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MINI REVIEW

PEPTIDES AS PROBES FOR G PROTEIN SIGNAL TRANSDUCTION

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Abstract—Triggered by agonist binding to cell surface receptors, the heterotrimeric G proteins dissociate into α and βγ subunits, each activating distinct second messenger pathways. Peptides from the primary sequences of receptors, G proteins, and effectors have been used to study the molecular interactions between these proteins. Receptor-derived peptides from the second, third and fourth intracellular loops and certain naturally occurring peptides antagonize G protein interactions and can directly activate G protein. These peptides bind to G protein sites that include the N and C terminal regions of the α subunit and a yet to be identified region of the β subunit. Peptides have also been useful in characterizing G protein–effector interactions. The identification of the contact sites between proteins involved in G protein signal transduction should aid in the development of non-peptide mimetic therapeutics which could specifically modify G protein-mediated cellular responses.

Key words: G protein, receptor, synthetic peptide, signal transduction, review.

INTRODUCTION

This review will focus on the use of peptides (naturally occurring, G protein-derived, or receptor-derived) to study receptor-G protein coupling and G protein activation. Peptides have been used to address a number of questions regarding receptor-G protein interactions including: which regions of receptors come in contact with the G protein; which residues in receptor are responsible for G protein activation; and which regions of the G protein are responsible for effector activation. The sites of contact between these proteins could provide targets for therapeutic drug design.

Most of the peptides used in such studies have been synthesized corresponding to specific sequences in the protein of interest (rational design). However, other peptide sources (including synthetic libraries or phage display libraries) could be potentially useful in the identification of peptides which selectively target the proteins involved in G protein signal transduction.

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MAPPING THE RECEPTOR-G PROTEIN INTERFACE

G proteins are heterotrimeric proteins composed of α, β, and γ subunits. Current modeling of the receptor-G protein interaction suggests that receptors bind to both the N and C terminal regions of the α subunit [1]. Data in support of this model include the ability of mutations in the C terminus of the α subunit [2, 3] and covalent modification of the C terminus of α_i by pertussis toxin to block receptor-G protein interactions [4]. Also, as will be discussed, peptides derived from the N and C terminus of the α subunit block receptor-G protein interactions [5]. Recent evidence suggests that the βγ subunit complex may also be important in coupling receptors to G proteins [6–10]. However, residues in either β or γ subunits that participate in G protein coupling to

specific G protein coupled receptors (GPCR) remain to be identified.

G protein-derived peptides

To explore the molecular interactions between receptors and G proteins, synthetic peptides corresponding to G protein sequences were examined for their ability to block receptor-G protein coupling. This approach is based on the idea that a peptide derived from a region of the G protein that is involved in receptor binding will bind to the receptor and compete with G protein binding. In 1988, Hamm *et al.* showed that peptides from the extreme N terminus (residues 8–23) and C terminus (residues 311–329 and 340–350) of the alpha subunit of the retinal rod G protein, transdutin (G_T), could block binding of G_T to rhodopsin. Subsequently, Palm *et al.* showed that a peptide derived from the corresponding C-terminal region of the α_s subunit (residues 379–394) blocked β_1-adrenergic receptor (β_1-AR) mediated activation of adenylyl cyclase [11]. These data were consistent with earlier mutagenesis which showed that replacement of a C-terminal Arg by a Pro in α_s blocked receptor-G protein interactions [3], and truncation of the C-terminal 5 amino acids of Scg1 (the α subunit involved in yeast mating) blocked pheromone receptor-Scg1 interactions [2]. Also, chimaeric α_q/α_{i2} subunits containing only the C-terminal 3 amino acids of α_{i2} signal through α_{i2} coupled receptors [12].

A more conventional use of synthetic peptides has been to generate antibodies against G protein-derived peptides to examine receptor-G protein interactions. Antibodies raised against C terminal peptides from alpha subunits block receptor-G protein interactions [13]. Using antibodies generated to decapeptides from the C-termini of various G protein subtypes, Simonds *et al.* showed that the α_2-adrenergic receptor (α_2-AR) mediates inhibition of adenylyl cyclase in platelet membranes through G_{i2} exclusively [14]. Using similar techniques, Gerhardt and Neubig showed that α_2-AR, when transfected into CHO-K1 cells, could signal through either G_{i2} or G_{i3} to mediate adenylyl cyclase inhibition [15]. Also Gutowski *et al.* used peptide-specific C-terminal antibodies against G_q to show that angiotensin (in neuroblastoma cells) and bradykinin (in rat liver cells) signal through that G protein to stimulate PIP_2 hydrolysis [16]. The use of peptide-derived antibodies in such studies has been reviewed recently [17].

Receptor-derived peptides

Rhodopsin, α_2 adrenergic, β adrenergic, and muscarinic receptors are members of a large receptor family that couples to various signal transduction pathways through G proteins. Sequence analysis predicts that these GPCR are structurally similar, consisting of seven membrane spanning regions, three cytoplasmic loops and an intracellular C-terminus [18]. Early studies employing site-directed mutagenesis and chimaeric receptor constructs have implicated the third cytoplasmic loop and possibly the second cytoplasmic loop and C-terminal tail in coupling these receptors to G proteins [19–25].

Receptor-derived peptides have proven useful in further examining the regions of GPCRs involved in receptor-G protein coupling. In 1989 Konig *et al.* reported that peptides derived from sequences in rhodopsin could block rhodopsin–G_T interactions [26]. Eleven peptides were synthesized comprising all putative aqueous-exposed regions of rhodopsin. Of these only three peptides, those corresponding to the second (i2) and third (i3) intracellular loops and the N-terminal region of the cytoplasmic tail (i4N), could block G_T binding to photoactivated rhodopsin. The Hill coefficients for competitive binding to G_T ranged from 1.8 to 2.4. Interestingly, combining any two of the active peptides reduced their IC_{50} 30-fold. Addition of the third active peptide further reduced the IC_{50} (two-fold) to a level within one order of magnitude of the affinity of intact rhodopsin for transducin. Previously, Franke *et al.* showed that replacement or deletion of an i2 region of rhodopsin or deletion of an i3 region of rhodopsin blocked rhodopsin-mediated G protein activation [20, 22]. The peptide data confirm and extend this mutagenesis study by providing evi-

dence that the i2, i3 and i4N regions of rhodopsin directly interact with G protein.

Shortly thereafter, Palm *et al.* used β_1-adrenergic receptor-derived peptides to examine G protein signal transduction. Their studies indicated that peptides derived from the i2, C-terminal part of i3 (i3C) and, to a lesser extent, the first intracellular loop (i1) all modified receptor-mediated adenylyl cyclase activity in turkey erythrocyte membranes [27]. The authors later reported that these peptides competed synergistically with the β_1-receptor for G protein binding [28]. In addition, we have shown that peptides from the i2, N-terminal i3 (i3N) and i3C regions of the α_2-AR could block α_2-AR-G protein interactions [29, 30]. Similar methods have been used to determine the regions involved in G protein coupling to other GPCRs including the β_2-adrenergic [31], the D_2 dopamine [32], and the N-formyl peptide receptor [33]. The ability of peptides derived from the i2, i3 and i4 regions of various GPCRs to block receptor-G protein interactions indicates that these specific receptor regions are involved in coupling receptors to G protein. However, it is not clear from these studies whether these peptides block coupling at the level of the receptor or the G protein.

G PROTEIN ACTIVATOR PEPTIDES

Ligand binding to a GPCR causes a conformational change in the receptor which activates G protein by stimulating the exchange of GDP for GTP. The activated GTP-bound G protein is then thought to dissociate into α-GTP and $\beta\gamma$ subunits, both of which interact with specific effector systems to propagate second messenger signals. Since α subunits have an intrinsic GTPase, receptor-mediated G protein activation can be measured by an increase in the apparent rate of GTP hydrolysis.

Naturally occurring G protein activators

In 1986 Higashijima *et al.* reported the first evidence that short peptides could directly activate G protein. The authors demonstrated that a wasp venom tetradecapeptide, mastoparan, induced histamine release from mast cells by direct G protein activation [34]. The effects of mastoparan were similar to receptor-mediated G protein activation in that mastoparan (i) enhanced GTPase activity at submicromolar Mg^{2+}; (ii) was more effective when the G protein was reconstituted in phospholipid vesicles; and (iii) had no effect after pertussis toxin ribosylation of G proteins [34]. These data support the concept that mastoparan, a cationic amphiphilic tetradecapeptide, binds to and stimulates G proteins in a manner similar to that of agonist liganded GPCRs.

Since the discovery of mastoparan a number of naturally occurring peptides and GPCR derived peptides have been shown to bind to and stimulate G proteins directly. Mast cell degranulating peptide (MCD, an amphiphilic 22 amino acid peptide derived from honey bee venom) also induces histamine release from mast cells and directly activates G protein [35, 36]. A similar mechanism has been proposed for the venom peptide melittin [37]. Other peptides including substance P [38, 39], bradykinin [40], dynorphin [37] and angiotensin II [37] have been shown to activate G proteins directly with increases in GTPase activity ranging from two- to three-fold for 100 μM dynorphin to four- to seven-fold for 100 μM angiotensin II.

Synthetic (receptor-derived) G protein activators

The first evidence that receptor-derived peptides could directly stimulate G proteins was reported by Palm *et al.* in 1989. The authors examined the ability of β_1-AR derived peptides to attenuate receptor-mediated adenylyl cyclase and noted that one peptide derived from the i3C region stimulated adenylyl cyclase in the absence of agonist [27]. These data suggested that this peptide can assume the conformation of an activated receptor thereby activating G_s protein signal transduction in a hormone-independent manner. Following this observation, Okamoto and Nishimoto examined the capacity of peptides derived from the cytoplasmic domain of the insulin-like growth factor-II (IGF-II) receptor to

directly activate G proteins. One tetradecapeptide (peptide 14) activated G_{i2} GTPase in a manner reminiscent of receptor-mediated G protein activation. An antibody raised against this peptide fully attenuated IGF-II receptor-mediated G protein activation, which further indicated that this region of the receptor was critical for IGF-II signalling [41–43]. Later, Dalman and Neubig showed that trideca- and tetradecapeptides from the i3N and i3C regions of the α_2-AR directly stimulated G_o/G_i protein GTPase in phospholipid vesicles [29, 44]. Okamoto and Nishimoto [45] and Ikezu *et al.* [46] also found that α_2-AR i2 and i3N, as well as i3C derived peptides all directly activated G_i and G_o proteins. The list of peptides which directly activate G proteins has grown to include peptides derived from the M_4 muscarinic receptor (i2 and i3C) [45], the β_2-AR (i3N and i3C) [31, 47] and the D_2-dopamine receptor (i3N) [48].

Structural relationships of activator peptides

Secondary structure may be an important determinant for peptide activity. The membrane and G protein bound conformations of mastoparan are amphiphilic alpha helices [49, 50]. Higashijima observed a correlation between the helical content of several mastoparan analogs and their ability to stimulate purified G proteins [37]. Other G protein activators including MCD and the i3N β_2-AR peptide [51] also form amphiphilic alpha helices. These data are consistent with the idea that i3N and/or i3C regions of the β adrenergic receptors form α helices within the tertiary structure of the protein [52]. However, the specific amino acid residues within the helices responsible for activation have yet to be identified. Higashijima *et al.* reported that the lysine residues within the helix of mastoparan were critical for activity and suggested that these basic residues bind to the G protein [37]. However, a study with mutated β_2-ARs suggests that the hydrophobic residues (not the basic residues) within the α helix of the i3N region are important in G protein activation [53]. Also, the ability of peptides to form an α helix is neither necessary nor sufficient for G protein activation. Oppi *et al.* demonstrated that of four MP analogs which formed α helices only two were active [54]. Voss *et al.* found no correlation between the helical content of peptides derived from dopamine receptor (D_1 and D_2) and β_1-AR and their ability to stimulate G_o/G_i or G_s proteins [48].

Okamoto and Nishimoto suggest that the primary amino acid sequence may be more important than secondary peptide structure in determining peptide activity [42]. They postulated that activator sequences fit the three following criteria: (i) 10–26 residues in length; (ii) two basic residues near the N-terminus; and (iii) either a BBXB or BBXXB (B = basic AA) sequence at the C-terminus. These requirements were defined by activator peptides derived from both the IGF-II receptor (peptide 14) and the β_2-AR (i3C) [41, 47]. The authors found regions in the α_2-AR and M_4AR that fitted these criteria and synthesized the corresponding peptides which could directly activate G_o and G_i proteins [45]. The i3C region of the α_2-AR also fits these criteria with a slight modification of rule (iii) to include a C-terminal aromatic residue [46]. Although these sequence criteria can be useful in defining potential G protein activating regions within receptors, not all G protein activators fit such requirements. Notably, the naturally occurring activators mastoparan, SP, bradykinin, MCD, melittin and bombolitin fit only the first (10–26 residues in length) of three proposed criteria for G protein activator sequences. Of the receptor-derived activators the i3N β_2-AR peptide [31] fits only the first criterion, whereas the i3C β_2-AR peptide [31] and i3c β_1-AR peptide [27] fit the first and second criteria and the i3N D_2 peptide [48] fits the first and third criteria.

In general, the G protein activator peptides are short cationic peptides, however, their sequence patterns, hydrophobicity, amphiphilicity and helical content vary. These data are consistent with the notion that the active sequences may bind to different regions on G protein.

Peptide modifications

High concentrations of some of these peptides

are required to activate G proteins. Most of the activator peptides have EC_{50}s in the 10–30 μM range, although a few (mastoparan, MCD, an i3C M_4 peptide, and a i3C α_2-AR peptide) have EC_{50}s around 300 nM. A number of studies have indicated that increasing the hydrophobicity of activator peptides can increase their potency. Okamoto *et al.* observed that addition of an 11 residue hydrophobic segment of the IGFII transmembrane domain to the IGFII activator peptide (peptide 14) increased the potency of the activator peptide over 300-fold [41]. Similarly, Nishimoto *et al.* reported that addition of a small hydrophobic transmembrane domain of Alzheimer amyloid protein (APP) to the APP G protein activator peptide increased its potency 10–20-fold [55]. Also, we have observed that the attachment of a hydrophobic diazopyruvoyl cross-linking agent to the i3C α_2-AR peptide enhances the potency of this peptide 15-fold [56]. The addition of a hydrophobic tail may anchor these activator peptides into the phospholipid membranes and stabilize their active conformation. Interestingly, both the extreme N- and C-terminals of the α subunit are hydrophobic. Binding to these regions could account for the enhanced affinity of the hydrophobic peptide analogs.

As mentioned previously, inclusion of two or more peptides from rhodopsin or the β_2-AR synergistically enhanced the potency of these peptides to block receptor-G protein interactions. Recently, Wade *et al.* have shown that a heterodimer of two activator peptides from the i3N and i3C regions of the α_2-AR is 10-fold more potent in activating G proteins than either monomer peptide [30]. The possibility exists that varying connector chain lengths of such heterodimers may further enhance the potency of these peptides.

Although modifications of the G activator peptides have enhanced their potency, they have not enhanced their efficacy. Mastoparan appears to be the most efficacious peptide with seven- to 20-fold increases in G_o/G_i GTPase and two- to four-fold increases in G_s GTPase [34]. Mastoparan appears to act as a full agonist in G_i stimulation, but a partial agonist in G_s stimulation. Most receptor-derived peptides increase GTPase two- to five-fold, which is consistent with the extent of receptor-mediated GTPase in reconstituted systems [57–59]. However, we have shown that the i3C peptide from the α_2-AR (which stimulates a two-fold increase in G_i GTPase) acts as a partial G_i agonist and thus blocks α_2-AR agonist promoted GTPase in platelet membranes and mastoparan promoted GTPase in lipid vesicles [29, 30, 44].

Peptide specificity

Lack of specificity for certain G protein subtypes is a major problem associated with the use of these small peptides. The G protein activator derived from IGF-II receptor (peptide 14) is the most selective of the activator peptides. Peptide 14 preferentially activated the inhibitory G proteins (G_{i2}>G_{il} = G_{i3}) and to a lesser extent the closely related G_o protein. Peptide 14 had no effect on either the stimulatory G protein (G_s), or two members of the low molecular weight G proteins, c-Ki-ras p21 and smg p25A [41]. As previously mentioned, mastoparan is selective for the G_i and G_o proteins but also stimulates G_s proteins [34]. Peptides derived from the β_2-AR [31] and the α_2-AR [46] were slightly more selective activators of G_s than G_i (β_s-AR) or G_i than G_s (α_2-AR). The identification of the peptide binding sites may aid in the rational design of more subtype selective G protein activators.

Peptide binding sites

Mastoparan appears to bind to a site in the N-terminal [60] and/or C-terminal region [61] of the α subunit. These regions are closely associated in the tertiary structure of the G protein [62]. The ability of pertussis toxin to attenuate GTPase stimulation by the IGF-II peptide 14 [41] and the i3N α_2-AR (unpublished observations, Taylor and Neubig) suggests that these peptides may bind in the C-terminal region of the α subunit. We recently found that the activator peptide i3C from the α_2-AR binds specifically to both the N-terminal region in the α subunit and a site on the β subunit [56, 63]. Also, the βγ subunit complex is required for i3C stimulated G protein activation [56]. Phillips and Cerione have determined that an

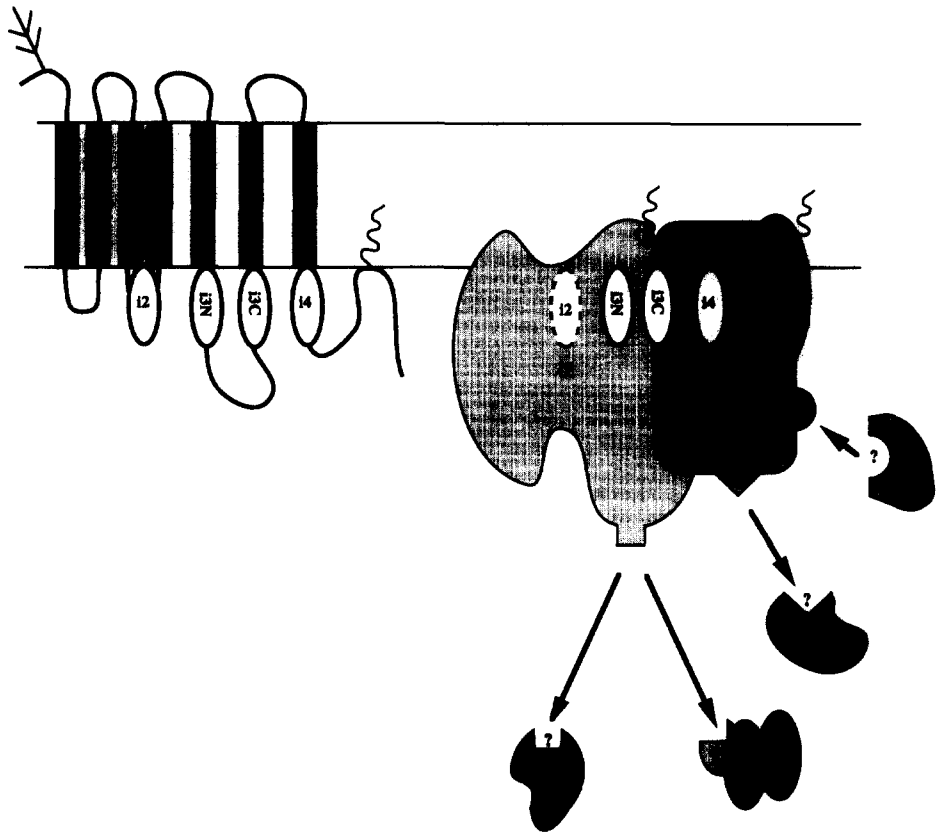

Fig. 1. Molecular interactions in G protein-mediated signal transduction. Mutagenesis and synthetic peptide studies provide evidence for a model of the sites of G protein interaction with receptors and effectors. The proposed second (i2), third (i3) and fourth (i4) intracellular loops of GPCRs are involved in coupling to G protein. The ovals represent putative sites of interaction of these regions of the receptor with G protein. Evidence in support of this model (see text) includes: (i) pertussis toxin blockade of GTPase stimulation by an i3N α_2-AR peptide; (ii) cross-linking of an i3C α_2-AR peptide to both the N-terminal region of the α subunit and a site on the β subunit; and (iii) binding of an i4 rhodopsin-derived peptide to a site on transducin $\beta\gamma$ subunit. The dashed oval surrounding the i2 peptide indicates that this binding site is less well defined. Peptide studies have also identified an effector (PDE) binding site on α_T (aa 293–314) as well as the corresponding site on the PDEγ subunit to which it binds (aa 24–45). A similar region on the α_s (aa 236–356) has been implicated in binding adenylyl cyclase. The $\beta\gamma$ subunit interacts with phospolipase C β (PLCβ) and the β-adrenergic receptor kinase (βARK), but the amino acids involved have yet to be identified. Many G protein–effector interactions (and possibly other receptor-G protein interactions) will likely be identified in the near future.

i4 rhodopsin-derived peptide also binds to a site on the $\beta\gamma$ subunit [6]. These data are consistent with recent findings which suggest that GPCRs bind to the $\beta\gamma$ subunit complex [6, 9, 64, 65] and signal through G proteins of unique $\beta\gamma$ subunit composition [7, 8]. Thus it appears that specificity of receptor–effector coupling in cells does not reside in the α subunit alone, but involves receptor recognition of $\beta\gamma$ subunits as well. Based on these data, a hypothetical organization of the receptor-derived peptide binding sites on G protein is shown in Fig. 1. Future studies to address the specific binding sites of such peptides should be useful both in mapping the receptor-G protein interface and in developing more potent and selective G protein activators.

EFFECTOR ACTIVATING PEPTIDES

Most recently, peptides corresponding to G protein sequences were synthesized to study G protein–effector interactions. The regions that mediate adenylyl cyclase activation by α_s have been deduced from α_i/α_s chimaera [66–68]. These studies indicated that four clusters of native α_s residues within a 121 amino acid region (aa 236–356) were required to observe functional coupling to adenylyl cyclase. In 1992 Rarick *et al.* showed that a peptide derived from a similar region of the α subunit of G_T (aa 293–314) directly activated cGMP-phosphodiesterase (PDE) [69]. Later studies, using a fluorescent probe, determined the specific residues within PDE (aa 24–45) which bind the α_T derived activator sequence [70]. These studies were confirmed by evidence that a peptide derived from PDE residues 24–45 cross-linked to a site on α_T within residues 306–310 [71]. Others have utilized peptides derived from low molecular weight G proteins to identify down-stream effectors [72–74], however the details of these studies are beyond the scope of this review.

CONCLUSIONS AND FUTURE DIRECTIONS

Peptides can be used to screen large areas of proteins to determine the critical regions involved in their protein–protein interactions. Clearly, the use of peptides has proven effective in determining the specific regions involved in receptor-G protein and certain G protein–effector interactions. Such data can be used to develop a structural map of the interactions between the major proteins in GPCR signal transduction (Fig. 1). A number of peptide studies suggest that the proposed second, third and fourth intracellular loops of GPCRs couple to G protein. The specific residues in the G protein to which these regions bind are yet to be determined, although both the N and C-terminus of the α subunit and a site on the β subunit have been implicated in receptor binding. The identification of the mutual contact sites between such proteins should aid in the development of therapeutic agents which could specifically target G protein signal transduction at multiple levels.

In light of recent evidence that certain diseases such as hyperthyroidism [75] and familial precocious puberty [76] may result from aberrant receptor-mediated G protein activation, the potential for G protein modulating peptides as models for therapeutic agents is clear. However, peptides themselves are unlikely to be suitable for administration as therapy. Two possible avenues could be explored to overcome potential problems of peptidases and the inability of these peptides to traverse target cell membranes. One area of interest is the growing field of peptidomimetics whereby non-peptide analogs with similar structures can be synthesized and tested for their abilities to mimic peptide activities [77]. The ability to place conformational constraints on non-peptide analogs can actually improve potency and selectivity as has been observed for certain opiate peptide antagonist analogs [78]. Gene therapy is another potential mechanism which can bypass problems associated with targeting these peptides to their sites of action [79]. Luttrell *et al.* employed a minigene strategy to show that expression of the α_{1B}-AR i3 loop in human embryonic kidney cells inhibited α_{iB} and α_{iC} but not M_i muscarinic or D_{iA} domamine receptor-mediated signal transduction [80]. Both approaches should be useful in the future development of therapeutic strategies to target the receptor-G protein interface.

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