Vol. 179, No. 1, 1991
August 30, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 10-16

Distinct Mode of G Protein Activation Due to Single Residue Substitution of Active IGF-II Receptor Peptide Arg^{2410}-Lys^{2423}: Evidence for Stimulation Acceptor Region Other than C-terminus of Giα

Takashi Okamoto, Yoshihiro Ohkuni, Etsuro Ogata, and Ikuo Nishimoto¶

Life Science Laboratory, Fourth Department of Internal Medicine
University of Tokyo School of Medicine, Tokyo 112, Japan

Received July 10, 1991

Arg^{2410}-Lys^{2423} (RVGLVRGEKARKGK, peptide 14) of the human insulin-like growth factor II receptor directly activates Gi and deletion of C-terminal 4 residues from peptide 14 nullifies this activity. A study was thus made of the effects of peptides modified in the C-terminal structure. RVGLVRGEKAAKGK and RVGLVRGEKARKGA scarcely activated Gi, whereas RVGLVRGEKARAGK (peptide A5) activated Gi as much as peptide 14 did. However, peptide A5 action did not depend on Mg^{2+} concentration and was little affected by pertussis toxin modification of Giα. Peptide A5 may thus recognize the region on Giα that is distinct from the extreme C-terminus. It is consequently considered that (i) the first and the last basic residues in the C-terminal motif of peptide 14 determine the capacity for recognition of Gi and (ii) there is a region different from the C-teminus of Giα, through which the C-terminal second basic residue-altered peptide 14 activates Gi in a Mg^{2+}-independent manner.

G proteins (guanine nucleotide-binding proteins) are a family of transducer proteins which regulate membrane effector activity in response to receptor-generated signals [1]. Extensive studies [2-4] suggest that several cytoplasmic domains of G protein-coupled receptors are involved in interactions with G proteins. Recently, a small peptide mastoparan, a honey bee venom, is shown to directly activate G proteins in a manner similar to that observed in ligand-activated receptors [5] and the N-terminal 24 residues of GAP-43 (growth-associated protein-43) to activate Go [6]. However, the sequence-function relationship for the effects of these peptides remains unclear. In contrast, IGF-IIR (insulin-like growth factor II receptor), which is only one demonstrated G protein-coupled receptor with a single transmembrane domain, is found to possess a G protein activator domain in its cytoplasmic region, whose activity depends on its primary structure [7].

IGF-II is a polypeptide structurally related to IGF-I and insulin [8] and acts on cellular metabolism and proliferation by binding to the cell surface IGF-IIR with high affinity in cultured cells [9-14]. In BALB/c 3T3 cells, IGF-II activates a calcium-permeable cation channel and stimulates DNA replication through IGF-IIR by a mechanism involving Gi [14-18]. Purified rat and human IGF-IIR can directly couple to purified Gi in phospholipid

¶To whom correspondence should be addressed.

0006-291X/91 $1.50

vesicles in response to IGF-II [19-21]. Based on the direct Gi-activating function of this receptor, Gi has been found to be directly activated by a 14-residue peptide (peptide 14) corresponding to Arg^{2410}-Lys^{2423} of human IGF-IIR [7]. The action of peptide 14 is determined by two basic residues at the N-terminus and the C-terminal structure, RKGK. The essential role of the C-terminal structure was surmised from the finding that N-terminal 10-residues of peptide 14 are totally without effect on Gi activation [7]. In this study, we extended the experiment of structure-function relationship of peptide 14 and clarified the role of the basic residues at the C-terminal structure by substituting each basic residue with an alanine residue. The basic residues were found to be related to the capacity of peptide 14 to recognize Gi and possibly there are at least two stimulation acceptor regions on Giα, stimulation of which leads to Gi activation in different manners in terms of Mg^{2+}-dependency and pertussis toxin sensitivity.

Experimental Procedures

Materials
The peptides used in this study were synthesized by the solid phase method and highly pure (≥ 99%) as described [7]. The lyophilized synthetic peptide was dissolved in distilled water. Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, USA). Gi used in this study was trimer Gi_{-2} purified from bovine brain, which was kindly provided by Dr. Toshiaki Katada (Tokyo Institute of Technology, Yokohama, Japan) [22]. Monomer Giα was $Gi_{-2}\alpha$ purified from bovine lung [23], which was kindly provided by Dr. Tomiko Asano (Institute for Developmental Research, Aichi, Japan). Nucleotides were from Boehringer Mannheim (Germany).

GTPγS binding assay
GTPγS (guanosine-5'-o-(3-thio-triphosphate)) binding to G proteins was assayed at 37 °C in the presence of 20 μM Mg^{2+} and 60 nM [^{35}S]GTPγS, as described [7]. GTPγS binding to peptides was negligible. The total amount of G proteins was measured as maximal GTPγS binding at room temperature. [^{35}S]GTPγS was purchased from Du Pont-New England Nuclear. In experiments to examine the effect of Mg^{2+}, Mg^{2+} concentration was set using a Mg-EDTA buffer as described [24].

ADP-ribosylation
Gi was ADP-ribosylated by 5 μg/ml preactivated pertussis toxin in the presence of 10 μM NAD at 30 °C for 15 min as already described [19]. As a control, Gi was incubated with pertussis toxin in the absence of NAD under the same conditions. NAD was from WAKO (Osaka, Japan).

Results and Discussion

We synthesized three peptides derived from peptide 14 (RVGLVRGEKARKGK): peptide A_4 (RVGLVRGEKAAKGK), peptide A_5 (RVGLVRGEKARAGK), and peptide A_6 (RVGLVRGEKARKGA). As shown in Fig. 1, peptide A_4 or A_6 had no stimulatory effect on the GTPγS binding rate of Gi, compared to the basal binding rate. This indicates that these two basic residues are essential for Gi activation induced by peptide 14. We previously suggested similar and essential roles of basic residues at the N-terminal region in peptide 14 [7]. In contrast, peptide A_5 stimulated GTPγS binding to Gi as effectively as peptide 14 did. The potency of peptide A_5 action on Gi was similar to that of peptide 14. The effect of peptide A_5 reached saturation at 100 μM at a binding level of ≈ 1 mole per 1 mole Gi, whereas the effect

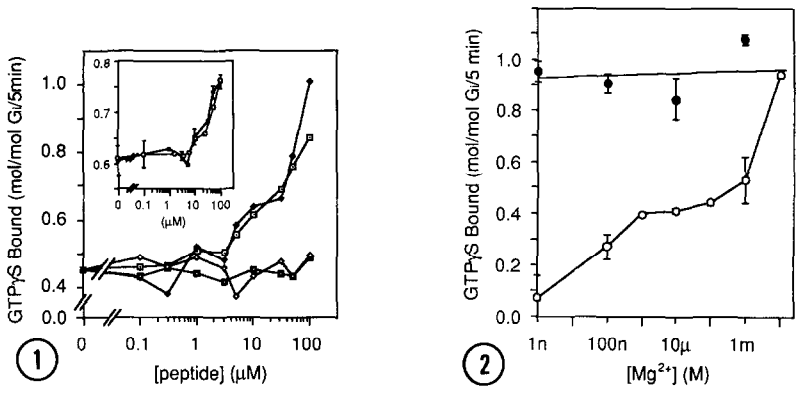


Figure 1. *Effects of peptides A4, A5, and A6 on Gi. **Inset**: effects of peptide A5 on the monomer Gi-2α.*

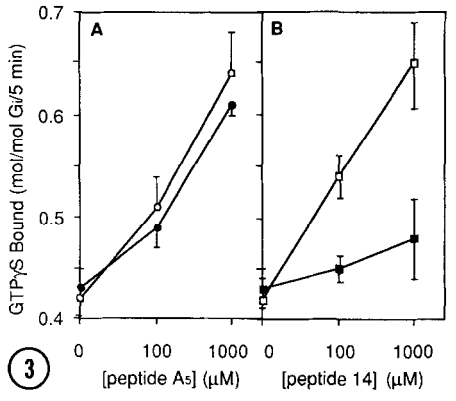
10 nM trimer Gi was incubated with increasing concentrations of peptide A4 (RVGLVRGEKAAKGK, ■), peptide A5 (RVGLVRGEKARAGK, ◆), peptide A6 (RVGLVRGEKARKGA, ◇), or peptide 14 (RVGLVRGEKARKGK, □) in the presence of 60 nM [35S]GTPγS at 37 °C for 5 min. GTPγS binding was measured. Values represent means of three experiments. S.E. was < 5 % of each value. *Inset*: 10 nM monomer Giα was incubated with increasing concentrations of peptide A5 (■) or peptide 14 (○) for 5 min, and GTPγS binding (mol/mol Gi/5 min) was measured. Values represent means of three experiments, whereas S.E. was < 5 % of each value.

Figure 2. *Effect of Mg^{2+} on peptide A5-induced GTPγS binding to Gi.*

10 nM Gi was incubated with (●) or without (○) 100 μM peptide A5 at various concentrations of Mg^{2+} for 5 min; and GTPγS binding to Gi was assayed. Mg^{2+} concentrations were determined using Mg-EDTA buffer. Values represent the means ± S.E. of three experiments.

of peptide 14 did so at 100 μM at a level of ≈ 0.9 mole per 1 mole Gi. Peptide A5 may thus have a Gi-2 activating ability, comparable to or larger than that of peptide 14. A similar observation was reported for mastoparan, INLKALAALAKKIL. INLKALAALAKAIL (underlined Ala is substituted from Lys of mastoparan) has a stimulatory effect larger than mastoparan [25]. Mastoparan may have a structure similar to a partial sequence of peptide 14, RVGLVRGEKARK. This 12-residue peptide from peptide 14 has weak action on Gi, comparable to that of mastoparan [7]. It is thus likely that the fifth basic residue of peptide 14 plays a role similar to that of the third basic residue of mastoparan.

To characterize peptide A5-induced Gi activation, we examined the effect of Mg^{2+} on the action of peptide A5. As shown in Fig. 2, peptide A5 fully activated Gi in a trace amount of Mg^{2+}; and peptide A5-stimulated GTPγS binding to Gi did not depend on Mg^{2+} at all. This contrasts the peptide 14 action on Gi, which clearly depends on Mg^{2+} concentration [7]. At a trace concentration of $Mg^{2+} \leq 100$ nM, peptide 14 cannot activate Gi at all and peptide 14 reduces the threshold concentration of Mg^{2+} required to activate Gi by 1/1000. This mode of action of peptide 14 is similar to that of G protein-coupled receptors [1]. In contrast, peptide A5 appeared to act on Gi in a manner different from that of these receptors.



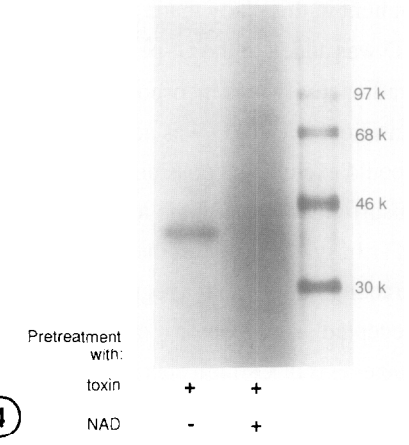


Figure 3. *Effect of pertussis toxin-induced ADP-ribosylation on peptide A5 action on Gi.* Gi was treated with 5 μg/ml pertussis toxin for 15 min at 30 °C in the absence or presence of 10 μM NAD. Each Gi (○, □: Gi preincubated with pertussis toxin in the absence of NAD; ●, ■: Gi preincubated with the toxin in the presence of NAD) was incubated with peptide A5 (*A*) or peptide 14 (*B*) at 37 °C for 5 min; and then GTPγS binding was measured. Values represent means ± S.E. of three experiments. Under the condition used, most of the Giα was ADP-ribosylated by pertussis toxin in the presence of NAD.

Figure 4. *Effect of pertussis toxin treatment on ADP-ribosylation of Giα.* Trimer Gi was treated with pertussis toxin in the absence (*left lane*) or presence (*middle lane*) of NAD, as described in the legend of figure 2. [^{32}P]NAD was then added to these mixtures, which were subjected to further incubation for 30 min. These samples were processed into SDS-polyacrylamide gel electrophoresis and autoradiography. The right lane indicates molecular mass of molecular weight markers.

What is the mechanism underlying the receptor-unlike mode of action of peptide A5 ? Receptors are considered to mainly recognize the C-terminal region of Giα proteins. This has been confirmed by the observation that receptor stimulation of Gi is inhibited by pertussis toxin, which ADP-ribosylates the Cys residue located at the fourth residue from the C-terminal end of Giα [26]. Pertussis toxin sensitivity is thus regarded as another stimulation characteristic of receptors, which prompted us to exmaine the effect of pertussis toxin on the action of peptide A5. When Gi was pretreated with pertussis toxin plus NAD, peptide 14 action on Gi was significantly reduced (≈ 70 % for the effect of 1 mM peptide 14) relative to that on Gi pretreated with the toxin in the absence of NAD (Fig. 3), as described previously [7]. In contrast, at 100 μM or 1 mM of peptide A5, the action of peptide A5 was reduced only by 40 or 20 %, respectively, when Gi was given prior treatment with pertussis toxin and NAD (Fig. 3). The reasons why the effects of 100 μM these peptides were rather small relative to those on pertussis toxin-untreated Gi and why the effects of these peptides did not reach saturation at 100 μM are unclear. However, similar results were obtained from Gi pretreated with pertussis toxin in the absence of NAD, and thus the reason appears to be not the ADP-ribosylation of Gi but rather its denaturation during preincubation. The experimental conditions of pertussis toxin

treatment in this study made it possible for most Giα to be ADP-ribosylated. When radioactive NAD was added to the Gi-pertussis toxin mixture which had been incubated with 10 μM NAD, no radioactivity was incorporated into the 40-kDa band of Giα (Fig. 4). In contrast, when 10 μM radioactive NAD was added to the control mixture, Giα was found to be labelled. Thus, we could exclude the possibility that the pertussis toxin insensitivity of peptide A5 action is due to insufficient effect of the toxin on Giα ADP-ribosylation. Since pertussis toxin is known to ADP-ribosylate Cys^{352} at the C-terminal end of Giα [26], peptide A5 appears to activate Gi by interacting mainly with a region other than the extreme C-terminus of Giα. The C-terminal area is accepted as a receptor recognition region on G proteins [27-30]. However, a study on the mutagenesis of Gs indicates there to possibly be another region on G proteins which receptors recognize [30]. It thus follows that peptide A5 may interact with such a putative receptor recognition region other than the C-terminal end.

To confirm that the region recognized by peptide A5 is located on the α subunit of Gi, we examined the effects of peptide A5 on the monomer Giα. As shown in the inset of Fig. 1, peptide A5 significantly increased the rate of GTPγS binding to the monomer Giα. The efficacy and the potency of peptide A5 action were comparable to those of peptide 14 on the monomer Giα. The region recognized by peptide A5 is thus situated on Giα not on Gβγ, and this indicates second stimulation acceptor region of Gi to possibly be on its α subunit.

Gi activation induced by low concentrations (≤ 10 μM) of peptide 14 is abolished by pertussis toxin treatment of Gi, whereas the activation by higher concentrations of peptide 14 is not completely blocked by the toxin, despite the fact that Giα has been completely ADP-ribosylated [7]. This is also the case with IGF-IIR. In IGF-IIR-Gi-2 vesicles, pertussis toxin completely blocks Gi-2 activation induced by low concentrations (< 10 nM) of IGF-II; but the toxin incompletely inhibits activation induced by IGF-II of higher concentrations [19]. Peptide 14 and IGF-IIR would thus appear to mainly recognize the C-terminal region of Giα at a low concentration of peptide 14 and at a low level of IGF-II stimulation, respectively. They may also interact with the second region at higher stimulation levels. Most receptor stimulation *in vivo* is not completely blocked by pertussis toxin [26]. Receptor recognition of the second stimulation acceptor region on G proteins accounts for such resistance against pertussis toxin.

Based on the presence of the second stimulation acceptor region affected by a short peptide, pertussis toxin insensitivity of receptor signaling may not alway result from the absence of Gi participation in that pathway. If one receptor recognizes only the second region on Gi, such receptor stimulation would lead to activation of Gi in a pertussis toxin-insensitive manner. These paradoxical receptors are possibly present; receptors containinig peptide A5-like sequences are good candidates for them. Conversely, even if pertussis toxin cannot affect the signaling pathway of receptor stimulation, the involvement of Gi in that pathway could not still be excluded.

This study also indicates that stimulation of the peptide A5 recognition region causes G protein activation in a Mg^{2+}-independent manner. This finding along with results from experiments using peptide 14 and pertussis toxin suggests that the stimulation of different regions on Giα leads to activation of the G protein in distinct manners. The stimulation of one

region, which probably includes Cys352, induces Mg^{2+}-dependent activation of Gi, while that of other regions, not affected by pertussis toxin, produces Mg^{2+}-independent activation. However, there is still the possibility that peptide binding to the former region depends on Mg^{2+} concentrations. Yet, to the best of our knowledge, this is the first report that functionally links the location of stimulation acceptor regions with Mg^{2+} dependency of G protein activation. This may provide a basis for the detailed clarification of the mechanism of G protein activation.

In summary, the present study clearly shows the first Arg and the last Lys in the C-terminal structure of peptide 14 to be essential for Gi activation and the second Lys to have a different role for distinguishing stimulation acceptor regions on Giα. There thus appear to be at least two regions on Giα, each leading to G protein activation. Peptide 14 may mainly interact with one stimulation acceptor region at the C-terminal end of Giα. Peptide A5 may interact with the other acceptor region. Although the latter region is still putative, Hamm *et al.* [31] suggest that two regions Asp 311-Val328 and Ile 340-Phe350, near the C-terminus and at the C-terminal end of transducin-1α, respectively, interact with rhodopsin. At present, whether the second region on Giα corresponds to the region near the C-terminus of transducin α remains unclear. However, it would be possible to determine receptor-interacting regions on Giα using the system of peptide 14, peptide A5, and authentic or mutant Giα. These stimulation acceptor regions may have similar structures, since the sequences of peptides 14 and A5 are the same except for one residue. These peptides are expected to provide clues for identifying receptor recognition regions on Gi and investigating the functional significance of the different locations of the recognition regions.

Acknowledgments-We thank Drs. Tomiko Asano and Toshiaki Katada for kindly providing monomer and trimer G proteins, respectively. This work was supported by grants from the Ministry of Education, Science and Culture of Japan, Workshop on Cell Calcium Signal in the Cardiovascular System, Toyo Jozo Co., Teijin Institute for Biomedical Research, Life Insurance Association of Japan, Napolex Corporation, and Japan Research Foundation for Clinical Pharmacology.

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