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Somatostatin displayed on filamentous phage as a receptor-specific agonist

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1 In search of methods to identify bio-active ligands specific for G protein-coupled receptors with seven transmembrane spanning regions, we have developed a filamentous phage-based selection and functional screening method.

2 First, methods for panning peptide phage on cells were established, using the hormone somatostatin as a model. Somatostatin was displayed on the surface of filamentous phage by cloning into phage(mid) vectors and fusion to either pIII or pVIII viral coat proteins. Peptide displaying phage bound to a polyclonal anti-somatostatin serum, and, more importantly, to several somatostatin receptor subtypes (Sst) expressed on transfected CHO-K1 cells, in a pattern which was dependent on the used display method. Binding was competed with somatostatin, with an IC50 in the nanomolar range. The phage were specifically enriched by panning on cells, establishing conditions for cell selections of phage libraries.

3 Binding of somatostatin displaying phage to sst_2 on a reporter cell line, in which binding of natural ligand reduces secretion of alkaline phosphatase (*via* a cyclic AMP responsive element sensitive promoter), proved that the phage particles act as receptor-specific agonists. Less than 100 phage particles per cell were required for this activity, which is approximately 1000 fold less than soluble somatostatin, suggesting that phage binding interferes with normal receptor desensitization and/or recycling.

4 The combination of biopanning of phage libraries on cells with functional screening of phage particles for receptor triggering activity, may be used to select novel, bio-active ligands from phage libraries of random peptides, antibody fragments, or libraries based on the natural receptor ligand.

Keywords: Filamentous phage; phage display; somatostatin receptors; G-protein coupled receptors; immunochemical detection; functional agonist; phage selection; receptor agonist

Introduction

In peptide phage display, methods to create vast libraries of peptide variants are linked to powerful selection strategies to isolate peptide ligands binding to any chosen antigen, including receptors, enzymes or viruses (Scott & Smith., 1990; Cwirla *et al*., 1990; Devlin *et al*., 1990; Winter *et al*., 1994). Its success hinges on the availability of highly purified preparations of antigen, and on the development of very sensitive screening methods for testing the selected peptides. Indeed, peptides specific for many globular proteins or peptides (Cwirla *et al*., 1990; Devlin *et al*., 1990) and purified domains of cell surface receptors (Doorbar & Winter 1994; Livnah *et al*., 1996) have been selected. Selections on impure antigens are significantly more difficult, due to the problem of enriching phage peptides specific for non-relevant structures. This makes the procedure particularly difficult for membrane receptors, that may either not be purified in quantities required for phage panning, or for which the target area is defined by multiple transmembrane regions. Nevertheless, there is a large group of receptors, the G-protein coupled receptors, that harbour a wide range of activities and have therefore functioned as target for ligand screening using chemical peptide and other libraries. These receptors often bind small ligands within their transmembrane helices, while on the extracellular side displaying a rather short and heavily glycosylated N-terminal region only. Access to novel receptor-binding and triggering phage peptide ligands may therefore be achieved only when whole cells or membrane isolates, carrying the native receptor on their surface, could be used for selection. This would have the advantage, over the use of combinatorial chemistry and screening tests, to access the vast complexity of phage libraries.

To assess whether such 7-TM receptors are accessible to ligands displayed on the surface of phage particles, and to establish methods for selection and screening for binding and function of phage libraries, we have initiated the study of a phage carrying somatostatin on its surface. We determined whether receptor-specific peptide phage could specifically bind to cells expressing the 7-TM receptor for the hormone, and, whether interaction was sufficient for its selection via cell panning, and whether detection via ELISA or FACS was feasible. Further, in view of developing functional screening assays, the triggering activity of the phage particles on receptor function was tested.

Somatostatin is a multifunctional peptide hormone, which regulates key cellular processes such as secretion, neurotransmission and proliferation, through interaction with at least five somatostatin receptors (Somatostatin and its receptors, 1995; Hoyer *et al*., 1995). The five cloned *sst*, all G protein-coupled seven-transmembrane receptors which are homologous in the transmembrane domains of the different receptor subtypes, appear to have similar pharmacological properties. Functional discrimination between the different receptors has been hampered by the shortage of *sst* subtype specific a(nta)gonists and the lack of monoclonal antibodies to the receptor. Extensive efforts with the traditional hybridoma technique for

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antibody production to *sst* has failed, as for many of the 7-TM receptors, not in the least due to the lack of immunogenicity of these receptors. Some antibodies have been made that recognize the denatured form of one of the five receptors (Theveniau *et al*., 1994; Schonbrunn *et al*., 1995), but none recognize the native receptor on the cell surface. *Sst* subtype specific agonists have emerged recently, but subtype specific antagonists have not yet been identified (Hoyer *et al*., 1995).

We have built a model, using somatostatin and its receptors, that establish methods to display the hormone on phage, and procedures for cell panning of phage libraries, binding assays, and receptor activation assays. Somatostatin was displayed on the surface of phage particles via fusion to pIII and pVIII phage coat proteins. Sensitive assays were developed for measuring binding of peptide phage to whole cell transfectants expressing a chosen subtype receptor. The somatostatin-14 displaying phage particles specifically bind to at least three of its five receptors expressed on the surface of transfected CHO-K1 cells, and, in a reporter gene system, phage act as agonists with unexpected sensitivity. These methods may be used to develop *sst* subtype specific ligands, which in some cases may be useful for effective imaging or therapy of tumours expressing high levels of somatostatin receptors. The methods and principles that we present here will be of general applicability for developing bio-active ligands for many other G-protein coupled transmembrane receptors.

Methods

Reagents

All reagents were, unless mentioned otherwise, obtained from Merck. Oligonucleotides were purchased from Eurogentec.

Cloning vectors

pCANTAB6 is a derivative of pHEN1 (Hoogenboom *et al*., 1991) carrying an additional Histidine stretch of six residues upstream of the c-*myc* sequence to allow Immobilized Metal Affinity Chromatography (IMAC) purification; the plasmid is further described by McGuinness *et al*., 1996. pHEN8 (Jacobsson & Frykberg, 1996) is a derivative of pHEN1 with geneIII replaced by geneVIII. fd-tet-SN is a derivative of fd-tet-DOG1 (Clackson *et al*., 1991) and carries *Sfi*I and *Not*I cloning sites upstream of its geneIII in the same configuration as in pHEN1. In all of these vectors the gene encoding the molecule to be displayed on either pIII on phagemid (pCANTAB6) or phage (fd-tet-SN), or on pVIII of the phagemid (pHEN8), is cloned as a *Sfi*I-*Not*I fragment in frame with upstream pelB or geneIII leader sequence, and downstream geneIII or geneVIII.

Construction and preparation of somatostatin displaying phage particles

The somatostatin binding domain was made by PCR using overhanging oligonucleotides: SOMBACK: 5′-TTA CTC GCG GCC CAG CCG GCC ATG GCC GCT GGC TGC AAG AAT TTC-3′ and SOMFOR: 5′-TTG TTC TGC GGC CGC ACA GGA TGT GAA AGT CTT CCA GAA GAA ATT CTT GCA GCC AGC-3; the oligonucleotides encode the 14 residue somatostatin and incorporate at their ends *Sfi*I and *Not*I restriction sites. PCR was performed using 2 mM $MgCl_2$ 25 mM KCl, 10 mM Tris-HCl, pH 8.3, 200 μM of deoxyribonucleoside triphosphates, 1 μM of oligonucleotide and 1 U Taq polymerase (Perkin Elmer) (94°C for 7 min followed by 25 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min). The PCR product was purified by electro elution, *Sfi*I-*Not*I digested and ligated with T4 DNA ligase (Promega), for 6 h at 15°C, into the phage(mid) vectors (see Figure 1). The constructs were transfected by electroporation into *E. coli* TG1 bacteria. Bacteria were plated on TYE-Agar (16 g/l bacto-tryptone, 10 g/l Yeast extract, 5 g/l NaCl and 16 g/l Agar containing the appropriate antibiotic: 100 μg ml^{-1} ampicillin, 2% glucose (AG), or 15 μg ml^{-1} tetracycline (T), and incubated overnight at 30°C. Individual clones were toothpicked and cultured overnight at 37°C in 2 × TY (TYE without agar) supplemented with AG or T. Phage were prepared according to Clackson *et al*., (1991) for fd-phage or according to Marks *et al*. (1991) for phagemids. Periplasmic fractions were prepared after 4 h of IPTG-induction at various temperatures; induced bacterial cultures were spun down and the pellet resuspended in 1 ml of ice-cold 1 mM EDTA in PBS. After incubation for 30 min, the periplasmic fraction was obtained by brief centrifugation.

Sequencing

DNA from phage(mid)s was phenol extracted, once iso-amyl alcohol washed and precipitated using 3 M NaAc/100% ethanol. After desalting over a G75 spin column, sequencing was done according to Sanger *et al*., (1977) with 10 μg of single-stranded template DNA. Semi-automatic sequencing was done on the ALF Express (Pharmacia). Sequence reactions were done according to the manufacturer's manual of the Autoread Sequencing Kit (Pharmacia) using oligonucleotides FD-P3: 5′-CTC TTC TGA GAT GAG TTT TTG-3′, or 'myc-tag' based MYC: 5′-CGT TAG TAA ATG AAT TTT CTG TAT GA-3′.

SDS–PAGE and immunoblotting

Phage (6×10^9 TU) was mixed with 2 × SDS-loading buffer (10% β-mercaptoethanol, 2% SDS, 30% Glyerol, 0.025% Bromophenol-blue, 0.05 M TRIS, pH 6.8) and denaturated by heating to 95°C for 5 min. Samples were run on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Schleicher & Schuell) by electroblotting at 100 mA for 45 min. The filter was blocked overnight at 4°C with PBS/4% Marvel (dry skimmed milk powder, Sainsbury's)/0.1% Tween 20 (PMT-buffer). Incubations with antibodies were done at RT; in between incubations the membrane was washed with PMT-buffer. The filter was incubated either with 1 : 10 diluted anti-pIII antibody (Tesar *et al*., 1995), 1 : 1 diluted anti-*myc* tag antibody 9E10 (Marks *et al*., 1991), or 1 : 800 diluted rabbit anti-somatostatin serum (30-1-0) (made in house using immunization with somatostatin-BSA conjugate, unpublished). Subsequently the filters were incubated with rabbit-anti-mouse peroxidase (Dako; for anti-pIII and 9E10 detection; diluted 1 : 200 in PMT-buffer), or goat anti-rabbit peroxidase (Dako; for anti-SOM detection; diluted 1 : 800). Both reactions were done for 1 h. Staining of the filter was done with 0.05% diaminobenzidine, 0.1% H_2O_2 in 0.05 M Tris-HCl, pH 7.6; the reaction was stopped by rinsing with water. The membrane was dried and the density of different lanes was measured using an LKB gel scanner.

Cell lines

CHO-K1 cells were cultured in Ham-F12 medium, 10% fetal calf serum, penicillin (50 IU ml^{-1}), streptomycin (50 μg ml^{-1}), 2 mM of glutamine under humidified conditions at 37°C, 5%

CO_2 and air. Sst_4 and sst_5 transfectants were cultured in the presence of 400 μg ml^{-1} of G418. Sst_2 transfected CHO-K1 cells were cultured in a mixture of Ham-F12 and DMEM medium, 10% fetal calf serum, penicillin (50 IU ml^{-1}), streptomycin (50 μg ml^{-1}), 4 mM of glutamine, 400 μg ml^{-1} G418 and 500 μg ml^{-1} (hygromycin under humidified conditions at 37°C, 5% CO_2 and air. The *sst* transfectants of the CHO-K1 cell line were made by transfection of pCIN (Rees *et al*., 1996) containing cloned human *sst* genes (sst_2, sst_4 or sst_5; sst_1 and sst_3 expressing cell lines were not available). Cells expressing high levels of the recombinant receptor were selected with G418 and subcloned by limiting dilution. Stability of receptor expression was checked by radioligand binding (using radiolabeled somatostatin) at regular intervals. The reporter cell line *sst2* # 86.1.3 was constructed by creating a CHO-K1 cell transfectant stably carrying a reporter plasmid with the secreted alkaline phosphatase gene (SPAP) under control of a minimal thymidine kinase promoter which is sensitive to the activity of cyclic AMP responsive elements. Details of the generation and analysis of the cell line and the vector will appear in a separate manuscript (Coote *et al*., in preparation).

Receptor density determinations

The somatostatin receptor content was determined using (3-[^{125}I]iodotyrosyl^{11})-Tyr^{11}-somatostatin (Amersham). Briefly, cells were grown in 6-well plates until near confluent. The monolayer was washed with TMBB buffer (170 mM Tris-HCl, 5 mM $MgCl_2$, 40 μg ml^{-1} bacitracin and 1% BSA, pH 7.4). Incubation was done in duplicate for 30 min at 25°C using 250 μl of a dilution series of labelled peptide ranging from 0.125 nM to 2 nM of ^{125}I-somatostatin in the presence or absence of a 1000 fold excess of unlabelled peptide. The reaction was stopped and cells were washed three times with ice-cold TMBB and once with ice-cold PBS. Cells were harvested by trypsinization and bound radioactivity was determined in a gamma counter. The receptor density and dissociation constant were calculated using Scatchard plot analysis.

Phage ELISA

Phage particles were analysed in ELISA essentially as described (Clackson *et al*., 1991). 96-Well microtiter plates were coated overnight at 4°C with 100 μl of a 1 in 800 dilution in NaHCO3 0.1 M, pH 9.6, of anti-somatostatin serum (30.1.0) made in rabbits by immunization with somatostatin-BSA. All further incubations were done at RT. In between incubations the wells were washed three times with PBS and three times with PBS/0.1% Tween-20. Plates were blocked for 30 min with 100 μl PBS/4% of Marvel. Phage incubation was done for 1 h with 100 μl of freshly prepared phage (10^{11} CFU) diluted in PBS/2% Marvel (2% MPBS). Bound phage particles were detected by incubations at RT with 1 : 500 diluted sheep-anti-fd serum (Pharmacia Biotech; 100 μl/well, 30 min), 1 : 2000 diluted rabbit anti-goat peroxidase (Dako; 100 μl, 30 min) both diluted in 2% MPBS and 100 μl 0.1 mg ml^{-1} 3,3′,5,5′-tetramethylbenzidine (TMB) in 0.11 M Citrate buffer, pH 5.5 with 0.003% hydrogen peroxide. The reaction was stopped after 10 min by adding 100 μl of 1 M H_2SO_4, and the OD_{450} measured.

Whole-cell ELISA

Cells were harvested by trypsinization, washed twice with PBS/10% FCS, sieved with a cell strainer (Falcon) and counted. Cells were spun and resuspended in 2% MPBS. From this suspension, 150 μl, containing 5×10^5 cells, were transferred under agitation to a 96-well plate with v-shaped bottom (Greiner). The plates were left for 30 min on a shaker (Titertek) and subsequently spun at $500 \times g$ for 3 min, followed by careful removal of the supernatant from eight wells at the time with a suction device. The cells were immediately resuspended in 100 μl of phage solution, dissolved in 2% MPBS, by five times carefully pipetting up and down using a multichannel pipette. After 1 h of incubation at RT with shaking, the plates were spun and the cells washed twice with 180 μl PBS per well. Detection of bound phage particles was carried out with 100 μl/well sheep anti-fd serum (Pharmacia Biotech; 1 : 5000 diluted in 2% MPBS; 30 min) and with 100 μl/well peroxidase-conjugated rabbit anti-goat immunoglobulin (Dako; 1 : 2000 diluted in 2% MPBS; 30 min). After the last wash, the cells were immediately transferred to a fresh plate pre incubated with 150 μl 2% MPBS/well. The cells were washed four times by resuspension and spinning. After the last wash the supernatant was removed and the cells were resuspended in 100 μl/well 0.1% TMB in 0.11 M Citrate buffer pH 5.5 with 0.003% hydrogen peroxide. After 45 min the plate was spun at $500 \times g$ for 10 min and the reaction quenched by transferring the supernatants carefully (without transferring the cells) to a new 96-well plate (Falcon), filled with 100 μl/well of 2N H_2SO_4. The OD_{450} was measured.

Flow cytometric analysis of phage binding

5×10^5 CHO-K1 cells or transfectants were harvested by trypsinization, washed with PBS, resuspended in 50 μl PBS/4% Marvel/0.1% BSA/0.1% (w/v) sodium azide (PMBA) and transferred to tubes (Falcon, 2058). All incubations were done at RT; in between incubations the cells were washed twice with 1 ml of PMBA. To the cell suspension 50 μl containing approximately 5×10^{11} CFU of (control or somatostatin displaying) phage were added. In case of IC_{50} determinations phage particles were added with a series of somatostatin dilutions. Incubations with antibodies were carried out at RT for 30 min with 100 μl sheep-anti-fd serum (Pharmacia Biotech; diluted 1 : 500 in PMBA). Cells were washed and subsequently incubated with 100 μl FITC-labelled rabbit-anti-goat immunoglobulin (Dako; 1 : 50 diluted in PMBA). After two final washes with PBS, cells were resuspended in 0.5 ml of PBS and analysed on a FACSort (Becton and Dickinson).

Fluorescence microscopy

CHO-K1 cells and transfectants were stained for microscopical analysis as described above for flow cytometric analysis. After staining the cells were diluted 1 : 10 in PBS/0.1% BSA to 200 μl and spun at 10,000 r.p.m. for 5 min on to gelatine-chromalum coated slides. The cytospins were air dried and covered with 50 μl DABCO/DAPI (0.5 μg ml^{-1} DAPI (Sigma) in 0.02 M Tris/HCl, pH 8.0, containing 0.02% diazobicyclo-octane (Sigma), 0.02% NaN_3, 90% v/v glycerol). For analysis and photography a confocal laser scanning microscope (Zeiss) was used.

Model enrichment

Sst_4 transfected CHO-K1-cells were harvested by trypsinization, resuspended in PBS and 2.5×10^6 cells were transferred to a cryo-vial. Tetracycline resistant fd-phage (CFU = 10^{11}) were mixed with somatostatin displaying phage particles in a ratio of 100 : 1. Cells and phage were combined in 0.5 ml and

incubated while rotating (Mixer 820; Swelab) for 1 h at RT. The cell suspensions were washed 10 times with 3 ml PBS/4% Marvel/0.1% BSA and 2 times with 3 ml PBS/0.1% BSA by centrifugation ($50 \times g$, 5 min) and resuspension. Phage particles were eluted by incubating cells with 666 μl 0.1 M TEA and neutralizing the supernatant with 333 μl 1 M Tris-HCl, pH 7.4. After spinning (14,000 r.p.m., 5 min), phage titers were determined according to (Clackson *et al*. (1991). Alternatively, 0.1 M HCl was used for elution (5 min incubation): cells were resuspended and incubated in 900 μl 0.1 M HCl (5 min); the supernatant was transferred to a new vial and neutralized with 100 μl 4.5 M Tris-HCl, pH 7.4. After centrifugation ($14{,}000 \times g$, 5 min) phage titers were determined.

Oxidizing/reducing SOM-phage

PEG-precipitated SOM-phage was treated with a mixture of 3 mM oxidized glutathione and 0.3 mM reduced glutathione (Sigma), in 50 mM Tris/HCl, pH 8.0 (Jespers *et al*., 1995). After PEG precipitation binding was measured in ELISA with anti-somatostatin serum.

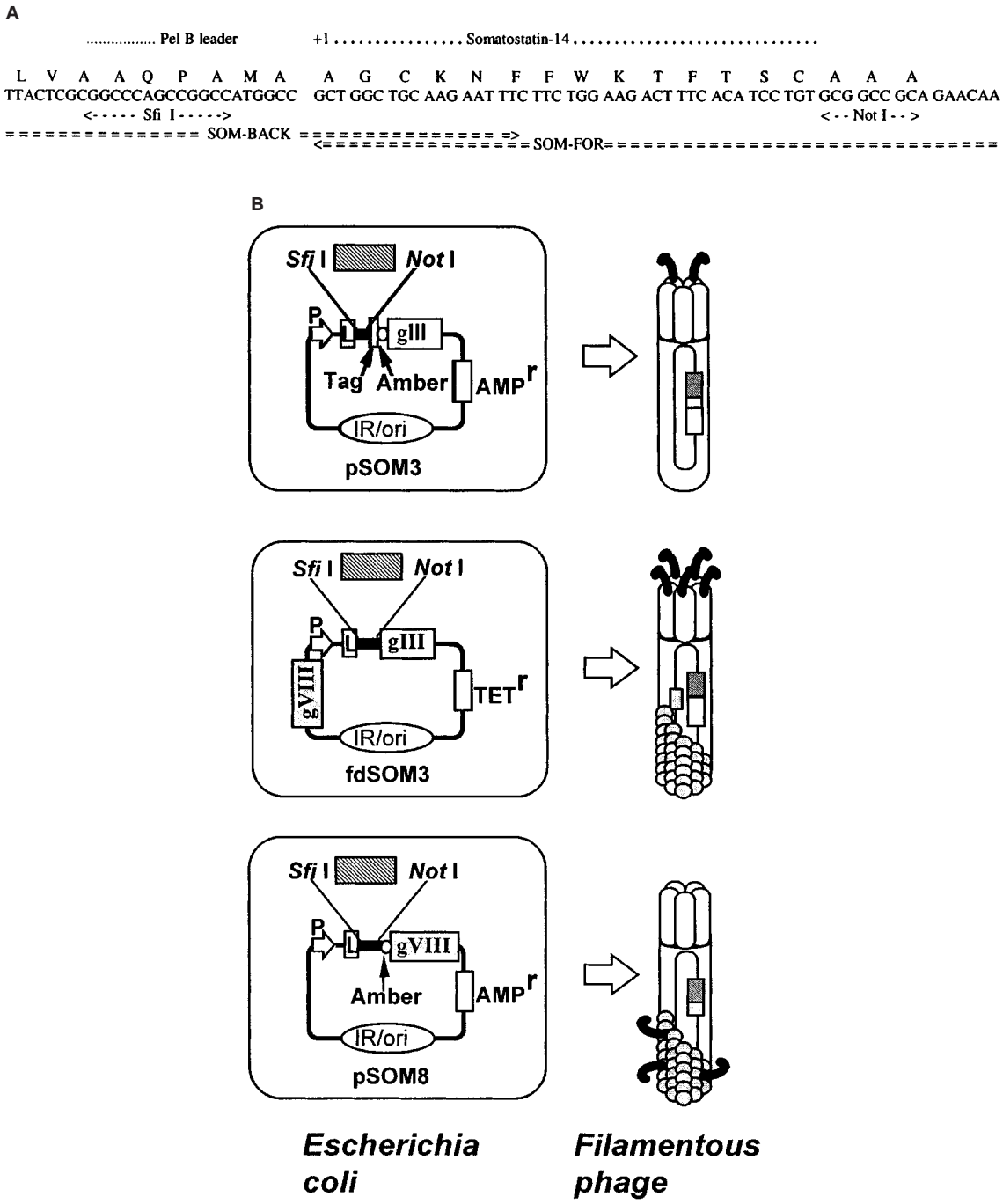

Figure 1 Design and vectors for somatostatin display. (A) The region encoded by the two oligonucleotides used for the somatostatin-14 gene cloning is indicated. After annealing and digestion of the fragment with *Sfi*I and *Not*I the somatostatin fragment was cloned in three different display vectors. (B) After cloning and phage production, somatostatin-14 is expressed as a fusion product on the surface of filamentous phage, anchored via pIII (top, middle) or pVIII (bottom). AMP^r, ampicillin resistance gene (β-lactamase), TET^r, tetracycline resistance gene; IR/ori, Intergenic region and origin or replication; L, Leader or signal sequence; P, *lac*Z promoter; Tag, sequence derived from *myc*, recognized by antibody 9E10.

Receptor reporter assay

CHO cells co-transfected with human sst_2 gene and the SPAP reporter gene were cultured in a 75 cm^2 flask to near confluency. The culture medium was replaced by medium containing 0.1% BSA instead of 10% FCS, and the cells were cultured for an additional 48 h. Cells were harvested, resuspended in 5 ml of medium with 0.1% BSA, and 50 μl aliquots containing 5×10^4 cells/well plated in a 96-well microtiter plate. After 1 h at 37°C, 10 μl of the phage solution or a dilution series of somatostatin-14 was added. After 30 min at 37°C, 10 μl of 70 μM forskolin (Calbiochem, CA) solution was added. Cells were incubated for 6 h at 37°C. Detection of SPAP activity was performed by adding 200 μl of 8 mg ml^{-1} p-Nitrophenylphosphate as the chromogenic substrate in 1 M diethanolamine, 0.28 M NaCl and 5 mM $MgCl_2$. The OD_{405} was measured in a microtiterplate reader (BIORAD, model-450).

Results

Cloning of somatostatin-14 for display on filamentous phage

For the display of somatostatin on the surface of filamentous phage, we have tested a variety of display vectors. Display of peptides may be achieved by fusion to the minor (pIII, pVI) or major coat proteins (pVIII) of filamentous phage such as fd or M13. Somatostatin has two bioactive forms: a 14-mer form, comprising a tetra-peptide loop constrained by a disulphide bridge and a longer version, a 28-mer peptide, containing an additional 14 residues. The receptor binding region of somatostatin is formed by four residues at the tip of the loop, leaving, in principle, both N-and C-termini of the peptide available for fusion to phage coat proteins. We decided to test the display of active somatostatin (the somatostatin-14 version) *via* fusion of the C-terminus to the phage coat, thus by fusion to pIII or pVIII. A somatostatin 14-mer encoded by a pair of overlapping synthetic oligonucleotides was extended, amplified by PCR and cloned either directly or indirectly into three different phage display vectors (Figure 1A,B). The cloning resulted in an in-frame fusion product between somatostatin-14 and the phage coat protein of choice. For display of somatostatin on the minor coat protein pIII, both phagemid vector pCANTAB6 and phage vector fd-tet-SN were used. In the latter case no helper phage is required to produce phage particles, and the somatostatin-pIII fusion product therefore is not competing with the pIII of the helper phage, effectively increasing the display efficiency (Hoogenboom *et al*., 1991). Due to the length of somatostatin, display on the major coat protein pVIII. on the other hand, appeared most feasible with a phagemid vector that allows incorporation of mainly wild-type pVIII as well as some fusion product; we have used pHEN8 (Jacobsson & Frykberg, 1996). The three display systems that were used, with their resulting fusion products, are summarized in Figure 1B. In all cases there is a secretion signal preceding the somatostatin sequence; upon cleavage by signal peptidases the matured fusion peptide should start with the first residue of somatostatin-14. The cloned DNA fragments were sequenced to confirm the correct insertion of the oligonucleotide pair. This cloning resulted in pCANTAB6-Somatostatin-14 (pSOM3), fd-tet-SN-Somatostatin-14 (fd-SOM3) and pHEN8-Somatostatin-14, (pSOM8).

Efficiency of display and effect on infectivity

In Western blot analysis the display efficiency of the phage particles from these three vectors was measured, using anti-pIII, anti-myc and anti-somatostatin antibodies (Figure 2). The data show that very efficient display can be achieved of somatostatin *via* pIII fusion: approximately 50% of the pIII-reactive product is the somatostatin fusion protein (Figure 2A). Although by the cloning of the somatostatin-14 gene into the display vector we do not expect a detectable change in molecular weight, the somatostatin-14 containing product is the one with a higher apparent molecular weight, as indicated by the Western blot with the anti-somatostatin serum (Figure 2A). This apparent size difference is most likely caused by the unusual behaviour on SDS-PAGE of pIII and its fusions (McCafferty *et al*., 1990). For fd-tet-SN and fd-SOM3, as predicted no change in the molecular weight of the 'wild type'

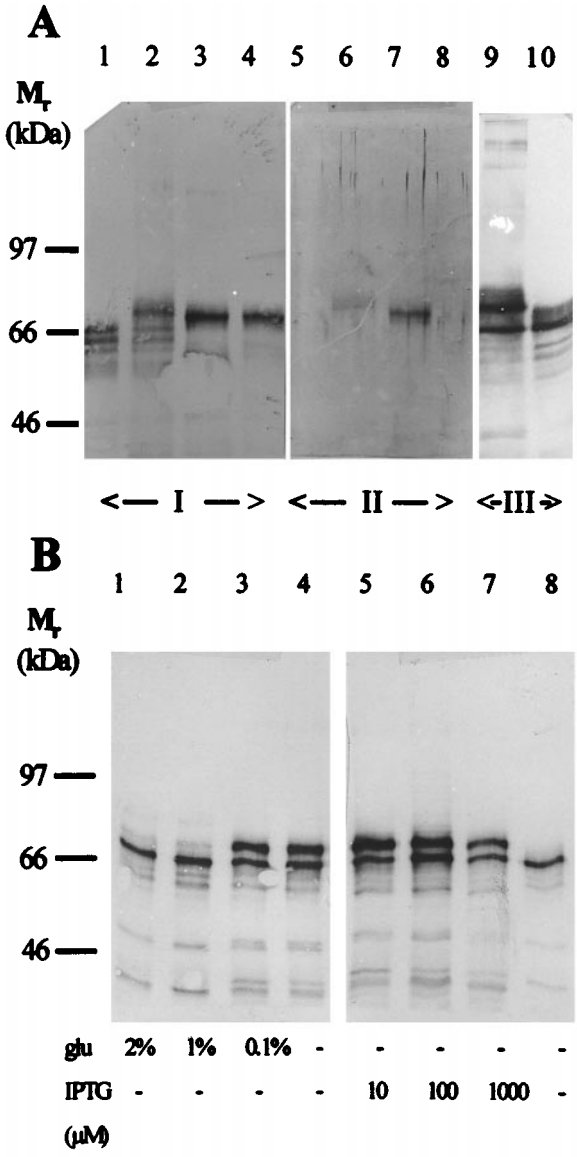

Figure 2 Western blot analysis of phage displaying somatostatin. (A) Display efficiency of phage derived from pSOM3 and fd-SOM3. Antibodies used: I. anti-pIII antibody (1 = pCANTAB6, 2 = pSOM3, 3 = fd-SOM3, 4 = fd-tet-SN); II. anti-SOM serum (5 = pCANTAB6, 6 = pSOM3, 7 = fd-SOM3, 8 = fd-tet-SN); and III. anti-*myc* tag antibody 9E10 (9 = pSOM3, 10 = pCANTAG6). (B) Influence of IPTG and glucose on the ratio of pIII versus fusion pIII of pSOM3 (lanes 1 to 7) and pCANTAB6 (lane 8) (detection as in (A).III).

pIII versus the somatostatin fusion is visible. Tinkering with the expression level of SOM-pIII may modulate the display efficiency; we therefore used glucose, the catabolic repressor of the *lac* promoter, and IPTG, its inducer, to suppress or induce respectively the expression of the somatostatin-pIII fusion product. No further increase in the ratio of fusion-p3 to wild-type p3 was noted by IPTG induction (Figure 2B); only a decrease in total phage yield was visible (most likely due to the toxicity of the pIII). Even under partial catabolic repression (1% glucose in the overnight culture), display is visible. Western blot of the fd-SOM3 derived phage showed an even higher display efficiency: now the majority of the pIII was fusion product, as expected (Figure 2A). Western blot with anti-pVIII and anti-somatostatin sera to detect display of phage derived from the pSOM8 version was not successful (results not shown), most likely because the number of pVIII fusion products is very small compared to the approximate 2700 copies of wild type protein per phage particle. The consequence is that the fusion product is obscured in Western blot. Other binding tests confirmed the display of somatostatin on these phage particles (see below).

The infectivity per particle of the different fusion phage was compared by calculating the number of physical particles per CFU using the formula described Yu & Smith (1996). As expected, only a fraction (20–30%) of the physical phage particles infects bacteria; there is no significant difference between the different fusion phage tested (results not shown). Display of somatostatin appears not to affect the infectivity of the phage.

Activity of somatostatin-phage: SOM-phage bind specifically to anti-somatostatin serum and to native sst expressed on cells

Somatostatin on phage(mid) particles is recognized by anti-somatostatin serum in ELISA (Figure 3). The aspecific background for somatostatin displayed on the phage particles for pSOM3 was consistently higher than for the fd-tet-SN phage-displayed version as measured in ELISA using BSA as coated antigen. However in all cases the majority of binding was competed off by soluble somatostatin to a level comparable to that obtained for wt-phage. Total binding of somatostatin displaying phage was determined using the same number of phage particles and proved to be equal for phage derived from fd-SOM3 and pSOM3, but lower in comparison to phage derived from pSOM8 (Figure 3). Avidity differences between phage of fd-SOM3 and pSOM3 are not seen, most likely because the display efficiency is sufficiently high to yield avid phage (with more than one copy of somatostatin-pIII) in both cases.

Binding to anti-somatostatin sera does not necessarily implicate binding to the native receptor of the hormone. Therefore, binding of phage was tested on CHO-cells

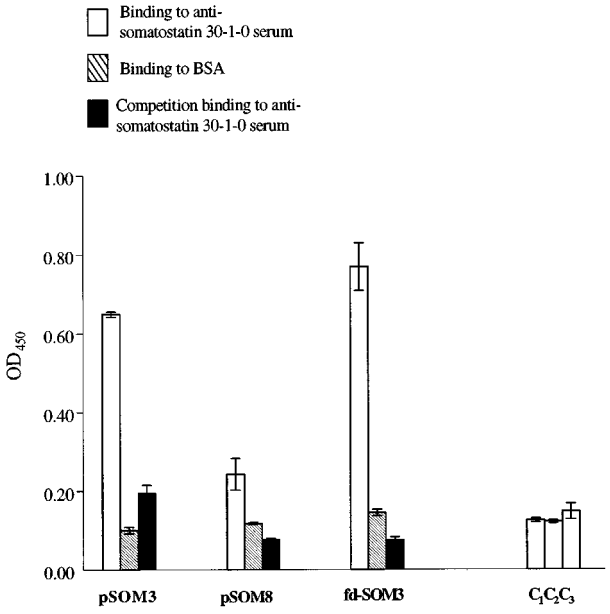

Figure 3 Binding of SOM-phage in ELISA. Phage were tested for binding to the immobilized anti-somatostatin polyclonal 30-1-0 serum and to BSA; binding to anti-SOM was also tested while competing with 1 μM somatostatin-14. ELISA results of corresponding control phage, pCANTAB6 (C1), pHEN8 (C2) and fd-tet-SN (C3) are shown.

Table 1 OD_{450} in whole CHO-K1 cell ELISA of SOM-phage[a]

Phage derived from	*CHO-K1*	*CHO-K1* sst_4	*CFU/well* ($\times 10^{11}$)
pCANTAB6	0.145	0.099	75
pSOM3	0.314	1.789	46
pSOM8	0.212	2.112	30
fd-tet-SN	0.112	0.073	16
fd-SOM3	0.215	0.882	19
no phage	0.073	0.071	–

[a]Number of cells/well is 0.9×10^6 for CHO-K1 and 1.2×10^6 for CHO-K1-*sst*$_4$.

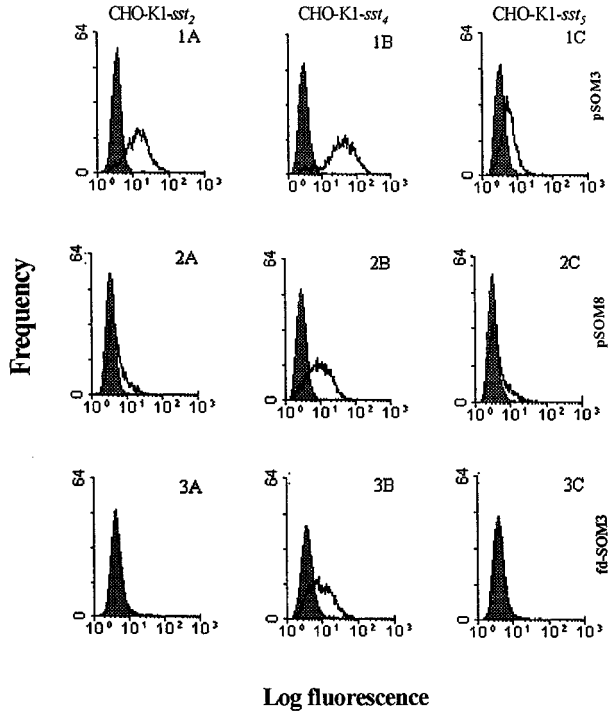

Figure 4 FACS analysis of SOM-phage binding. Flow cytometric analysis of CHO-K1 cells transfected with *sst*$_2$ (A), *sst*$_4$ (B) and *sst*$_5$ (C) receptor subtype genes by incubation with pSOM3 (1), pSOM8 (2) and fd-SOM3 (3), with/without (shaded/non-shaded) addition of 1 μM somatostatin-14. Bound phage particles incubated with cells (A–C) were detected with anti-fd antibodies and a sheep anti-fd FITC conjugate. Specificity was confirmed by competition with an excess of free somatostatin.

transfected with sst_2, sst_4 or sst_5, expressing high levels of receptor. Receptor densities were determined to be 200,000 binding sites/cell for the sst_2 and sst_4 transfectants and 150,000 for sst_5. The results depicted in Table 1 show that somatostatin displaying phage specifically binds cells expressing *sst*. In contrast, vector-derived phage not displaying the hormone, pCANTAB6, pHEN8, and fd-tet-SN, do not bind these cells at all. Binding is specific, because the addition of an excess of somatostatin abolishes binding completely.

In the second binding assay, flow cytometry utilizing anti-fd serum and FITC-conjugates, binding of pSOM3 phage to all transfectants was confirmed (Figure 4). Phage derived from fd-SOM3 and pSOM8 detect somatostatin receptors expressed by *sst* transfected CHO-cells; these receptors all possess high affinity molar dissociation constants for free somatostatin as measured in receptor density determinations. Two different test systems were used: a whole cell ELISA and a flow cytometric assay. In the whole cell ELISA, transfectants were incubated with phage and bound particles detected with receptor present on the sst_4 transfectant. Receptors on sst_2 and sst_5 transfectants were also detected by pSOM8 phage, to a lesser extent, but not by fd-SOM3 phage. The specificity of binding was confirmed by competition with 1 μM soluble somatostatin (Figure 4). As in the whole cell ELISA, phage particles not displaying the hormone do not bind to the cells. Dilution of the number of input phage gave a direct decrease of the number of positive cells in FACS (Figure 5A) and in OD^{450} in the whole cell ELISA (Figure 5B), thus the limiting factor for binding is not the number of receptors, but the number of phage.

A titration with competing somatostatin yielded an IC50 for sst_4 of approximately 15 nM for pSOM3 and 30 nM for pSOM8 phage (Figure 6). These values are 20 to 100 fold lower than the reported K_d values for free somatostatin. This means that the pIII-fusion appears to have reduced the affinity for the receptor, without loss of specificity of binding. When taking into account that approximately 50% of the pIII protein is fused to somatostatin (Figure 2A) in the presence of five copies of pIII per phage, statistically 87% of the pSOM3 phage carry two or more copies of somatostatin. Since phage with avid binding may be bound 10 to 100 fold stronger than phage with monovalent interaction, the indicated values should not directly be compared with absolute affinity constants for the free somatostatin-*sst* interaction. A competition experiment using free somatostatin to compete off pSOM3 derived phage already bound to sst_4 transfected CHO-cells, shows a different 'affinity': in FACS a slight reduction (from 98 to 91%) was noted after 80 min competition with 1 μM

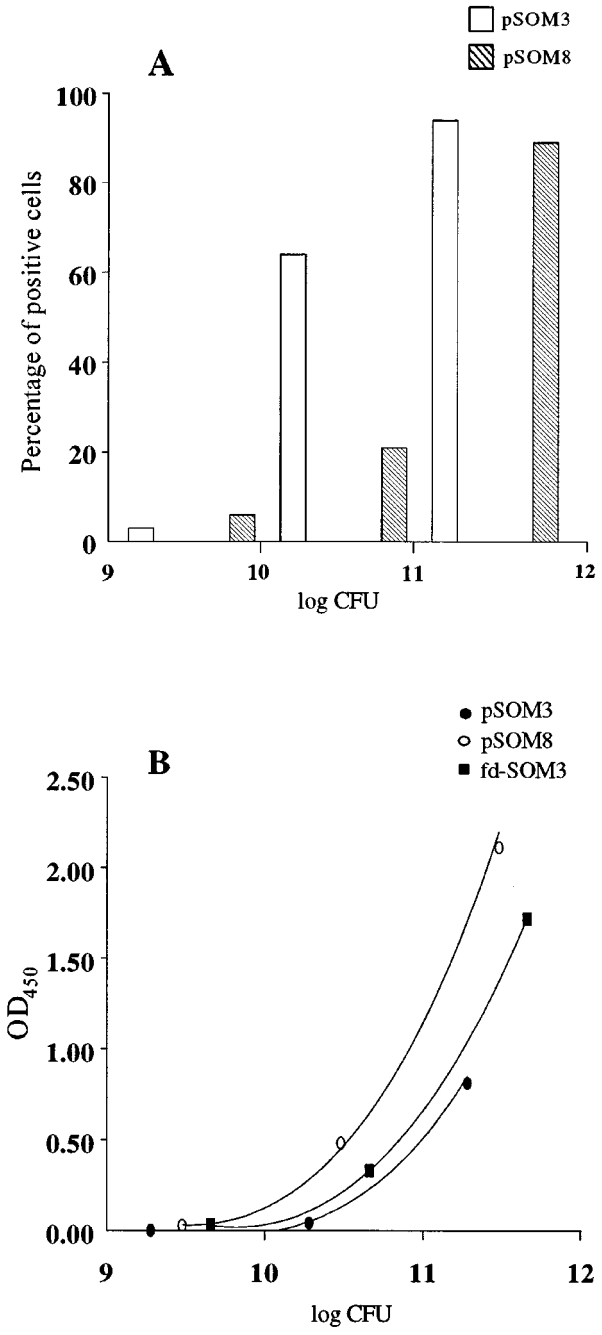

Figure 5 Binding versus phage titre. (A) Percentage of positive CHO-K1-sst_4 cells in flow cytometry versus the number of SOM-phage (log CFU); pSOM3-phage and pSOM8-phage. (B) OD_{450} in anti-SOM ELISA versus the number of SOM-phage; pSOM3-phage and pSOM8-phage; fd-SOM3 (black).

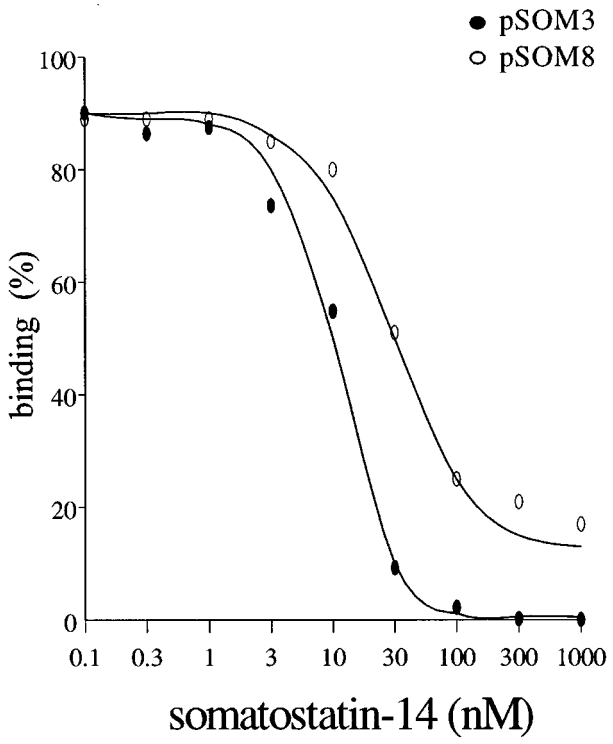

Figure 6 IC_{50} values for binding of SOM-phage to sst_4 determined by flow cytometry. Competition for binding to CHO-K1-sst_4 cells of pSOM3-phage or pSOM8-phage with free somatostatin-14.

somatostatin, suggesting that the pSOM3 derived phage *sst* interaction either shows a very slow dissociation rate, or that avidity binding or post-receptor binding events indeed stabilize the interaction.

Further binding to sst_4 transfectants was visualized using fluorescence microscopy (Figure 7). This revealed a pattern of intense dots, between a few and 20–30 per cell. Similar fluorescence pictures were obtained when staining lymphocytes with other phage antibodies (anti-CD25; anti-CD30; unpublished work). The total amount of receptors per cell was found to be in the 10^5 range, thus either the receptors reside in a few concentrated clusters, or, if one dot is one phage, only very few phage were bound per cell.

It was unclear why such a low amount of phage bind to the receptor expressing cells, taking into account that a 25 fold excess of phage particles was added compared to 2×10^5 receptor binding sites/cell. Possibly the somatostatin binding site on the native receptor is not easily accessible by the phage particles, due to glycosylation of the extracellular N-terminal region of the receptor, causing steric hindrance for somatos-

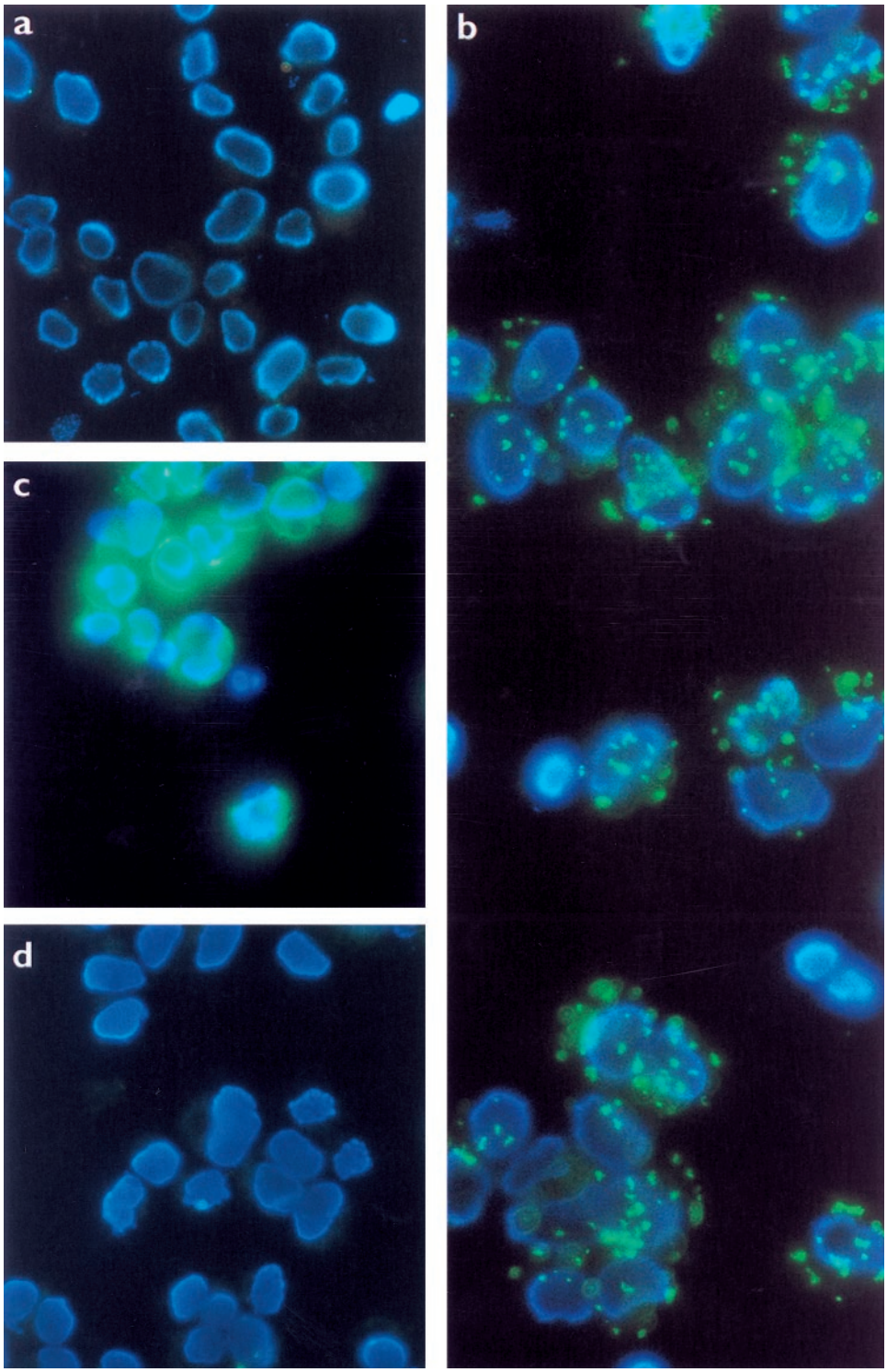

Figure 7 Fluorescence microscopy of SOM-phage. Binding of pSOM3 phage to CHO-K1 cells (a), to CHO-K1-sst_4 cells (b), and to CHO-K1-sst_4 cells with competition of 1 μM somatostatin (d). (c) represents binding of a non relevant but specific CHO-K1 binding phage antibody for comparison. Nuclear counter staining with DAPI; magnification: 400×.

Table 2 Model enrichment of SOM-phage on cells[a]

Input phage (CFU per ml)	*Number of wash steps*	*Enrichment factor*	*Recovery for pSOM (% of input)*
fd-tet-SN:2.4×10^{11} pSOM3:8.8×10^{9}	20	108	0.0009
fd-tet-SN:2.5×10^{11} pSOM8:8.4×10^{9}	20	106	0.0006
fd-tet-SN:9.2×10^{10} pSOM3:1.4×10^{9}	12	52	0.003
fd-tet-SN:4.9×10^{10} pSOM8:3.8×10^{9}	12	104	0.002

[a]Phage were mixed, panned on cells and the cells washed; bound phage were eluted with 0.1 M TEA and titrated on ampicillin or tetracycline containing plates, to determine the ratio of pSOM over control fd-tet-SN phage.

tatin phage but not for free hormone. Indeed, the receptors appear to be N-glycosylated (Reisine *et al*., 1992). Differential glycosylation could lead to a fraction of the receptors to be accessible only. This was tested by incubating sst_2 and sst_4 CHO-cells with tunicamycin for 24 and 48 h, and measuring the percentage of pSOM3 or pSOM8 phage bound to the cells. As a control the total receptor expression level was tested with radiolabelled somatostatin. Phage binding increased approximately 2 fold, with a constant total receptor expression level (results not shown), indicating that overall accessibility of the receptor for SOM-phage appeared to have increased only minimally.

Finally, a reason for poor display quality may be a lack of correctly formed disulphide-bridges. Somatostatin is a cyclic disulphide-bridged hormone, in which the S-S bridge constraint is needed to establish a high affinity interaction with its receptors. To test whether mis-matching/non-bridging of the SH-groups of the SOM-cysteines was reducing the percentage of active pSOM-3 phage, phage were treated with a mixture of reduced/oxidized glutathione and tested in ELISA for binding to anti-SOM serum. No significant improvement in binding was found when comparing untreated phage (OD_{450} of 0.47) with treated phage (OD_{450} of 0.45); controls included phage treated with DTT (OD_{450} of 0.28) and DTT and glutathione (OD_{450} of 0.38). The polyclonal serum might recognize linear as well as S-S bridged somatostatin; therefore, these phage were tested in flow cytometric analysis with sst_4 cells. Only minor shifts in binding were noted (results not shown).

Model enrichment studies

The specific but low quantity of binding of these phage prompted us to perform enrichment studies on cells expressing the receptors. This would answer queries about whether SOM-phage can be useful to allow the development and improvement of cell selection procedures of peptide phage from display libraries. We tested enrichment of somatostatin phage (pSOM3 and pSOM8) over appropriate control phage (fd-tet-SN) by biopanning on sst_4 transfected CHO-K1 cells. These cells were found to be excellent carriers of functional receptors, and to be relatively resistant to the experimental protocol of phage panning using detached cells in suspension. The use of control phage with an another antibiotic resistance marker than their somatostatin displaying counterparts allows the determination of the enrichment factor from the ratio of ampicillin and tetracycline resistant bacteria after infection and plating. From the data in Table 2 it is apparent that only a small fraction of the input SOM-phage is recovered ($<0.003\%$). Enrichment for the specific phage, is significant: a factor of 50–100 in one round of selection. Similar results were obtained using elution with 0.1 M HCl, which, unlike the triethylamine used in the first experiment, does not lead to cell lysis (data not shown). For comparison, in a similar model enrichment experiment on cells, with a phage scFv antibody with similar affinity (10 nM) and antigen density (10^5 copies per cell), up to 3–6% of the input population was retrieved, with enrichments of 10^4 (Roovers and Hoogenboom, in preparation). By comparing the recovery for SOM-phage with those for the antibody-phage, the active fraction would be approximately 100 fold below the level found for the cited antibody phage. This was independent of the type of display, as pSOM3 and pSOM8 behave similarly.

Somatostatin-phage act as agonists

Despite the low efficiency, pSOM3-derived phage can specifically bind to three out of three tested receptors in a highly specific way. To examine whether the SOM-phage can also trigger the receptor's natural signal transduction and act as agonists, we have used a reporter cell system made from CHO-K1 cells transfected with sst_2 and a reporter gene driven off a CRE-sensitive promoter. The principle is that adenylate cyclase activity (*via* protein G coupling) is inhibited after binding of somatostatin or SOM-phage to its receptor. This results in reduction of cyclic AMP levels, which ultimately leads to less cyclic AMP response element binding protein (CREB). After binding of CREB to the CRE domain in the genome, a reduction of secereted alkaline phosphatase activity (SPAP), *via* a CRE-sensitive promoter, is observed (Coote *et al*., in preparation). The use of forskolin as an activator of adenylate cyclase allows the detection of SOM-phage or somatostatin mediated inhibition. A clear concentration related agonistic effect of both pSOM3 and pSOM8 derived phage compared to control phage pCANTAB6 and pHEN8 respectively, is observed (Figure 8), indicating that phage particles can be used to trigger receptor-ligand interactions. As expected, this effect was still visible after heat inactivation carried out just before substrate was added; indeed, this step denatures endogenous alkaline phosphatases, but leaves the activity of the heat-resistant SPAP unaffected (results not shown). Further, SOM-phage particles did not have any effect on SPAP levels when forskolin was omitted (not shown). For pSOM3 derived phage this effect has an IC^{50} slightly higher than the measured 'affinity' of the sst_4 receptor interaction, between 1–3 nM; for pSOM8 phage the effect is not as pronounced, but overall binding to sst_2 was found to be reduced when compared to sst_4 in FACS analysis as well (Figure 4). The specificity of the receptor triggering was confirmed by replacing forskolin with salmon calcitonin, which will trigger adenylyl cyclase levels *via* the endogeneous salmon calcitonin receptor present on all CHO-K1 cells, independent of somatostatin (Coote *et al*., in preparation). Indeed, SOM-

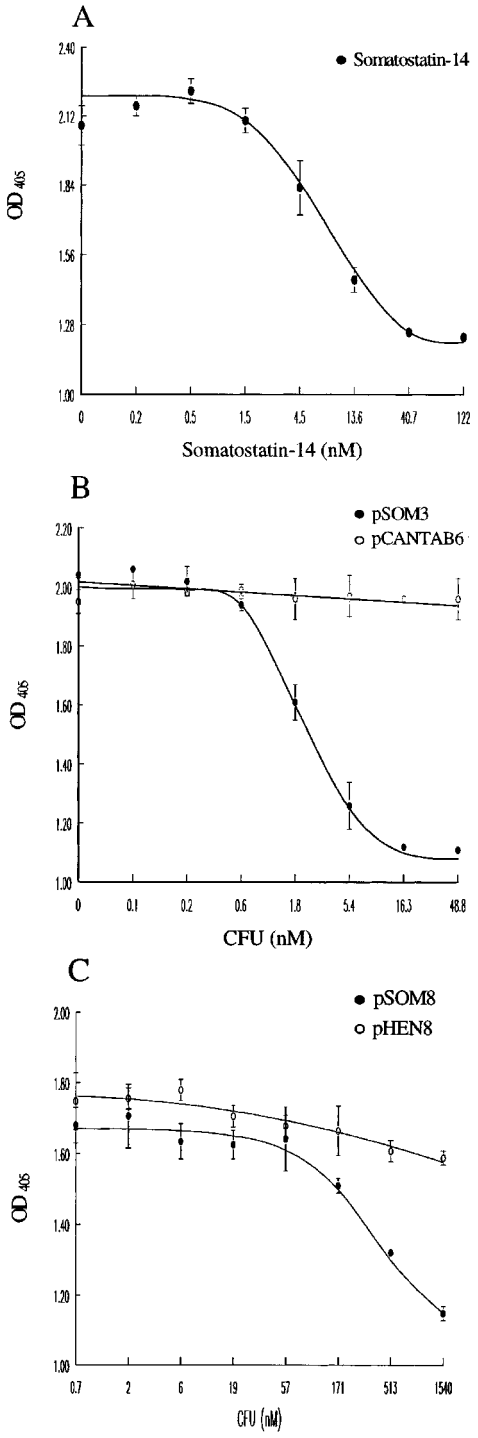

Figure 8 Reporter assay. Inhibition of SPAP activity in CHO-K1-*sst*$_2$ cells, by somatostatin-14 (A), by pSOM3 and pCANTAB6 (B) or by pSOM8 and pHEN8 (C).

phage did not at all affect SPAP levels in cells stimulated with calcitonin (not shown), showing that the effect is not due to phage-mediated toxicity or non-specific binding. Finally, the fraction of SOM-phage that did not bind to the reporter cells was negative in the reporter assay. The SOM-phage preparation therefore appears to contain a small subset of phage that bind with high specificity and reasonable affinity to *sst*, and trigger reduction of intracellular cyclic AMP levels. Of the many applications that have now been described with peptide phage libraries, this is one of the first demonstrations of a direct 7-TM-receptor-triggering peptide-phage.

Discussion

Our data prove that phage carrying peptide ligands can access, bind and trigger G-protein coupled 7-TM receptors in a specific manner. We have made three different phage particles displaying somatostatin-14 which specifically bind all three tested receptors of the hormone, *sst*$_2$, *sst*$_4$ and *sst*$_5$. Binding to *sst*$_1$ and *sst*$_3$ was not tested, but may be expected, because *sst*$_1$ is in sequence and ligand selectivity is closely related to *sst*$_4$, while *sst*$_3$ is part of the *sst*$_{2/3/5}$ subfamily. Bound phage particles are detected by immunofluorescence as discrete dots on the cell surface of CHO-K1 cells with 150,000–200,000 copies of *sst*$_2$, *sst*$_4$ or *sst*$_5$. This dot-like pattern is most likely not caused by incomplete binding of phage, owing to low concentration of phage displayed somatostatin. The IC50 of SOM on phage for *sst*$_4$ is in the 15 nM range, which is close to the receptor concentration expressed on *sst* transfected CHO-K1 cells (calculated to be in the range of 1–10 nM). Whether this binding pattern correlates with the clustering of receptors remains to be determined.

The binding specificity was confirmed by whole cell ELISA and FACS. These assays indicate that there is a clear difference in how SOM-phage are bound by different receptors. All three forms of the SOM-phage bind to *sst*$_4$. pSOM3 phage also bind to the other two receptors subtypes, whereas pSOM8 phage bind only weakly to *sst*$_2$ and *sst*$_5$ expressing CHO-K1 cells. No detectable binding for fd-SOM3 particles to the latter two receptor subtypes is observed. Since the effects of format and thus avidity on *sst*$_4$ binding are marginal, the differences between the binding of these phage to the other receptors most likely is caused by a difference in linker between somatostatin and pIII. Indeed, both fd-SOM3 and pHEN8 carry a short linker between somatostatin and pIII (of just a few amino acids), while pSOM3 incorporates a stretch of over 25 amino acids. This short linker may interfere with binding of somatostatin to *sst*$_{2/5}$, possibly caused in part by steric hindrance due to N-terminal glycosylation of these receptors.

So far, *sst* are visualized using radio nuclide-coupled somatostatin analogues (23, 24). It is not clear yet whether SOM-phage can be used to detect the receptor in human cells or tissue, and when it is expressed at lower levels (approximately 2,000–20,000 copies per cell). The large size of the phage particles excludes their *in vivo* use, but the potentially huge amplification factor may be interesting for *in vitro* diagnosis. Experiments to establish whether we can use the phage to detect *sst* on tumor cell lines are underway; phage themselves may thus prove to be valuable receptor specific probes.

The total display efficiency of somatostatin is high (approximately 50% of the pIII product is a peptide fusion) and cannot easily be regulated by manipulating the concentration of promoter-inducer. The control of the display level would help in defining the degree of avidity in function of the application, without having to clone the ligand in different display vectors (Livnah *et al*., 1996). Our data indicate that phagemid display should not be considered close to a monovalent display, as this appears ligand-dependent. Thus phagemid display does not guarantee monovalent display (Lowman *et al*., 1991; Wright *et al*., 1995). When a reduction of display efficiency is required, further manipulation of the regulation of the *lac* promoter may be attempted (Krebber *et al*., 1997), or more tightly regulated promoters used (Lutz & Bujard, 1997), an amber codon or protease sensitive site may be introduced between the displayed ligand and geneIII, or alternative display systems which are less efficient, such as those based on pVI (Jespers *et al*., 1995), may be considered.

From our experiments it appears that only a small fraction of SOM-phage binds to cells; the majority appears to be not active. We carefully examined which factor may cause this poor quality of display, but as yet cannot highlight its exact cause. We are not able to drastically improve the quality of the displayed somatostatin by *in vitro* formation of the disulphide bridge. The accessibility of the receptors by deglycosylation did not dramatically enhance binding. We cannot completely exclude fusion protein processing problems by the *E. coli* signal peptidase; the differences in pIII-SOM and wt-pIII fusion products seen in Western blots are larger than expected, but only for pSOM3 and pSOM8, not for fd-SOM3. This has also been noted by others who examined peptide-pIII fusions (Szardengings *et al*., 1997). Although three different signal sequences were used in the three different SOM-phage, the carboxy terminal end of the signal sequence ('Pro-Ala-Met-Ala') is identical and follows the consensus for signal peptidase cleavage sites. The first residue of the mature somatostatin is an Alanine; although this is not the best residue at the +1 position for cleavage (ideally this should be a charged residue), it has been used successfully by others for display of somatostatin on phage (Wright *et al*., 1995).

Presumably as a result of the small percentage of active somatostatin-displaying phage, the enrichment on sst_4 transfectants was 100 fold per round of panning at best, which is 100 fold lower than what is found for phage antibodies with a similar binding affinity and target antigen density. Avidity did not seem to play a major role in the enrichment: display via pVIII fusion or in phage versus phagemid did not affect enrichment nor yield. These enrichment factors are similar to what has been reported for panning of naïve antibody libraries on purified antigens (Marks *et al*., 1991), and better than what was achieved by panning of somatostatin-displaying phage on anti-somatostatin serum (Krebber *et al*., 1997). Thus receptor-specific peptides may be isolated by cell-panning phage peptide libraries using the conditions presented herein.

Somatostatin induces multiple cellular responses and individual somatostatin receptors are capable of coupling to several effector systems. We report that the SOM-phage themselves act as agonists in a sensitive reporter cell assay by lowering the cyclic AMP concentration via reducing adenylyl cyclase activity. It remains to be seen whether other sst_2-mediated signal transduction pathways are similarly triggered. May the reporter assay system be used for detecting quantitative differences between phage clones, and for a direct comparison with the effect of the original hormone? The IC_{50}'s of pSOM3 and pSOM8 derived phage in this assay indeed follow the trend seen in binding to sst_2 in FACS (Figure 4); also, the IC_{50} for the pSOM3 derived phage is similar to the 'affinity' for the sst_4. However, for a direct quantitative comparison in the reporter assay system, (i) the phage concentrations need to be adjusted for the number of physical phage particles, (ii) the number of somatostatin molecules per phage involved in the receptor interaction needs to be established, and (iii) the fraction of phage binding to the receptor has to be measured. Since these parameters will have to be determined for each new phage clone independently, a quantitative comparison cannot be made easily. Most importantly, the fate of the bound phage may be very different from that of the natural ligand. After binding free somatostatin, sst_2 has recently been reported to be desensitized, internalized and phosphorylated (Hipkin *et al*., 1997). It has also been suggested that in some cell lines ligand binding would protect the receptor from degradation, which would increase its half life (Schonbrunn *et al*., 1995). The IC_{50} for the phage-mediated agonistic effect is approximately 10 nM (calculated on the basis of the titre of infectious phage), corresponding to the 2×10^6 phage particles present per cell. This compares with 10^5 molecules (per cell) of somatostatin at the IC50 value (0.5 nM, Figure 8). However, taking into account that, at the phage concentration used at the IC_{50} point, only 10–100 phage particles per cell are visible in immunofluorescence, it may well be that phage binding allows multiple triggering, leading to a thousand fold amplification of the G-protein mediated cyclic AMP reduction. It remains to be determined whether the phage-mediated effect is caused by preventing receptor internalization, and/or phosphorylation, thus desensitization of sst_2.

In summary we have demonstrated that bacteriophage displaying peptides can specifically bind and trigger G-protein coupled 7-TM receptors. It should now be feasible to isolate receptor-specific peptides from phage libraries, using panning on cells that overexpress the target receptor. This could be done using linear or cyclic random peptide libraries, in which case cell sorting or subtraction methods need to be applied to remove peptides binding to irrelevant antigens. Alternatively, if it is known which residues of the ligand interact with the receptor, the peptide library may be designed to retain those residues, and randomize the neighbouring residues. Such libraries are expected to have a propensity to bind the original receptor, reducing the need for differential selection (Szardengings *et al*., 1997). Our finding of the agonist phage pave the way for developing high throughput screening assays for discriminating inhibitors (antagonists) from activators (agonists) of receptor binding peptide or antibody-phage, selected *via* bio-panning phage libraries on cells.

As a consequence of the phage acting as receptor-specific agonist, using phage directly for functional analysis may reduce extensive chemical synthesis of selected peptide variants. After selection for binding, individual peptides phage may be screened for receptor triggering effects, to separate, in the selected population, agonists from antagonists from irrelevant peptides. The applicability of this screening method depends on the nature of the ligand and receptor accessibility, and the method of triggering of the receptor. Phage has been reported to be able to directly trigger receptor dimerisation, for example with phage carrying Epidermal Growth Factor (Souriau *et al*., 1997). To date only one other study has addressed phage-mediated triggering of 7-TM receptors (Szardengings *et al*., 1997): in this elegant study the melanocortin receptor was triggered using one of the receptor's natural ligands displayed on phage.

In the functional screening assay, we have chosen to detect secreted alkaline phosphatase. In the future more sensitive reporter systems could be used, for example using luciferase (Souriau *et al*., 1997) or Green Fluorescent Protein as reporter genes. With such potentially very sensitive reporter systems, it may eventually be possible to sort cells which carry an a(nta)gonistic phage particle. Such sorting procedure could allow the direct selection of phage particles with agonist or antagonist activity for a given receptor directly from the phage library. With new reporter genes and sensitive fluorescent read out methods under development (Broach & Thorner, 1996), we envisage that such 'functional selection' schemes will be useful tools for drug discovery. Finally, this method may be used for identifying peptide ligands for orphan receptors (such as the many related opioid receptors), for which a function but not a natural ligand is known.

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