Probing Biomolecular Interactions of Glutathione Transferase M2-2 by using Peptide Phage Display

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The interactions between biomolecules and human glutathione transferase M2-2 (GST M2-2) were probed by using 9- and 15-mer combinatorial peptide libraries displayed on phage. The peptide libraries were based on random DNA sequences fused to gIII, *a gene that expresses a phage coat protein and thus causes the peptides to be displayed on the surface of phage particles. A peptide sequence was enriched through binding to GST M2-2, which indicated a successful selection. Binding studies with the peptide displayed on phage showed binding specificity. The sequence of the peptide had similarities to segments of proteins in the Swiss-Prot Database, to c-Jun N-terminal kinase (JNK), and to the protein Bcl3. JNK is linked to the regulation of the transcription factor AP-1. Use of cell-based assays of the transcriptional activity of AP-1 allowed a novel coactivation function of GST M2-2 to be demonstrated. Specificity in the activation was indicated by the lack of effect of GST A1-1. No coactivator function of GST M2-2 could be demonstrated in assays with Bcl3. These results suggest that GST M2-2 has biological roles in addition to catalysis of detoxication reactions, and demonstrate the potential of phage display in functional genomics research.*

KEYWORDS:

combinatorial chemistry · enzymes · peptides · phage display · transferases

Introduction

Glutathione transferases

Glutathione transferases (GSTs) are a family of enzymes with important biological functions. GSTs are believed to play a major role in cellular defense against a variety of endogenous and xenobiotic electrophilic toxins, which are mutagenic and cause cancer and several other degenerative diseases. The enzymes catalyze the conjugation of the tripeptide glutathione (GSH, γ-Glu-Cys-Gly) to these harmful compounds, a reaction that usually makes the toxins chemically unreactive and water soluble.[1] The mammalian soluble GSTs are divided into classes[2] named Alpha, Kappa, Mu, Pi, Omega, Sigma, Theta, and Zeta based on their primary structures.[2–4] Three-dimensional structures of members of each class have been determined.[3] The soluble GSTs exist as dimers, and each subunit provides an active site with determinants for binding GSH (G-site) and the hydrophobic electrophiles (H-site). The expression levels of the different GSTs are tissue specific. In adult human liver, 80% of the total GST proteins are Alpha class members, while the Pi-class enzyme GST P1-1 is present in essentially all tissues except the liver. GST P1-1 is also a major protein expressed in many tumor cells.[5–8]

Beside the established enzymatic function, it has been suggested that GSTs may act as the intracellular binding protein, "ligandin", in a manner similar to albumin in blood plasma.[9–10] GSTs bind noncovalently poorly water-soluble, hydrophobic compounds such as bilirubin, bile salts, steroids, heme, fatty acids, and so on. There is evidence for a third binding pocket between the subunits,[11–12] which, in addition to the two active sites, could be a binding site for nonsubstrate ligands. Binding outside the active site may occur for rat GST M1-1 (GST Yb1), which has been shown to bind both a cyclolinopeptide A analogue and a linear rennin-inhibiting peptide[13] without affecting the GST activity measured with the most commonly used substrate, 1-chloro-2,4-dinitrobenzene. It was also reported that the uptake of bile acids in isolated liver cells was competitively inhibited by both the cyclic and the linear peptide.

In the present investigation the binding properties of human GST M2-2 are explored by screening against random 9- and 15-mer peptide libraries displayed on phage. The results were expected to reveal novel interactions between human GST M2-2 and peptides or other ligands.

Phage display

In the mid 1980s Smith fused foreign DNA that encodes a small antigenic region of a protein to DNA that encodes the *gene-III* minor coat protein in the filamentous f1 phage genome.[14] Five copies of this coat protein are normally present on the surface of

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filamentous phage of types f1, fd, and M13. The fusion provides a physical link between the phenotype and the genotype; in other words, the phage particle on which the peptide is displayed also contains the particular DNA that encodes the amino acid sequence of the peptide. The phage that presented the antigenic peptide were easily isolated from other phage by binding to antibodies with affinity for the exposed epitope, a selection procedure called "panning". Moreover, these phage could be amplified in *Escherichia coli* and subjected to another round of selection to enrich the gene encoding the chosen peptide.

This first scheme displayed multiple copies of the peptide on a phage particle (polyvalent display) and selected for micromolar-affinity interactions. Polyvalency can be obtained even more effectively by fusion of the peptide to the *gene VIII* product, a major coat protein.[15] The phage display technology has been further developed for monovalent display selection of nanomolar- to picomolar-affinity binders. The monovalency is accomplished by fusion of the protein or peptide onto the *gene III* product carried in a phagemid and provision of a large amount of wild-type protein III expressed from an M13 helper phage.[16] The combination of polyvalent display with monovalent display provides the means to select peptides that have high affinity for a target protein.[17]

Activating protein 1 (AP-1) and c-Jun N-terminal kinase (JNK)

AP-1 transcription factors are early response genes that are involved in transcriptional regulatory processes such as cell proliferation and apoptosis of cells in response to cellular stress.[18–19] The AP-1 structure that binds to DNA is a protein dimer that consists of a Fos family member and a Jun family member. AP-1 components are activated by numerous stimuli, which include growth factors, antigen binding by B and T lymphocytes, neurotransmitters, ionization and UV irradiation, cytokines, and many others.[19]

JNK, also called stress-activated protein kinase, is a mitogen-activated protein kinase (MAPK). JNK activates AP-1 by phosphorylation of the N-terminal region of c-Jun.[20] There are three genes that encode JNKs, *JNK1*, *JNK2*, and *JNK3*.[21]

In the present work the polyvalent peptide display introduced by Smith[22] was used to explore the ligandin function of human GST M2-2. The data obtained suggest that GST M2-2 serves as an AP-1 coactivator.

Results

Peptide libraries

The two peptide libraries used were constructed to contain 9 or 15 random amino acid sequences flanked by Cys residues. This design leads to the creation of a disulfide bridge in the displayed peptides and thereby constrains the variant sequences to cyclic structures.[23] The rationale for this approach is that the entropy loss for binding of a cyclic peptide is less than the entropy loss for binding of a linear and unconstrained peptide, thus cyclization promotes binding. However, the fact that in the present case one of the libraries contained both linear and cyclic peptides was potentially an advantage, since an earlier report indicated that both linear and cyclic peptides interact with rat GST M1-1.[13]

Phage peptide selection

Immobilized GST M2-2 was used to select binding sequences from a mixture of the two peptide libraries displayed on phage. The fraction of phage particles specifically binding to the target GST was determined by division of the number of phage particles recovered after a selection round by the number of phage particles subjected to selection. The fraction that bound to GST M2-2 increased 100-fold after the third round, which indicates specific enrichment (Table 1). Selection with GST M2-2 was discontinued after three rounds because the sequencing results showed a predominant clone (Table 2) and already clearly indicated enrichment.

Table 1. *The fraction of phage that bound specifically (in phage/out phage) in sequential rounds of adsoption to immobilized GST M2-2.*[a]

Target protein	First round	Second round	Third round
GST M2-2	2.7×10^{-8}	1.6×10^{-8}	1.2×10^{-6}

[a] 50×10^6 colony forming units (cfu) from each peptide library were mixed together and added to microtiter plates coated with GST M2-2. Unbound phage were removed and the plates were washed 10 times. Specifically bound phage were then acid eluted and the cfu were counted ("out phage"). "In phage" refers to the number of phage present at the beginning of a selection round.

Table 2. *Peptide sequences of GST-binding phage.*[a]

Phage clone	Variant peptide sequence	Number of identical clones/total number of clones sequenced
Pm1	AEDLELCA**GVVRGPSRG**ACTS	5/7
Pm2	AEDLELCA**ALARAARLG**ACTS	1/7
Pm3	AEDLELCA**AENRFDADLRSSALA**ACTS	1/7

[a] Peptide sequences were expressed on phage selected with GST M2-2. The peptide sequences shown were obtained by sequencing phage clones that were picked at random after the third round of selection. Bold font identifies sequences derived from the randomized portion of the peptide libraries.

Seven phage clones were chosen at random for sequencing after the last round of panning. All the peptides analyzed contained the two Cys residues that characterize cyclic peptides. Thus, GST M2-2 appears to have selectivity for sterically constrained cyclic peptides. Five clones of the seven selected were identical (Table 2), which shows an obvious enrichment of one particular peptide (Pm1: GVVRGPSRG).

Binding studies with phage-displayed peptide

In order to investigate the specificity of the binding of peptides to GST M2-2, Pm1 displayed on phage was compared with an

unrelated control peptide (Pneg), a 15-mer linear peptide (V-1:19) with an affinity for mouse microglia.[24] Pm1 was found to bind approximately 30-fold more tightly to GST M2-2 than does Pneg (Figure 1). Furthermore, binding experiments showed a decreased recovery of phage particles displaying Pm1 peptide when GST M2-2 was added in solution to compete with the immobilized GST M2-2 (Figure 1). These results provide evidence that GST M2-2 interacts with Pm1 in a specific manner.

Table 3. *Protein sequences with similarities to the GST-M2-2-binding peptide. The Swiss-Prot Database was searched for human proteins.*

Phage clones	Query	Hits	Human protein	Recognized site (amino acid residues)
Pm1	GVVRG-PSR-GA	GVIRGQPSPLGA	JNK1	408 – 420
Pm1	GVVRG-PSRGA	GVVKDQPSDA	JNK2	408 – 417
Pm1	GVVRG-PSR-GA	GVVKGQPSPSGA	JNK3	408 – 419
Pm1	AGVVRGPSRGA	AGVLRGPGR	Bcl3	427 – 435

Possible interaction between GST M2-2 and known protein sequences

The Swiss-Prot Database was searched for sequences similar to the GST-selected peptide in order to identify proteins that could potentially interact with GST M2-2. The C-terminal sequence of JNKs and the Pm1 sequence are highly similar (Table 3). Eight or nine out of twelve residues in JNK1 and JNK3 were identical to those in Pm1 and one residue was a conservative replacement. Similarities with JNK2 were also noted. The strong sequence similarity in the C-termini of the JNKs means that the selectivity, if any, of the putative interaction between JNKs and GST M2-2 cannot be deduced from the available data.

Pm1 also showed a similarity to the C-terminal region of Bcl3, which indicates that there might be an interaction between GST M2-2 and Bcl3. Seven residues of eleven were identical and one residue was a conservative replacement.

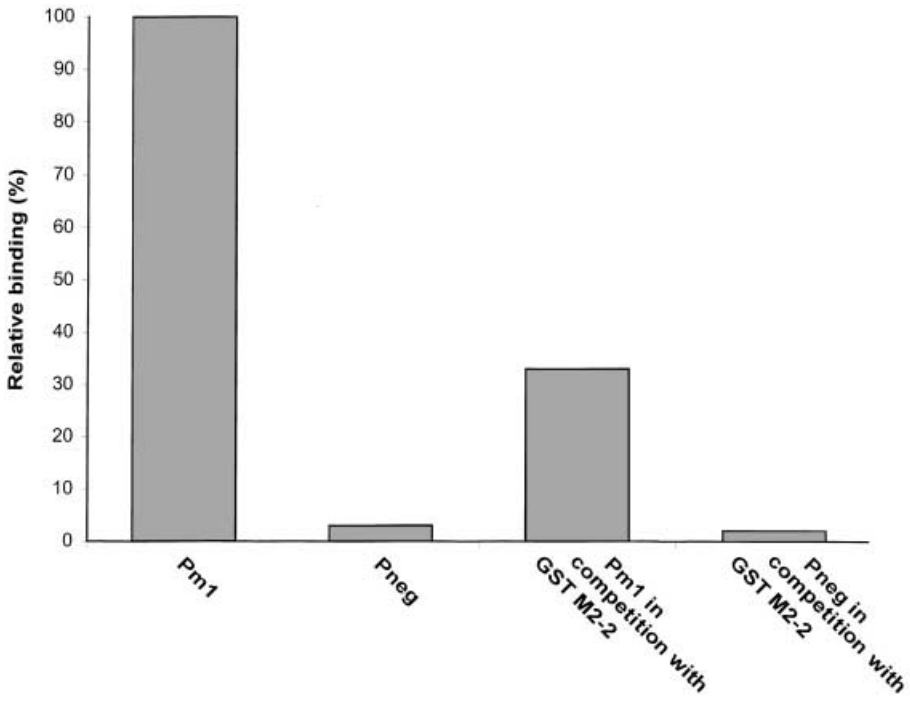

Figure 1. *Selective binding of phage that display peptide Pm1 to immobilized GST M2-2. The specificity was demonstrated by comparison with the negative control phage Pneg, as well as by competition with GST M2-2 in solution. GST M2-2 (100 μg) was immobilized in a microtiter plate well prior to addition of Pm1 or Pneg phage (50 μL). After 10 washes the phage were acid eluted and neutralized then used to infect a fresh culture of* E. coli *K91 cells. The ratio of "in phage" to "out phage" was calculated. For competition studies, GST M2-2 (50 μg) was added after 6 washes with PBS – Tween 20 (0.05 % (v/v)). 4 additional washes were made before the acid elution. Relative binding values are plotted with the positive control (Pm1) given as 100 %.*

Effect of GST M2-2 and JNK on AP-1

AP-1 is a dimeric transcription factor composed of Fos and Jun protein subunits. The transcriptional activity of c-Jun is strongly activated upon the phosphorylation of two serine residues in the activation domain of the JNK.[20] Luciferase reporter gene experiments showed that the transcriptional activity of AP-1 is induced upon stimulation of cells with phorbol 12-myristate 13-acetate (PMA), as expected (Figure 2 A). Overexpression of GST M2-2 upregulates AP-1 transcription almost fourfold (Figure 2 A, C). This coactivation effect increased with the amount of GST M2-2 DNA used for transcription. Specificity of the effect of human GST M2-2 on AP-1 transcription was demonstrated by overexpression of a related enzyme, GST A1-1. With this alternative GST no significant stimulation of transcriptional activity was noted (Figure 2 B). All the in vivo experiments were repeated three times independently in a human embryonic kidney 293 cell line as well as in human embryonic retinoblast 911 cells.

Effect of GST M2-2 and Bcl3 on AP-1 transcriptional activity

Bcl3 is a nuclear protein and a member of the IκB family that was first isolated as a gene translocated into the immunoglobulin α locus and is highly expressed in B-cell chronic lymphocytic leukemias.[25] Bcl3 has been shown to act as a transcriptional coactivator of AP-1 and the retinoid X receptor.[26–27] Our studies in cell lines were based on transcriptional activity of AP-1 upon PMA stimulation. The presence of Bcl3 increased the transcriptional activity, but no significant additional effect was obtained by GST M2-2 (Figure 2 D).

Discussion

Libraries in which random peptides are displayed on phage were employed in an attempt to discover

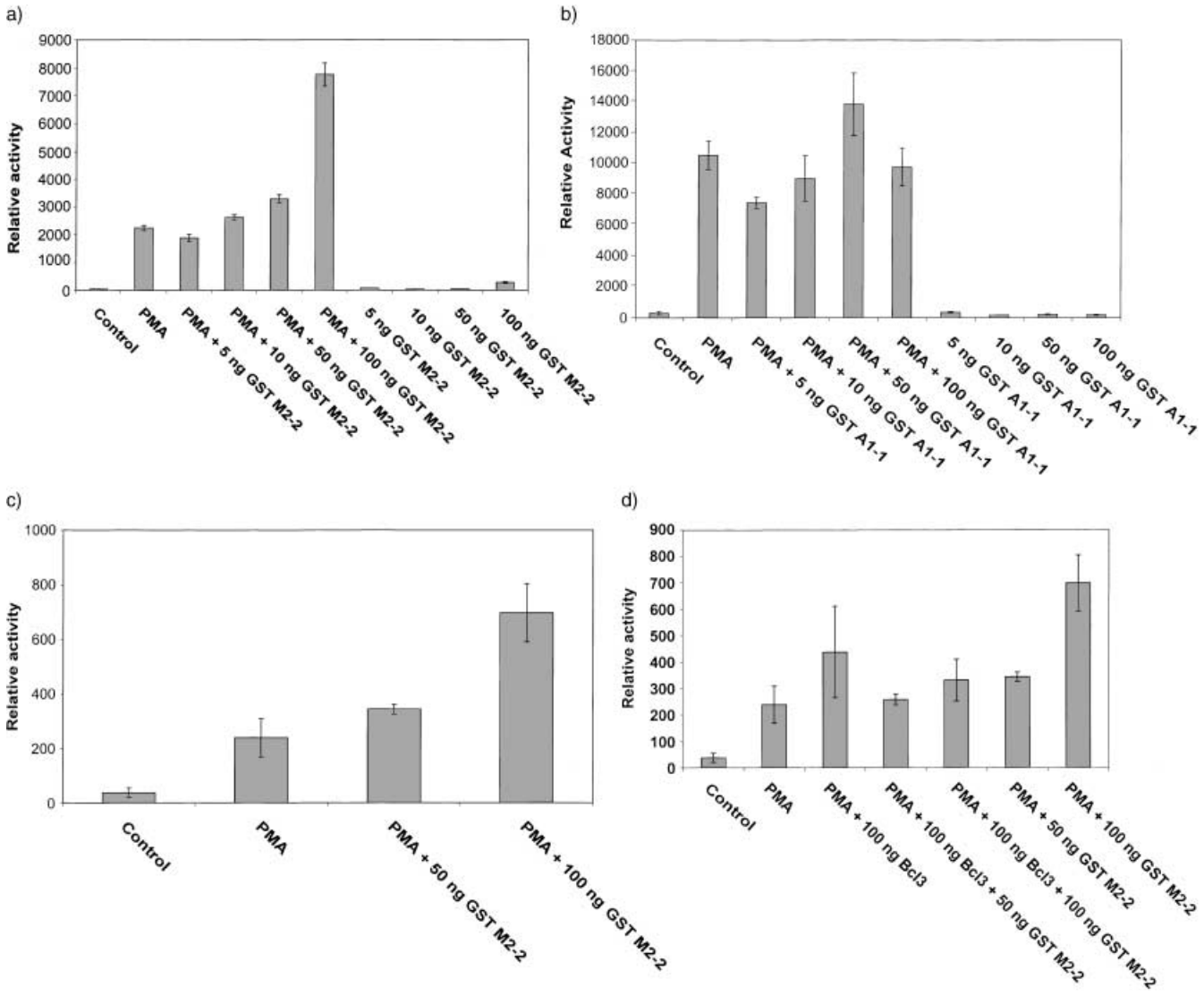

Figure 2. *a) 293 cells were cotransfected with luciferase reporter plasmids psctGST M2-2, pcDNA3, and pcDNAβ-gal. After 24 h of transfection, the cells were treated with PMA (100 ng mL^{-1}) for an additional 24 h before the cell lysates were assayed for luciferase activity. b) 293 cells were cotransfected with luciferase reporter plasmids psctGST A1-1, pcDNA3, and pcDNAβ-gal. After 24 h of transfection, the cells were treated with PMA (100 ng mL^{-1}) for an additional 24 h before the cell lysates were assayed for luciferase activity. c) 911 cells were treated as described for 293 cells in (a). d) 911 cells were cotransfected with luciferase reporter plasmid, psctGST M2-2, pcDNA3, pBcl3, and pcDNAβ-gal. After 24 h of transfection, cells were treated with PMA (50 ng mL^{-1}) for an additional 24 h before the cell lysates were assayed for luciferase activity. Each sample was normalized according to the activity of β-galactoside.*

interactions of glutathione transferase M2-2 with biomolecules. The fact that the fraction of specifically binding phage particles increases approximately 100-fold after three rounds of panning with GST M2-2 as the target protein is an indication of a successful selection procedure (Table 1).

GSTs have previously been proposed to serve as an intracellular carrier protein known as ligandin, which interacts with a wide variety of ligands such as bilirubin, heme, steroids, and fatty acids.[9–10] The results of the present study show that GST M2-2 is capable of binding peptides and in particular a constrained cyclic peptide sequence (Pm1).

The similarity of the GST-M2-2-binding peptide (Pm1) and the C termini of JNKs suggests interaction between GST M2-2 and JNKs, which indicates that GST M2-2 may be involved in cellular regulation as a coactivator of transcription factors. JNK occurs in three distinct isoforms: JNK1 and JNK2 are expressed ubiquitously, while JNK3 is expressed primarily in the brain, heart, and testis. Interestingly, GST M2-2 is a major GST in brain, testis, and heart,[28] which could indicate that the regulatory interaction of GST M2-2 and JNK3 is highly specific and tissue selective. All three *JNK* genes are expressed both with and without the C-terminal sequence[21] as a result of alternative splicing. The reason for the splicing event is unknown. Our data indicates that GST M2-2 binds to the C-terminal part of JNK, therefore it appears possible that truncation of JNK serves to make it insensitive to regulation by GST M2-2.

Other lines of evidence give support to the notion that GSTs are linked to cellular signaling. Pi-class GSTs have been proposed to be involved in protection of cells against reactive oxygen species by binding to JNK1.[29] JNK itself is activated by cytokines

(for example, TNFα), environmental stress, (such as UV radiation), and exposure to chemicals (for example, PMA).[30–31] Wang and co-workers have shown that Pi-class GSTs associate with the C-terminal half of JNK (residues 200 – 424).[32] The present work suggests that the region of JNK binding to GST M2-2 is not spread out over a large part of JNK, but is essentially restricted to residues 408 – 420. In contrast to GST P1-1, which inhibits transcriptional activity of AP-1,[33] GST M2-2 seems to coactivate AP-1 transcription in the human embryonic kidney cell line 293 and human embryonic retinoblast 911 cells stimulated with PMA. The binding site of JNK to GSTs seems in both cases to be restricted to the C-terminal region of JNK. However, the transcriptional outcome of this interaction appears to differ depending on whether GST P1-1 or GST M2-2 is binding to JNK. The difference in transcriptional consequences suggest that different GSTs display distinct regulatory functions, that is, inhibition and coactivation. Such transcriptional activity adds a new dimension to the cytoprotective role of GSTs.

The sequence similarity shown in Table 3 between Pm1 and the C-terminal region of Bcl3 suggests that GST M2-2 could also modulate the transcriptional activity of Bcl3. It was confirmed (Figure 2 D) that Bcl3 augments the activation of AP-1 by PMA as previously reported.[25] However, addition of GST M2-2 did not significantly influence the coactivation effect of Bcl3 (Figure 2 D). The lack of additivity suggests that GST M2-2 and Bcl3 might compete in the coactivation of AP-1. Alternatively, a direct interaction between GST M2-2 and Bcl3 may inhibit their respective effect on AP-1.

Earlier work that involved peptide phage display has proved useful in the identification of peptide ligands, for mapping epitopes of monoclonal and polyclonal antibodies, and in elucidation of substrate sites for proteases or kinases.[34–36] In the present investigation, peptide phage display has been adopted to search for novel biomolecular interactions with glutathione transferase M2-2.

The results of this investigation support the view that GSTs have a variety of functions in the cellular protection system. In addition to catalysis of detoxication reactions, GSTs seem to be involved in the response to cellular stress through interactions with different proteins that affect gene transcription. Subsequent detailed characterization of such interactions in vitro and in vivo are required to establish their biological importance.

Experimental Section

Random peptide libraries displayed on phage: The cyclic peptides libraries consisted of 9 and 15 random amino acid residues, respectively, flanked by Ala and Cys at the N- and C-terminal ends. The peptides were fused to the N terminus *gIII* product of a filamentous phage: N-term – AEDLELCA$(X)_{9/15}$ACTS – gIII. The phage vector fUSE2, which carries the tetracycline resistance gene, was modified (into the fAST vector) and used for construction of the libraries as previously described.[23] 30% of the 9-mer library consisted of linear peptides with a PALGTETS linker replacing the ACTS sequence at the C terminus.

Glutathione transferase M2-2: Recombinant human GST M2-2 was expressed and purified as previously described.[37]

Selection of phage-displayed peptides: The target GST protein (100 μg) in phosphate buffer saline (PBS; 50 μL) was immobilized on the plastic surface of individual wells of a 96-well Nunc-Immuno Plate (Maxisorp; Nunc Brand Products, Roskilde, Denmark). The plate was incubated at 4 °C overnight. The wells were then blocked with nonfat dried milk (5% (w/v)) in PBS for 1 hour at RT before the addition of the phage libraries. 50×10^6 cfu from each library were mixed together and added to a well preblocked with nonfat dried milk (150 μL, 5% (w/v)) in PBS and the library was incubated for 1 hour at RT prior to transfer to GST-M2-2-coated microtiter wells. The phage were incubated in the protein-coated wells at RT for 2 hours. The unbound phage were removed and the plates were washed 10 times with PBS (300 μL) that contained Tween 20 (polysorbate 20; 0.05% (v/v)). Specifically bound phage were then acid eluted with glycine – HCl buffer (100 μL; 0.1 M, pH 2.2) and neutralized with tris(hydroxymethyl)aminomethane (Tris) base solution (6 μL; 2 M). The eluted phage were used to infect K91 *E. coli* cells grown in Luria – Bertani (LB) broth that contained tetracycline (10 μg mL^{-1}; $OD_{600} = 0.6$). Aliquots of 100 and 10 μL were plated on LB agar plates that contained tetracycline (10 μg mL^{-1}) and the remainder was propagated at 37 °C overnight in LB liquid medium (50 mL) that contained tetracycline (10 μg mL^{-1}). The amplified phage were precipitated and used for another round of selection. Three rounds of selection were conducted with GST M2-2.

Precipitation of phage: Cultures of phage-infected bacteria grown overnight were centrifuged at 3950 × g for 15 min to remove the cells. In order to precipitate the phage, polyethylene glycol (PEG 8000; 4% (w/v)) and NaCl (3% (w/v)) were added to the supernatant. The mixture was incubated for 30 min on ice before centrifugation at 9900 × g for 30 min. The pellet was resuspended in PBS (1 mL), and the suspension was centrifuged at 16000 × g for 5 min to remove cell debris.

DNA sequencing: Phage selected for binding to GST M2-2 were chosen for DNA sequencing after the third round of panning. The nucleotide sequences were determined by using the Big Dye kit (Perkin Elmer, MA, USA) or the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (USB Corporation, Cleveland, OH, USA) and were analyzed on an ABI375 sequencer (Perkin Elmer, MA, USA).

Competitive binding studies with phage-displayed peptide: Four microtiter wells were coated with GST M2-2 (100 μg) and kept at 4 °C overnight. The wells were then blocked with nonfat dried milk (5% (w/v)) in PBS prior to addition of phage-displayed peptide (10^9 cfu). The selected on-phage peptide Pm1 was added to two of the wells, while a nonbinding phage-displayed peptide was added to the two other wells. After 6 washes with PBS that contained Tween 20 (0.05% (w/v)), competing GST M2-2 (50 μg) was added, followed by 3 washes with PBS that contained Tween 20 (0.05% (w/v)) and 1 wash with PBS. The phage were acid eluted by addition of glycine – HCl buffer (100 μL; 0.1 M, pH 2.2), neutralized with Tris solution (6 μL; 2 M), and quantified by infection of freshly cultured K91 *E. coli*.

Incorporation of GST M2-2 and GST A1-1 into a pSTC HA tagged expression vector: The GST M2-2[37] and GST A1-1[38] coding sequences were amplified by PCR, digested with the endonucleases BamHI (New England Biolabs Inc.) and XbaI (New England Biolabs Inc.), and purified on 1% agarose gel. The sequences were then ligated over 1 h at RT into pSTC HA3.X556 tagged expression vector (a derivative of a vector kindly provided by Dr. Rusconi, Universität Zurich, Switzerland), which was digested with the same restriction enzymes as above. The expression of GST M2-2 and GST A1-1 was confirmed by Western blot analysis.

Transfection of 293 cells and 911 cells, lysis, and luciferase assays: The human embryonic kidney 293 cells were maintained at 37 °C in minimal essential medium with L-glutamine (Life Technologies, Inc.) supplemented with fetal calf serum (10% (v/v)). The human embryonic retinoblast 911 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with fetal calf serum (10% (v/v)).

The cells were grown in 35 mm dishes to 30–40% confluence and then transfected by using the FuGENE 6 transfection reagent (Roche, IN, USA). 20–24 h before harvesting, the cells were stimulated with PMA (50 or 100 ng mL^{-}). Transfection efficiencies were normalized by using the activity of β-galactoside. The transcriptional activity was assayed by using the luciferase assay system (Promega, WI, USA). The Bcl3 expression vector was kindly provided by Dr. Claus Scheidereit, Max-Delbrück Center, Berlin, Germany and the AP-1 reporter construct was a gift from Dr. Hans Wolf-Watz, Umeå University, Sweden.

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