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Role of Sst2 in Modulating G Protein-Coupled Receptor Signaling

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Sst2 formally acts as an inhibitor of G protein-coupled receptor signaling in yeast perhaps stabilizing a G protein/unactivated receptor complex. desTrp1,Ala3 α-factor (dTA-αf), normally a competitive antagonist, activated responses in an *sst2* strain. The antagonist to agonist switch was consistent with an Sst2 effect on receptor/G protein coupling, but not with an Sst2 role in global reduction of signaling. Response to α-factor, assayed by growth arrest, was independent of level of expressed surface receptor over a 40-fold range in an *SST2*$^+$ strain, consistent with coupling of only a subset of receptors to G protein. In contrast, in an *sst2* strain, response to α-factor was proportional to receptor expression, consistent with participation of all receptors in signaling. The wt α-factor receptor inhibited signaling in response to dTA-αf when introduced into a CKC4 chimeric receptor strain. The dominant-negative effect of the receptor might reflect sequestration of G protein.

The yeast protein Sst2 is the original member of the RGS family of proteins that includes the mammalian proteins and *C. elegans* EGL-10 (1-5). These proteins are involved in the regulation of signaling through heterotrimeric G proteins, though the actual mechanism by which they act remains obscure (6). Deletions of the *sst2* gene in *S. cerevisiae* confer increased sensitivity to pheromone and a prolongation of response, suggesting that the role of the wt Sst2 product in signaling is inhibitory (1,2). Sst2 and homologs interact genetically (2,3,6) and physically (4) with G_α. The presence or absence of Sst2 does not affect levels of yeast G protein though (6). The identification of mutations in the yeast receptor gene *STE2* which genetically act as if they facilitate or interfere with the Sst2 process suggests that receptor as well as G protein might play a role in Sst2 function (7,8).

The interactions of heterotrimeric G proteins with receptors are complex with many potential points of control. The stability of the unactivated receptor/G protein complex has profound consequences, especially if the receptor is in excess (9,10) as may be the case in *S. cerevisiae* (11,12) (Sen and Marsh, manuscript in preparation). If the receptor/G protein complex is weak before receptor activation (the common condition), then a single activated receptor is capable of activating many G proteins.

If the unactivated receptor/G protein interaction is tight (preassociation) and receptor is in excess of G protein, then only receptors associated with G protein can signal. Receptors without access to G protein act as a separate pool of receptors incapable of signaling. A partial agonist capable of activation of only a small fraction of receptors might fully activate a system in which G protein had access to all receptors, but would only poorly activate a system in which most receptors lacked access to G protein. We present genetic evidence suggesting that the Sst2 product may act to stabilize the unactivated receptor/G protein complex.

MATERIALS AND METHODS

Strains and culture conditions. Strains were derivatives of LM23-3az [*bar1, SST2*$^+$] or AS21 [*ste2, BAR1, sst2::URA3*] unless otherwise defined (2,13). RC631 was an *sst2, BAR1*$^+$ strain. Standard culture conditions were used (14).

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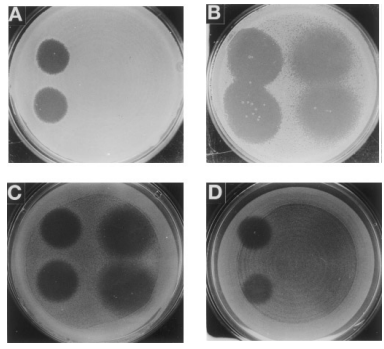

FIG. 1. Growth inhibition in response to α-factor and dTA-α-factor. Lawns of cells on Petri plates were spotted in duplicate (above and below) with peptide. α-factor 0.1 μg was spotted on the left and dTA-α-factor 10.0 μg was spotted on the right, in a volume $\leqslant$5 μl and incubated about 24 h. A, LM23-3az, *SST2*$^+$; B, RC631, *sst2;* C, LM103(YCpCKC4), CKC4/*ste2;* D, LM23-3az(YCpCKC4), CKC4/*STE2*$^+$.

Growth arrest assays. Standard growth arrest assays on solid media were used (15). Preparations of synthetic α-factor or des Trp1, Ala3, α-factor (dTA-α-factor) were spotted in a volume of 2μl to 10μl onto lawns of cells as previously described and incubated for approximately 24h at 30°C. Only the relative level of response could be compared in the *SST2*$^+$ and *sst2* strains since they differed in expression of the α-factor peptidase Bar1.

Surface expression of α-factor receptor. The α-factor receptor was expressed from three plasmids expected to yield different surface levels. The plasmid pAB539 contains *STE2* cloned on the high-copy 2μ-based vector YEp13 and overexpresses α-factor receptor (16). The plasmid pAS100 is a CEN-based plasmid derived from the vector pRS315 and expected to yield expression levels similar to those of the chromosomal copy of *STE2*. The plasmid pAS3 is similar to pAB539, but contained a deletion of a *Hind*III fragment of promoter DNA required for efficient receptor expression (17).

Determination of surface receptor expression. Ligand binding assays were performed using synthetic [^{3}H] α-factor as previously described (15,18). Level of actual receptor expression was determined, for accuracy, in the LM102 background which contains the *bar1* mutation preventing [^{3}H] α-factor degradation during the assay.

RESULTS AND DISCUSSION

Consequences of the stability of the preassociated receptor/G protein complex on patterns of signaling and response. The model that we test here is that the role of Sst2 is to stabilize the unactivated receptor/G protein complex and that at least some of its effects are mediated through this stabilization. Typically G proteins sample many receptors for one that might lead to coupling. A single activated receptor can activate many G proteins. To use a striking example, in a photoreceptor cell with rhodopsin molecules in excess of the G protein transducin, a single activated rhodopsin molecule activates many transducin molecules (19). Only a transient high-affinity complex is formed during coupling. A 'preassociation' situation is possible however in which unactivated receptor and G protein form a relatively stable 'preassociated' complex so that an individual G protein can only couple to the receptor with which it is preassociated. Activation of a small proportion of receptors is insufficient for response in the preassociated situation unlike the random coupling situation. The following experiments were designed to test aspects of signaling expected to differ in the 'tight complex' and 'loose complex' situations. We also tested the model that Sst2 played a role in promoting formation of a 'tight complex' between G protein and receptor.

Sst2 inhibition of response to the peptide ligand dTA-α-factor. The ligand dTA-α-factor has been widely used as an α-factor antagonist (18,20,21). In a number of studies it has been shown to efficiently bind the α-factor receptor and to act as a competitive inhibitor of responses. Strikingly, we observed that in an *sst2* strain, dTA-α-factor acted as a potent agonist (Fig. 1), promoting growth arrest, though not quite as completely as α-factor. In the wt control (*SST2*$^+$)

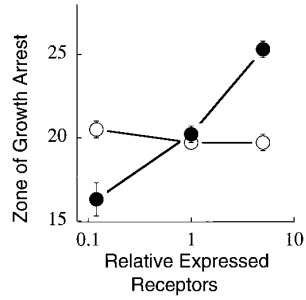

FIG. 2. Sensitivity to α-factor as a function of surface receptor expression. Level of receptor expression was adjusted using various plasmid constructs and was determined using [^{3}H] α-factor binding (see text). Size zone of growth arrest by 0.1 μl α-factor was determined in triplicate. LM102 (*SST2*$^+$) with various *STE2* constructs ○; AS21 (*sst2*) with *STE2* constructs.

strain the 100-fold excess of dTA-α-factor failed to cause growth arrest (Fig. 1) and in fact inhibited responses to α-factor (not shown)(20,21).

Since dTA-α-factor acted as an antagonist in the presence of Sst2 but as an agonist in its absence, the role of Sst2 must be more complex than general signal reduction. Because dTA-α-factor has many structural features of an α-factor agonist, and acts as an agonist on some mutant and chimeric receptors (such as CKC4) it has been previously proposed that dTA-α-factor has weak partial agonist potential (13,21) rather than being an absolute antagonist. A synergistic α-factor-related peptide that lacks agonist activity for a wt strain also exhibits agonist activity for an *sst2* strain but not an *SST2*$^+$ strain (22). Thus Sst2 may act by a mechanism that specifically inhibits signaling by weak agonists more than signaling by full agonists.

Role of level of receptor expression in response. The sensitivity of strains to α-factor as a function of level of receptor was determined. Strains expressed the wt receptor from a *CEN* vector (normal level), a vector with a promoter mutation (reduced expression), or from a multicopy 2μ-based plasmid (increased) expression. Levels of surface receptor were determined using [^{3}H] α-factor binding assays and normalized to the level of the *CEN*-based *STE2* construct which is approximately the same as that of *STE2* expressed from the genome (15).

In a *SST2*$^+$ background response was remarkably independent of surface expression over nearly a 50-fold range of receptor levels from 5-fold above the normal surface expression level to 8-fold below the normal level (Fig. 2). It had been previously noted that overexpression of the α-factor receptor did not increase response to α-factor (11,12). The simplest explanation for our result is that only a fraction of the receptors in a normal cell are able to couple to responses and that the rest are 'excess'. Reduction of the level of receptor below 5% of the normal level led to smaller, turbid, fuzzy-edged growth arrest zones in response to α-factor (not shown) suggesting that a threshold of receptor level exists. Lack of receptor expression completely blocks α-factor-induced growth arrest (15-17).

In the absence of Sst2 the result was different. Response to α-factor increased as the number of surface receptors increased (Fig. 2). Thus the *sst2* strain stood in contrast to the *SST2*$^+$ strain in which response was insensitive to surface receptor number. For most mammalian cell types and receptors, sensitivity to a ligand reflects the amount of receptor expressed, as predicted by the 'ternary model' for activation of G protein by liganded receptor (23). Thus receptor/G protein coupling seemed more typical in the absence of Sst2.

Dominant negative activity of wt receptor. The ability of wt receptor to act in a dominant negative fashion was determined. An unactivated receptor capable of forming a 'preassociated

complex' complex with G protein could inhibit signaling by sequestering G protein away from activated receptors. The chimeric receptor CKC4 confers sensitivity to dTA-α-factor even in a *SST2*$^+$ background (21). Intracellular residues are wt in the CKC4 receptor so interaction with G protein is predicted to be normal (21). The CKC4 receptor is activated by α-factor confering a slightly higher sensitivity than the wt receptor. Figure 1(C,D) shows that though the strain expressing only CKC4 receptor was sensitive to dTA-αf, the presence of the wt α-factor receptor inhibited response to dTA-αf. Both CKC4 strains responded well to α-factor, though the presence of the wt receptor again reduced response (Fig. 1). Thus the wt receptor confered an inhibitory, dominant negative, effect on signaling from the CKC4 receptor.

To determine if Sst2 was required for the dominant negative inhibition of signaling from CKC4 we attempted to construct an *sst2* strain expressing the CKC4 receptor by transforming our *sst2* strain with the CKC4 construct. Unfortunately no viable colonies with the proper genotype were recovered despite the fact that control transformations were efficient. The non-viability of the CKC4 *sst2* may reflect amplification of the weak constitutive signaling of CKC4 (21) in the *sst2* background leading to growth arrest.

Does Sst2 act to tether G protein to receptor? The model we propose to explain these data is that Sst2 acts to stabilize the complex of G protein with the unactivated receptor. Such a complex can be termed 'preassociated' since receptor and G protein are functionally linked but still require ligand association for signaling. In contrast, 'precoupled' complexes of receptor and G protein are capable of signaling in the absence of ligand (9,10,24). In the presence of Sst2, only activation of the specific receptor molecule with which a particular G protein heterotrimer is associated will lead to G protein activation. In this model Sst2 also promotes desensitization by sequestering G protein and preventing an activated receptor from participating in multiple rounds of G protein activation. Sst2 might easily play additional roles as well. A specific prediction of this model, testable using biochemical approaches, is that Sst2 interacts with both receptor and G protein, consistent with genetic observations (6-8,25).

Given that in many cells a single G_α subtype can couple to more than one type of receptor, a mechanism to tether G protein to a specific receptor could not only change the overall signaling properties of the cell, but could also play an important role in integration of signals from a variety of different receptors.

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