[Ca^{2+}]_i$ with the increase in $[Ca^{2+}]_i$ induced *via* adenosine A_3 receptors (A_3R), which are endogenously expressed in RBL-2H3-hm1 cells. The A_3R-agonist NECA induced a PTX-sensitive increase in $[Ca^{2+}]_i$ in RBL-2H3-hm1 cells. NECA-stimulated photolabeling with the stable GTP analog [α-^{32}P]GTP azidoanilide indicated a stimulation of both $G\alpha_{i2}$ and $G\alpha_{i3}$ by A_3 receptors, however, twice as much of the GTP analog was incorporated into $G\alpha_{i3}$ compared to $G\alpha_{i2}$. Injection of antisense oligonucleotides annealing to the mRNAs of G-protein subunits indicate that the A_3 receptor couples to the G-protein heterotrimer $G_{i3}\beta_2\gamma_2$ to activate PLC-β. Figure 1 summaries the specific heterotrimeric G proteins which we found coupling receptors to phospholipase C.

We confirmed these results by using a new technique, called „ballistomagnetic transfer", to introduce antisense oligonucleotides into cells (18). The oligonucleotides are attached to magnetic beads and gold particles and shot into cells. In a second step, the hit cells are separated by magnetic sorting from missed cells, resulting in a high rate of cells carrying antisense oligonucleotides. Cells of the magnetic fraction transfected by ballistic transfer of antisense oligonucleotides to the mRNAs encoding $G\alpha_{i3}$ showed largely reduced NECA-induced $[Ca^{2+}]_i$ peaks compared to cells of the non-magnetic fraction; the carbachol-induced signalling pathway was not affected. Using indirect immunofluorescence, we could show suppression of targetted $G\alpha_{i3}$ after ballistic transfer of anti-α_{i3} antisense oligonucleotides.
Thus, microinjection and ballistic transfer of antisense oligonucleotides in combination with electrophysiological or fluorometric detection of changes in hormonal effects provide a powerful and important tool for the identification of signal transduction cascades. Targetting the individual G-protein subunits by the antisense knock-out has led to the identification of high specificity in receptor-G protein and G protein-effector coupling in the genuine membrane environment.

REFERENCES

1. Hélène, C. and Toulmé, J.-J.: Specific regulation of genen expression by antisense, sense and antigene nucleic acids. Biochim. Biophys. Acta **1049**, 99-125 (1990)
2. Crooke, S.T.: Progress toward oligonucleotide therapeutics: pharmacodynamic properties. FASEB J. **7**, 533-539 (1993)
3. Milligan, J. F., Matteucci, M.D. and Martin, J.C.: Current concepts in antisense drug design. J. Med. Chem. **36**, 1923-1937 (1993)
4. Stein, C.A. and Cheng, Y.-C.: Antisense oligonucleotides as therapeutic agents - is the bullet really magical? Science **261**, 1004-1012 (1993)
5. Marshall, W.S. and Caruthers, M.H.: Phosphorothioate DNA as a potential therapeutic drug. Science **259**, 1564-1570 (1993)

6. Alberts, P.R. and Morris, S.J.: Antisense knockouts: molecular scalpells for the dissection of signal transduction. Trends Pharmacol. Sci. **15**, 250-254 (1994)
7. Kalkbrenner, F., Dippel, E., Wittig, B. and Schultz, G.: Specificity of the receptor-G-protein interaction: Using antisense techniques to identify the function of G protein-subunits. Biochem. Biophys. Acta in press (1996)
8. Baertschi, A.J.: Antisense oligonucleotide strategies in physiology. Mol. Cell. Endocrinol. **101**, R15-R24 (1994)
9. Kleuss, C., Schultz, G. and Wittig, B.: Microinjection of antisense oligonucleotides to assess G-protein subunit function. Meth. Enzymol. **237**, 345-355 (1994)
10. Degtiar, V.E., Wittig, B., Schultz, G. and Kalkbrenner, F.: Microinjection of antisense oligonucleotides and electrophysiological recording of whole cell currents as tools to identify specific G-protein subtypes coupling hormone receptors to voltage-gated calcium channels. Meth. Mol. Biol in press (1996).
11. Kleuss, C., Hescheler, J., Ewel, C., Rosenthal, W., Schultz, G. and Wittig, B.: Assignment of G-protein subtypes to specific receptors inducing inhibition of calcium currents. Nature **353**, 43-48 (1991)
12. Kleuss, C., Scherübel, H., Hescheler, J., Schultz, G. and Wittig, B.: Different β-subunits determine G-protein interaction with transmembrane receptors. Nature **358**, 424-426 (1992)
13. Kleuss, C., Scherübel, H., Hescheler, J., Schultz, G. and Wittig, B.: Selectivity in signal transduction determined by γ subunits of heterotrimeric G proteins. Science **258**, 832-834 (1993)
14. Degtiar, V.E., Wittig, B., Schultz, G. and Kalkbrenner, F.: A specific heterotrimer couples somatostatin receptors to voltage-gated calcium channels in RINm5F cells. FEBS Lettr., **380**, 137-141 (1996)
15. Kalkbrenner, F., Degtiar, V.E., Schenker, M., Brendel, S., Zobel, A., Hescheler, J., Wittig, B. and Schultz, G.: Subunit composition of G_o proteins functionally coupling galanin receptors to voltage-gated calcium channels. EMBO J. **14**, 4728-4737 (1995)
17. Gollasch, M., Kleuss, C., Hescheler, J., Wittig, B. and Schultz, G.: G_{i2} and protein kinase C are required for thyrotropin-releasing hormone-induced stimulation of voltage-dependent Ca^{2+} channels in rat pituitary GH_3 cells. Proc. Natl. Acad. Sci. U.S.A. **90**, 6265-6269 (1993)
17. Dippel, E., Kalkbrenner, F., Wittig, B. and Schultz, G.: The muscarinic m1 receptor couples to specific G-protein heterotrimers to increase the cytosolic calcium concentration. Proc. Natl. Acad. Sci. U.S.A., **93**, 1391-1396 (1996)
18. Kalkbrenner, F., Dippel, E., Schroff, M., Wittig, B. and Schultz, G. (1996): Intranuclear microinjection and ballistomagnetic transfer of antisense oligonucleotides as tools to determine receptor interaction of G-protein heterotrimers causing phospholipase C-β activation. Meth. Mol. Biol. in press.