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# Isolation of a putative receptor from *Zea mays* microsomal membranes that interacts with the G-protein, GPα1

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**Abstract** The C-terminal region of a heterotrimeric G-protein α-subunit is known to be one of the principal determinants governing its interaction with its cognate receptor. Use of an oligopeptide corresponding to the fifteen C-terminal residues of the *Arabidopsis* Gα-subunit (GPα1), as an affinity ligand, led to the resolution of a tightly binding 37 kDa membrane polypeptide from detergent solubilised *Zea* microsomal fraction membranes. An identical polypeptide bound tightly to an affinity matrix containing recombinant GPα1 protein as ligand: binding and release of this 37 kDa protein was dependent on the activation state of GPα1 which was regulated by inclusion or omission of the G-protein activator $AlF_4^-$. Finally, the isolated 37 kDa protein was labelled with the lectin concanavalin A, indicating it to be glycosylated. These data are consistent with the identity of the 37 kDa membrane polypeptide as a receptor that interacts with the *Zea* homologue of GPα1.

*Key words:* Receptor; G-protein; Synthetic peptide; Gα-subunit

## 1. Introduction

The heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) comprise a superfamily of proteins [1–3] that serve to transduce and amplify the signals which are initially perceived by integral plasma membrane receptor proteins. Within the last few years, considerable evidence has emerged for the operation of G-protein-linked pathways within plant cells. The earlier efforts included the measurement of high affinity binding of GTP and its analogues [4–6] and immunological detection, using antisera raised against animal Gα-subunits [7] or against synthetic peptides corresponding to regions of animal Gα-subunit sequence [4,8,9,10]. In addition, Gα subunit homologues have been cloned from *Arabidopsis* [11] and *L. esculentum* [12] whilst proteins showing elements of structural similarity to Gβ-subunits have been found in *Chlamydomonas* [13] and higher plants [14,15]. These topics have been fully reviewed by Terryn et al. [16] and Millner [17].

There has been rather less progress in identifying the receptors and effectors that respectively activate or respond to G-proteins in plants. However, based on the effects of a mastoparan analogue, which stimulated binding of GTPγS to plant plasma membranes [18] it is likely that at least some of the receptors belong to the 7-transmembrane span receptor (7TMS) superfamily. The latter, which in animal cells characteristically possess seven transmembrane spanning helices, are typified by rhodopsin and the adrenergic receptors [19] and form a superfamily that is somewhat larger than that containing the G-protein α-subunits [20]. The failure to identify plant homologues by classical cloning strategies is probably due to the extreme diversity of primary amino acid sequence even between closely related 7TMS receptors [21,22] and means that other approaches may be necessary to identify plant G-protein linked receptors. The strategy used in the present communication is based on current understanding of the protein domains, within Gα subunits, that specify Gα-receptor interaction.

Approaches involving preparation of chimaeric Gα subunits [23], use of bacterial exotoxins [24] and mutagenesis studies [25] have all indicated regions of amino acid sequence that play key roles in governing the interaction of Gα-subunits with their specific receptor, effector and Gβγ complexes (see [26,27] for reviews). From these studies, the principal stretches of contiguous sequence which specify interaction of the Gα subunit with receptor and effector have been shown to be localised to the extreme C-terminus and close to the C-terminus respectively. Most importantly, synthetic oligopeptides which correspond to these regions have been shown to be active in vitro. A peptide corresponding to the C-terminus of Gαs was shown to block the activation of adenylate cyclase mediated by the β-adrenergic receptor [28]. In other work, a peptide corresponding to a region close to the C-terminus of Gαt was able to directly activate cGMP-phosphodiesterase [29]. In the present work, a peptide representing the receptor interaction region (Fig. 1) of the *Arabidopsis* G-protein, GPα1 and active recombinant GPα1 have been used to resolve a putative GPα1-receptor from *Zea mays*

## 2. Materials and methods

### 2.1. Materials

Peptide synthesis reagents were purchased from Novabiochem, whilst SulfoLink resin was obtained from Pierce and Warriner, Chester, UK. The clone encoding protein GPα1 was a generous gift of Dr. H. Ma, Cold Spring Harbour Laboratory. All other reagents were of AnalaR or equivalent purity and were purchased from the Sigma Chemical Co. or Fisons plc.

### 2.2. Peptide synthesis

Automated synthesis of the peptide Cys-$G_{Ara2}$ [(C)DETRRRNLLEAGLL], which corresponds to the C-terminus of the *Arabidopsis* protein GPα1 [11] with an N-terminal additional cysteine (C) to facilitate coupling to SulfoLink resin, was performed using a Millipore 9050

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*Abbreviations:* ConA, concanavalin A; DTT, dithiothreitol; Fmoc, 9-fluorenylmethoxycarbonyl; rGPα1, recombinant GPα1; MEGA9, nonanoyl-*N*-methylglucamide; PMSF, phenylmethyl-sulphonyl-fluoride.


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peptide synthesizer utilising Fmoc-based synthetic chemistry [30]. The peptide was purified by reverse phase h.p.l.c. and sequence confirmation performed by solid phase microsequencing.

*2.3. Preparation of recombinant GPα1*

The GPα1 protein was over-expressed within *E. coli* [31] using the vector pT7.7 [32] which contains the T7 gene promoter and ribosome binding site. A detailed description indicating preparation of the expression construct, and purification and characterisation of the recombinant protein will be presented eleswhere.

*2.4. Preparation of affinity media*

Peptide Cys-$G_{Ara2}$ was coupled to SulfoLink, thiol-directed resin, basically according to the manufacturers instructions. Briefly, approximately 2.7 μmol peptide was reacted with each ml of gel. Following incubation of peptide in 0.1 M $NaH_2PO_4$ pH 6.0, 5 mM EDTA plus 0.1 M DTT for 3 h at 37°C, the peptide was desalted into 50 mM Tris-HCl, pH 8.5 and 5 mM EDTA (coupling buffer) via chromatography on Sephadex G-10. The reduced peptide was then allowed to react with SulfoLink gel in coupling buffer for 45 min prior to removal of unlinked peptide and subsequent blocking of unreacted gel by addition of 50 mM cysteine in coupling buffer. Finally, the gel was washed sequentially with 0.1 M NaCl and then 0.05% (w/v) $NaN_3$, in which the affinity resin was then stored. Estimation of peptide that remained uncoupled indicated a substitution level of 2.4 μmol Cys-$G_{Ara2}$ peptide·$ml^{-1}$ gel.

Coupling of rGPα1 to SulfoLink gel was performed essentially as described above except that procedures were carried out at 4°C. After storage in TEMD buffer, comprising 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $Mg(CH_3CO_2)_2$ and 1 mM DTT, approximately 800 μg (18.6 nmol) of rGPα1 was desalted into TEMN buffer, comprising 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $Mg(CH_3CO_2)_2$ and 100 mM NaCl. Subsequent reactions, including blocking of unreacted SulfoLink with cysteine and storage in the presence of $NaN_3$, was performed in TEMN medium. Estimation of non-coupled rGPα1 yielded a substitution level of 11 nmol·$ml^{-1}$ gel.

*2.5. Preparation and detergent solubilisation of* Zea *membranes*

Microsomal fraction membrane vesicles were prepared from etiolated *Zea* (var. Clipper) epicotyls essentially according to White et al. [6]. Membranes equivalent to 80 mg protein were resuspended at a concentration of 4 mg·$ml^{-1}$ in TEMD buffer, pH 8.0., plus 1% (w/v) sodium cholate, 30 mM MEGA9, 0.1 mM PMSF, and 1 μM each of pepstatin A and leupeptin. The membranes were maintained on ice and stirred gently prior to recovery of solubilised proteins by centrifugation at 440,000 × $g_{av}$ in a Beckman TLA 100.4 rotor for 50 min at 4°C.

*2.6. Affinity chromatography of* Zea *membrane proteins*

Detergent solubilised membrane proteins equivalent to 30 mg protein, were diluted three-fold in TEMD buffer, pH 8.0 supplemented with 0.33% (w/v) sodium cholate and 10 mM MEGA9 (TCM buffer). The diluted solution was recirculated overnight through a 1 ml Cys-$G_{Ara2}$ column at a linear flow of 0.13 ml·$min^{-1}$ prior to sequential elution with 15 ml of TCM buffer, a linear 20 ml gradient of 0 to 2 M NaCl in TCM, and finally, 5 ml of 4 M NaBr in TCM.

In other experiments, detergent solubilised membrane proteins were chromatographed on a 1 ml rGPα1 affinity column. Approximately 15–20 mg solubilised proteins were loaded onto the column in AMF buffer, comprising TCM buffer plus 30 μM $AlCl_3$, 50 mM $MgCl_2$ and 10 mM NaF, by recirculation overnight through the rGPα1 column at a linear flow rate of 0.13 ml·$min^{-1}$. Elution was then performed with 10 ml of AMF, a linear 0 to 1 M gradient of NaCl in AMF and 3 ml of 2 M NaCl in AMF. Elution was then continued with 8 ml of TCM buffer and 3 ml of 2 M NaCl in TCM.

*2.7. Electrophoresis and protein blotting*

Prior to electrophoresis, proteins were solubilised by incubation for 3 to 5 min at 95°C in 60 mM Tris, pH 6.8, 3% (w/v) SDS, 10% (w/v) sucrose, 2% (v/v) 2-mercaptoethanol and 0.005% (w/v) of Bromophenol blue. Proteins were then electrophoresed on 8–18% (w/v) linear gradient SDS-polyacrylamide gels according to Laemmli [33] prior to staining with silver [34] or Coomassie brilliant blue R-250 [35]. Alternatively, proteins were concentrated by precipitation with cold acetone, to a final concentration of 70% (v/v), and electrophoresed on a 12% SDS-polyacrylamide gel before transfer [36] to nitrocellulose membrane for subsequent ConA labelling, or to Fluorotrans (Pall Biosupport) PVDF membranes for subsequent protein microsequencing.

*2.8. Con A affinity labelling*

Labelling of blotted *Zea* proteins recovered from affinity chromatography runs, was carried out basically according to Faye and Chrispeels [37]. Blots were incubated sequentially with 3% (w/v) gelatin in 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl (TBS buffer) for 1 h; in TBS supplemented with 1% (w/v) gelatin, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ (TBS/M buffer) plus 25 μg·$ml^{-1}$ Con A for 1 h and finally in TBS/M plus 50 μg·$ml^{-1}$ horseradish peroxidase for 1 h. Between incubations blots were washed thoroughly, twice with TBS then four times 15 min with TBS/M plus 0.1% (v/v) Tween 20. Finally, Con A labelled proteins were visualised by addition of 0.06% (w/v) 4-chloro-1-napthol plus 0.03% (v/v) hydrogen peroxide in TBS. Controls were treated identically, except for inclusion of 200 mM α-methyl mannoside in all solutions excepting the 3% (w/v) gelatin in TBS step.

## 3. Results

Use of the GPα1 C-terminal peptide, Cys-$G_{ara2}$, as an affinity ligand, yielded a number of proteins which possessed varying degrees of affinity for the peptide resin (Fig. 2). A subset of proteins was eluted from the Cys-$G_{ara2}$ peptide column using NaCl up to a concentration of approximately 1 M. However, a polypeptide of molecular mass 37 kDa, was eluted throughout the 0–2 M NaCl gradient and was only completely released with more stringent washing procedures in which chaotropic agents were employed, i.e. 4 M NaBr. The latter eluant resulted in the elution of additional polypeptides with molecular masses between 85 and 18 kDa. In other experiments, the 37 kDa protein remained bound to the Cys-$G_{ara2}$ column when free peptide Cys-$G_{ara2}$ was applied at concentrations of 1 mM and 2.4 mM and application of 4.8 mM Cys-$G_{ara2}$ was required for elution (data not shown).

Fractions eluted by NaCl, and containing the 37 kDa entity, were pooled prior to concentration, about twenty-fold, by acetone precipitation. Microsequencing of the 37 kDa protein allowed assignment of most of the 16 N-terminal residues (Table 1) which contained predominantly hydrophobic residues. However, the sequence obtained showed no significant homology with any other sequence within the OWL composite protein database (Akrigg et al., 1988). Con A blotting of the concentrated material indicated the presence of a protein, also with an $M_r$ of 37 kDa, which possessed a mannose- or glucose-rich glycosyl moiety (Fig. 3, lane 2).

When affinity chromatography of detergent-solubilised

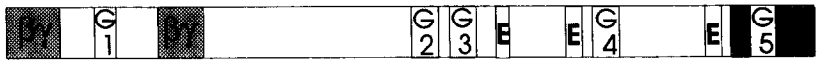


Fig. 1. Schematic of domain structure of a G protein α subunit. The approximate locations of the major contiguous domains which function in binding the βγ complex (βγ), interaction with the the receptor (R) and interaction with the effector (E) are shown. In addition the regions (G1 to G5) which are highly conserved within all Gα subunits are indicated; G1 is often referred to as the 'Gα$_c$' region. A more extensive description of the Gα subunit domain structure is given in Conklin and Bourne [1993]. The $G_{Ara2}$ peptide used as affinity ligand in this study corresponds to the extreme C-terminal R domain sequence of GPα1.

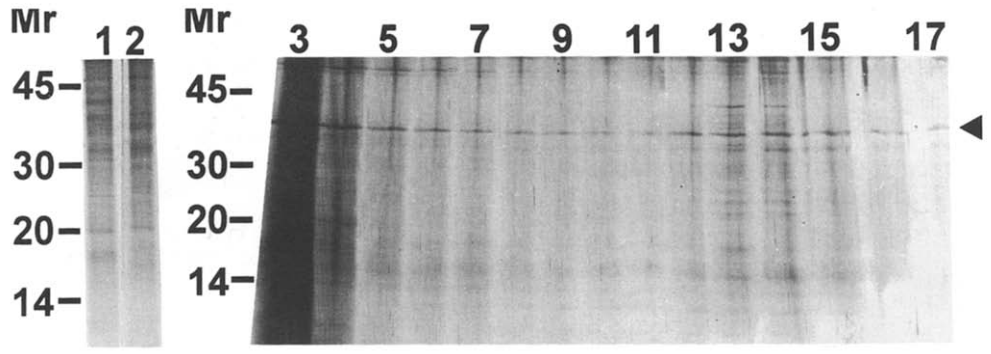


Fig. 2. Separation of detergent solubilised *Zea* microsomal membrane proteins on $G_{Ara2}$ peptide affinity resin. Sodium cholate/MEGA9 solubilised *Zea* microsomal membrane proteins equivalent to 15–25 mg protein were recirculated through a 1 ml $G_{Ara2}$ peptide column and then eluted with a 20 ml, 0 M to 2 M NaCl gradient in TCM buffer and then 5 ml of 4 M NaBr in TCM. Fractions of 1 ml were collected. After electrophoresis of the eluted proteins on a 8% to 18% (w/v) linear gradient SDS-polyacrylamide gradient gel, the gel was stained with silver; alternate fractions are labelled. Lane 1, 1 $\mu$g microsomal membrane proteins; lane 2, 1 $\mu$g detergent solubilised proteins; lanes 3 to 12, 25 $\mu$l of alternate fractions from the 0 M to 2 M NaCl gradient; lanes 13 to 17, fractions eluted with NaBr. (◀) 37 kDa putative receptor. The $M_r$ values of marker proteins, indicated, are in kDa.

*Z. mays* microsomal membrane proteins was performed on the rGP$\alpha$1 matrix, a small number of proteins bound tightly in AMF buffer. The $AlF_4^-$ ion, within the AMF buffer is known to activate G proteins and normally causes dissociation of the $\alpha$ subunit and $\beta\gamma$ complex [40]. After washing the column to remove AMF, three polypeptides with $M_r$ values of 20, 25 and 37 kDa, respectively, were eluted by the final 2 M NaCl wash (Fig. 4, lanes 19–21). The 37 kDa protein was found to possess an identical N-terminal sequence to that of the 37 kDa protein resolved by the Cys-$G_{Ara2}$ peptide column (Table 1) whilst there was insufficient of the 20 and 25 kDa proteins available to permit microsequencing.

## 4. Discussion

It is clear from work on animal G$\alpha$ subunits, that the predominant domain which governs interaction of the G$\alpha$ subunit with its cognate receptor is a contiguous region (Fig. 1) at the extreme C- terminus of the G$\alpha$ polypeptide (reviewed in [26,27]). Moreover, relatively short synthetic oligopeptides, which correspond to this region, have been shown to specifically block receptor: $G_\alpha$interaction [28] presumably by binding to the recognition site present on the receptor. Accordingly, the Cys-$G_{Ara2}$ peptide whose sequence corresponded to the C-terminus of the *Arabidopsis* G-protein GP$\alpha$1, would be expected to show high selectivity when used as ligand for isolation of its corresponding receptor. As we have previously detected a homologue to protein GP$\alpha$1 in *Zea* microsomal fraction membranes and the latter were readily available in high yield from chlorophyll free, i.e. etiolated tissue, these membranes were employed as starting material for the resolution of a putative GP$\alpha$1-receptor.

The data shown in Fig. 2 clearly indicate that some of the 37 kDa polypeptide was bound very tightly to the $G_{Ara2}$ peptide ligand and was only completely removed by the application of the chaotrope, NaBr. In fact, the 37 kDa polypeptide was eluted from the column across a range of NaCl concentrations. This is not surprising as some of the 37 kDa protein would be expected to interact with the $G_{Ara2}$ peptide both nonspecifically, due to the charged residues present in $G_{Ara2}(Asp^-, 2Glu^-, 3Arg^+$ and C-terminal carboxy group) and specifically when the $G_{Ara2}$ was present in the appropriate conformation for correct interaction to occur. Sequencing of the N-terminus of the 37 kDa polypeptide yielded 16 residues of amino acid sequence

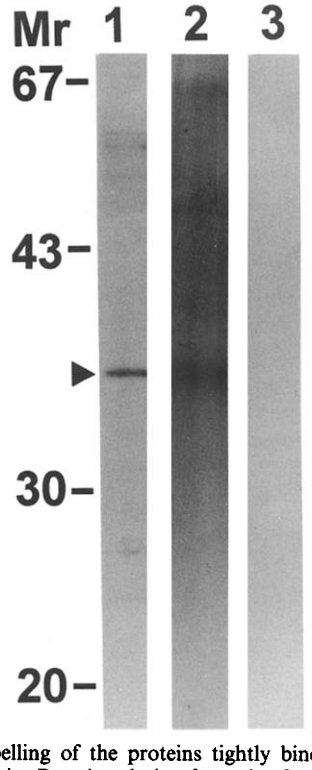


Fig. 3. ConA labelling of the proteins tightly binding to the $G_{Ara2}$ peptide affinity resin. Proteins eluting from the $G_{Ara2}$ peptide column between 1 M and 2 M NaCl were concentrated 20-fold by acetone precipitation. After resuspension in sample buffer, proteins originating from approximately 1 ml of the pooled fractions were electrophoresed on an 8% to 18% (w/v) SDS-polyacrylamide gradient gel. The proteins were either stained with Coomassie blue, or blotted to nitrocellulose membrane prior to labelling with 25 $\mu g \cdot ml^{-1}$ of Con A and then horseradish peroxidase (see section 2) prior to visualisation with 4-chloro-1-napthol plus $H_2O_2$. Lane 1, Coomassie blue stained proteins; lane 2, Con A labelled proteins; lane 3, control – as for lane 2 but with 200 mM $\alpha$-methyl-mannoside included in all solutions after blotting. (▶) 37 kDa putative receptor. The $M_r$ values of marker proteins, indicated, are in kDa.

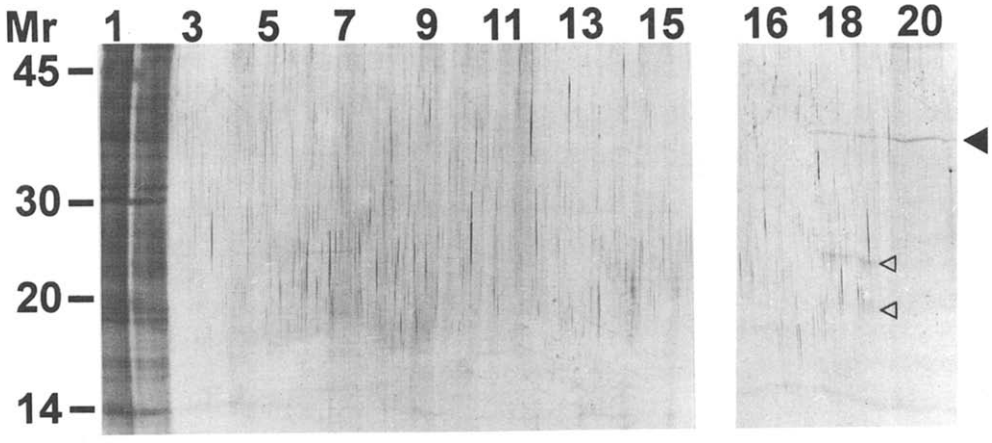


Fig. 4. Separation of detergent solubilised *Zea* microsomal membrane proteins on rGPα1-affinity matrix. Sodium cholate/MEGA9 solubilised *Zea* microsomal membrane proteins equivalent to 8–10 mg protein and in TCM + AMF buffer (see section 2), were recirculated through a 1 ml rGPα1 column and then eluted sequentially with a 10 ml, 0 M to 1 M NaCl gradient in TCM + AMF buffer, 3 ml of 2 M NaCl in TCM + AMF, 8 ml TCM (AMF absent to deactivate rGPα1) and finally 2 M NaCl in TCM (−AMF). Fractions of 1 ml were collected, except for the 2 M NaCl in TCM + AMF and TCM (−AMF) where a single 3ml fraction and 2 ml fractions were collected, respectively. After electrophoresis of the eluted proteins on a 8% to 18% (w/v) linear gradient SDS-polyacrylamide gradient gel, the gel was stained with silver; alternate fractions are labelled. Lane 1, 1 μg solubilised proteins; lane 2, 5 μl unbound material; lanes 3–11, 25 μl from 0 M to 1 M NaCl gradient fractions; lane 12, 25 μl from 2 M NaCl in TMF + AMF fraction; lanes 13–17, 25 μl from TCM (−AMF) fractions; lanes 18–20, 25 μl from TCM + 2 M NaCl (−AMF) fractions. (◀), 37 kDa putative receptor; (◁), 25 kDa and 20 kDa proteins. The $M_r$ values of marker proteins, indicated, are in kDa.

(Table 1) which did not display significant homology to any current database entry. This is not surprising, since homology between members of 7TMS superfamily is primarily at the secondary structural level, with even closely related 7TMS receptors displaying quite poor similarity at the level of primary sequence [21,22]. Glycosylation of the 37 kDa polypeptide, as indicated by its crossreaction with the α-D-mannosyl and α-D-glucosyl-binding lectin ConA, is consistent with its proposed identity as a 7TMS receptor since members of this superfamily are known to be N-terminally glycosylated [20], although the short stretch of N-terminal sequence obtained (Table 1) did not reveal an obvious glycosylation site. However, the first residue of the 37 kDa protein could not be equivocally determined and it unclear as to how close the sequence obtained was to the true N-terminal residue.

In support of the contention that the 37 kDa polypeptide represents a receptor coupled to GPα1 are the data obtained using rGPα1 as an affinity ligand. In this experiment (Fig. 4) it was possible to modulate the affinity of the 37 kDa polypeptide for the $G_{Ara2}$ ligand by performing the chromatography in the presence or absence of the G-protein activator $AlF_4^-$ [40]. The conformational change brought about within Gα subunits by $AlF_4^-$ binding is considered to be identical to that caused by binding of nonhydrolysable GTP analogues such as GTPγS [38]. Under conditions where $AlF_4^-$ was present, i.e. rGPα1 was activated, the 37 kDa polypeptide, whose N-terminal 15 residues were identical to those of the 37 kDa entity which was resolved by the $G_{Ara2}$-peptide column (Table 1), remained tightly bound to the rGPα1 even under very stringent conditions with 2 M NaCl present. Only when $AlF_4^-$ was removed, and presumably the rGPα1 deactivated, was the 37 kDa protein released by the 2 M NaCl applied. This indicates an increased affinity between the 37 kDa protein and rGPα1 when the latter was activated which would represent the converse situation to that found in animal G-proteins. However, as suggested originally by Bourne et al. [3] there is no obvious reason why the active G-protein species should not be the GDP-liganded form in which case the $AlF_4$ ion would be expected to increase receptor: Gα affinity. An alternative possibility is that the 37 kDa polypeptide is involved in dissociating activated GPα1 from its cognate receptor in some way. Our efforts in cloning the cDNA which encodes the putative 37 kDa receptor should resolve these points. Finally, we do not know yet the identities of the 20 and 25 kDa proteins that were released from rGPα1 at high NaCl concentration when $AlF_4^-$ had been removed.

Table 1
N-terminal sequence of the 37 kDa putative receptor protein

| Affinity ligand | Sequence of 37 kDa protein |
|---|---|
| $G_{Ara2}$ | $_1$**XFPVFEVSMMAAKIAY**$_{16}$ |
| rGPα1 | $_1$**XFPVFEVSMXAAKIA**$_{15}$ |

After chromatography of the detergent solubilised *Zea* microsomal membrane proteins on Cys-$G_{Ara2}$ peptide or rGPα1 affinity matrices (see legends to Figs. 2 and 4), fractions containing the 37 kDa protein were concentrated, subjected to SDS-PAGE and the 37 kDa protein then transferred to PVDF membrane for microsequencing. In the N-terminal sequences shown, subscripted numbers indicate the sequencer cycle whilst X indicates an unidentified residue.

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