Molecular forceps from combinatorial libraries prevent the farnesylation of Ras by binding to its carboxyl terminus

Dennis L Dong, Ruiping Liu, Rosemarie Sherlock, Michael H Wigler and H Peter Nestler

Introduction: Ras is one of the major oncogenes. In order to function properly it has to undergo post-translational processing at its carboxyl terminus. It has been shown that inhibitors of farnesyl transferase, the first enzyme in the processing chain, can suppress the transforming activity of oncogenic Ras.

Results: We have identified molecular forceps, branched peptidic molecules, from combinatorial libraries that bind to the carboxyl terminus of Ras and interfere with its farnesylation without inhibiting the farnesyl transferase. The active molecules were selected by a screening against the carboxy-terminal octapeptide of Ras.

Conclusions: The implications of our findings are twofold. First, we demonstrate that it is possible to prevent enzymatic transformations by blocking the enzyme's access to its substrate using a synthetic small molecule to mask the substrate. Second, we show that it is feasible to derive molecules from combinatorial libraries that bind a specific epitope on a protein by selecting these molecules with the isolated peptide epitope.

Address: Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA.

Correspondence: H Peter Nestler
E-mail: nestler@cshl.org

Key words: combinatorial libraries, epitope binding, farnesylation, molecular recognition, Ras oncogene

Received: **4 November 1998**
Revisions requested: **11 December 1998**
Revisions received: **7 January 1999**
Accepted: **13 January 1999**

Published: **12 February 1999**

Chemistry & Biology March 1999, **6**:133–141
http://biomednet.com/elecref/1074552100600133

Introduction

Knowledge of the determinants of noncovalent intermolecular interactions in biological systems can sometimes be used to modulate biological activities by chemical intervention. Past efforts have been hampered by the need to synthesize and investigate structures individually, but the recent development of combinatorial library techniques has lifted this restriction. Combinatorial chemistry allows the simultaneous controlled synthesis of thousands to millions of compounds, the subsequent simultaneous assay of their activity and the determination of the structure of active compounds. Virtually any sequence of chemical reactions can be employed to synthesize diverse and complex libraries of molecules. Such libraries have been mostly used for drug discovery and optimization [1–7] (M. Lebl and Z. Leblova, Dynamic database of references in molecular diversity; http://www.Sz.com/divinfo/) but they have also found application in the studies of molecular recognition and the development of artificial receptors [8–12]. In our initial studies, we elaborated libraries of small molecules with branched peptidic structure ('molecular forceps') and showed examples of highly specific interactions between these molecular forceps and peptide ligands in organic solvents [13–15]. Here, we describe how we have employed these systems to derive artificial binding pockets that bind proteins of physiological importance at specific functional sites in aqueous media. Such moieties can be used as synthetic surrogates for monoclonal antibodies, diagnostics to detect proteins in western blots or in cells, affinity agents on supports to allow the convenient purification of protein or agents that modulate the biological function of proteins by preventing their physiological interactions. In the present case we use such molecules to inhibit the processing of Ras proteins at their carboxyl termini.

We have chosen Ras as the first target protein for several reasons. Ras proteins play a central role in normal cellular physiology and mutant Ras proteins in the pathophysiology of tumors [16,17]. Moreover, much is known about the structure and function of Ras proteins. They are required for the transduction of signals from many membrane receptors, including tyrosine kinases and some G-protein-linked receptors, and are representatives of a large and highly conserved family of guanine-nucleotide-binding proteins, the Ras superfamily, that participate in a wide variety of cellular phenomena. Point mutations that activate the oncogenic potential of Ras genes are commonly found in human tumors. These point mutations usually cause amino-acid substitutions at either position 12 or 61, leading to impaired GTP hydrolysis. Ras proteins are initially synthesized in the cytoplasm, where they undergo a series of post-translational modifications at their carboxy-terminal sequence, the CaaX box (where 'C' is cysteine, 'a' an aliphatic amino acid and 'X' either serine or methionine), resulting in their farnesylation, and subsequent cleavage of the terminal tripeptide and carboxy-methylation [18,19]. The processed proteins become localized to the cell membrane, a step that is essential for their functioning [20–22].

Oncogenic Ras proteins have been considered to be logical targets for the development of cancer therapeutics. One approach has been to find agents that block Ras processing by inhibiting farnesyl transferase (FTase) [23–26]. In fact, such agents can induce morphological reversion of cells transformed by Ras at concentrations that do not arrest normal cell growth. The results with FTase inhibitors suggest that, in general, the level of an inhibitor needed to block the transforming activity of Ras will not necessarily reduce normal Ras activity below levels sufficient for normal cell growth [23,24,27]. Moreover, nonfarnesylated oncogenic H-Ras–Val12 has been shown to sequester Raf to the cytosol [28]. FTase inhibitors might therefore be generally useful as anticancer agents. One potential obstacle to using such agents as drugs is that they might block the farnesylation of other critical proteins in humans, such as the γ subunit of transducin and nuclear lamins.

On the other hand, a small molecule that binds to the carboxyl terminus of a particular Ras might prevent FTase from acting upon this Ras without inhibiting the activity of the transferase on other substrates. Indeed, the precise CaaX sequences and the two amino-acid residues preceding them distinguish the members of the Ras family from each other, and from other substrates for farnesyl and geranyl geranyl transferases. Our ability to find synthetic molecular forceps from encoded combinatorial libraries that distinguish peptides differing by single amino-acid substitutions in organic phase [13] raised our hopes of finding molecules that selectively bind to the CaaX box of one particular Ras protein in aqueous phase and therefore might specifically prevent the processing of Ras.

Results and discussion

Preparation of the encoded combinatorial library of molecular forceps

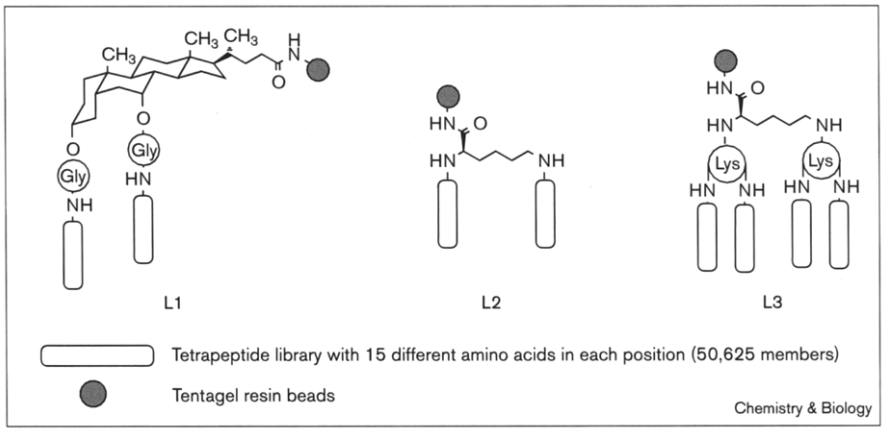
In earlier studies, we and others could show that the recognition of peptides and their discrimination with single amino-acid resolution does not require complex macromolecular structures but can be achieved with molecules that display two short peptide chains on a rigid scaffold [13,29]. We discovered that molecules presenting two tripeptides linked by a flexible pentamethylene linker can bind pentapeptides, although with less affinity and specificity than the rigid molecules [15]. These early experiments were performed in organic solvents. We expected, however, that similar interactions should occur in aqueous surroundings and that molecular forceps should be able to recognize epitopes on physiologically important proteins and interfere with their biological functions.

As it was not obvious whether a flexible or rigid structure would be advantageous for the interactions with a particular target, we used both the rigid chenodeoxycholic acid scaffold with a glycine spacer (L1) and a flexible lysine (L2) as cores to generate a symmetrical library of molecular forceps (MF) on Tentagel beads. We also used a trilysine core (L3) to study the influence of increased molecular surface on the strength of the interactions. These molecular forceps contained glycine and both enantiomers of alanine, valine, serine, proline, asparagine, glutamine and lysine at the four variable amino-acid positions (Figure 1). The library was generated using split synthesis [30,31] and the reaction history of each bead was recorded using the molecular catechol tags described by Nestler, Still and coworkers [32].

Ras proteins and the carboxy-terminal octapeptide of Ras bind to members of the library

Antibodies coupled to alkaline phosphatase have been frequently used in the screening of combinatorial libraries for beads binding to proteins [30,33]. This procedure is cumbersome, however, and requires multiple steps that might be unsuitable for the detection of interactions with lower affinities. Proteins have, therefore, sometimes been labeled directly with dyes, especially fluorescein, that can

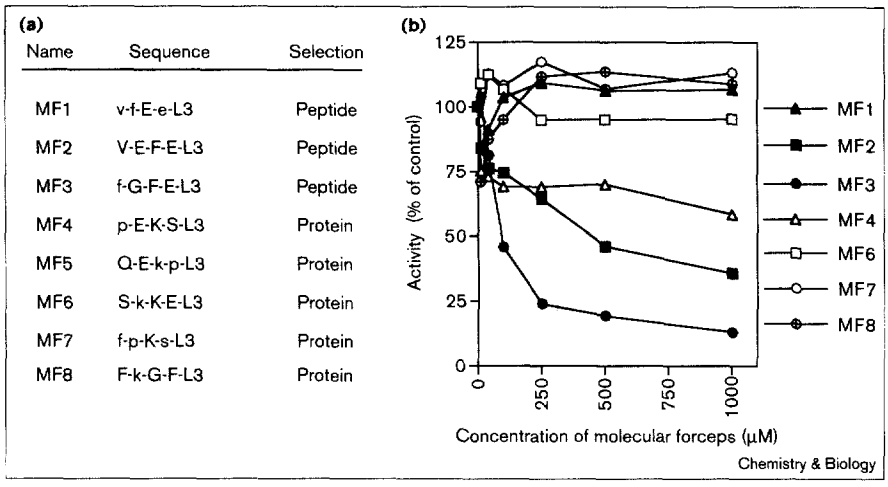
Figure 1



Branched molecular forceps library with three different linkers. The tetrapeptide libraries consisting of 15 L- and D-amino acids at each position were synthesized using split synthesis. The structures of the three different linkers are shown, one with chenodeoxy cholic acid (L1, steroid) as a scaffold, two others (L2 and L3) using lysines as flexible pentamethylene linkers. The total diversity of the library is $3 \times 15^4 = 151{,}875$ molecules.

Figure 2

Investigation of resynthesized molecular forceps on the activity of FTase. Selected molecular forceps from peptide and protein screenings were resynthesized. They were assayed for their effects on the activity of yeast FTase using purified H-Ras–Val12 protein as substrate. **(a)** The list of both positive (MF1–MF7) and negative (MF8) molecular forceps, and their selections; MF1–MF3 were selected from the His-tagged CaaX-peptide screening, and MF4–MF8 were chosen from FM–GST–H-Ras–Val12 protein screening. (Uppercase letters define L-amino acids, lowercase letters are the D-enantiomers.) **(b)** FTase activity assay in the presence of different concentrations of molecular forceps. The concentration for each molecular forceps ranges from 0 to 1000 μM.



be detected directly [34,35]. In our screenings, we employed fluorescein-5-maleimide (FM) to conjugate fluorescein to purified glutathione S-methyl transferase (GST) and a GST–H-Ras–Val12 fusion protein. First, about 7000 library beads were extensively incubated with FM–GST and the green fluorescent beads were removed to eliminate false positive binders. The remaining unstained beads were then incubated with FM–GST–H-Ras–Val12. The green fluorescent beads were picked up and the encoding tags were analyzed using gas chromatography (GC) [32]. Among the about 7000 library beads used in the screening, we found 65 relatively strong green beads. From GC analysis, we obtained 30 readable sequences (data not shown). All these positive molecules have either L2 or L3 as core template, and no molecules based on the L1 scaffold were found. From the 30 sequences, we selected four molecular forceps, $(p\text{-}E\text{-}K\text{-}S)_4L3$ ('MF4'), $(Q\text{-}E\text{-}k\text{-}p)_4L3$ ('MF5'), $(S\text{-}k\text{-}K\text{-}E)_4L3$ ('MF6'), and $(f\text{-}p\text{-}K\text{-}s)_4L3$ ('MF7') for further studies (single-letter amino-acid code is used; lower case letters are D enantiomers and upper case letters are L enantiomers). We decided to use one molecular forceps from a bead that failed to bind to FM–GST and FM–GST–H-Ras–Val12, $(F\text{-}k\text{-}G\text{-}F)_4L3$ ('MF8'), as a putative negative control (Figure 2a).

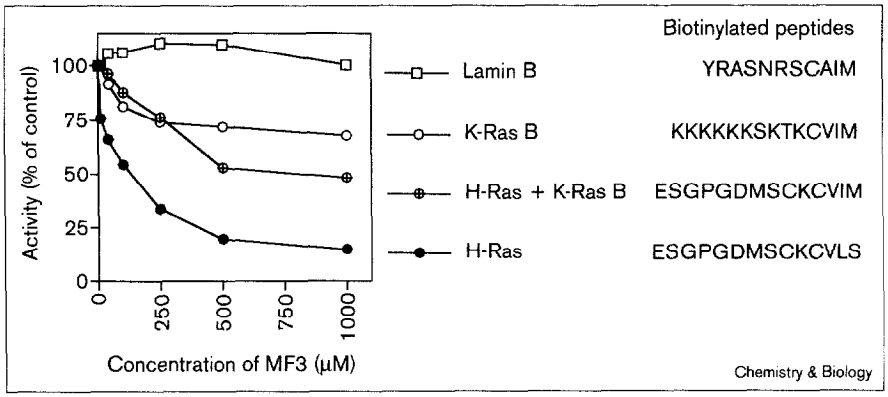
Selecting molecular forceps from libraries for Ras binding does not insure that these forceps will recognize the carboxyl terminus of Ras. We therefore sought to screen molecular forceps libraries with the isolated carboxy-terminal peptide from Ras to select molecular forceps that bind this epitope specifically.

We and others have used the dye-labeling strategy for the screening of small peptides against receptor libraries in organic solutions [13,15,36,37]. When hydrophilic dyes are conjugated with short peptides, however, the characteristics of the dye dominate in the screening and only library members interacting with the dye are selected [38]. To circumvent this problem, we have developed a method using the hexahistidine tag (His tag) on peptides and proteins to detect binding on beads [39]. The His tag interacts with ions of transition metals, such as copper. Copper ions can be used to catalyze the oxidation of benzidine to the blue dye Benzidine Blue. Consequently, beads that bind to the His-tagged peptides or proteins can therefore be distinguished by their purplish brown color when appropriately treated. We linked the His tag to the carboxy-terminal decapeptide of H-Ras to create a binding site for copper ions. The resulting peptide had the sequence HHHHHH-GSMSCK-CVLS (using single-letter amino-acid code). We screened about 10,000 library beads against this His-tagged Ras carboxy-terminal peptide and obtained 30 different structures after decoding of the selected beads. Figure 2a lists those 'hit' molecules, $(v\text{-}f\text{-}E\text{-}e\text{-})_4L3$ ('MF1'), $(V\text{-}E\text{-}F\text{-}E\text{-})_4L3$ ('MF2'), and $(f\text{-}G\text{-}F\text{-}E\text{-})_4L3$ ('MF3'), that we chose for further studies. Interestingly, as in the protein assay, most positive molecular forceps contained the trilysine linker (L3), and the remainder were two-arm lysine forceps (L2). As L3 was the predominant scaffold in the peptide as well as the protein screening, we focused our attention on molecular forceps based on this motif. We cannot offer any convincing rationalization for the failure to observe the steroid scaffold in our screening, but there are many possible explanations: failure of synthesis, mechanical collapse of the beads around the steroids, adhesion of the steroid to the hydrophobic core of the beads or collapse of the peptide chains on the steroid template in water.

Molecular forceps that bind the Ras carboxy-terminal sequence impede the farnesylation of Ras

Three molecular forceps from the His-tagged peptide assay and four forceps from the protein screen (MF1–MF7), as well as one from a bead that did not bind (MF8)

Figure 3



Effect of MF3 on the farnesylation of different CaaX peptides. *N*-Biotinylated peptides containing the CaaX motif were synthesized and used as substrates for FTase assays in presence of different concentrations of MF3. Sequence names with two proteins designate chimeric sequences, (i.e. H-Ras + K-Ras).

were resynthesized on Tentagel-NH_2 to retest and confirm their interactions with the Ras protein. All positive forceps (MF1–MF7) bind to the fluorescein-labeled GST–Ras (data not shown). For further studies in solution we prepared molecular forceps, cleaved them from the resin and purified them using high-performance liquid chromatography (HPLC).

The resynthesized molecular forceps (MF1–MF8, except MF5, which was not soluble) were tested for the inhibition of farnesylation by yeast FTase (the expression plasmids were the generous gift of F. Tamanoi) [40]. As expected, MF8, our negative selection, showed no inhibition on the farnesylation of Ras protein (Figure 2b). MF6 and MF7 from the protein screening also showed no effect, as did MF1, which we had obtained from the peptide screening. MF4 shows a weak impact on the farnesylation with an IC_{50} higher than 1 mM. The two molecular forceps MF2 and MF3 from the screening against the peptide epitope provided the best inhibition (Figure 2b). The IC_{50} values for MF2 and MF3 are about 400 μM and 100 μM, respectively. Additional experiments showed that neither the two-armed forceps derived from MF3 (which corresponds to the molecular forceps based on L2) nor the isolated tetrapeptide arm of MF3 has any effect on the farnesylation of Ras.

Impeding the farnesylation with MF3 is sequence dependent

As discussed in the introduction, many cellular proteins are processed by FTase. We reasoned that, if our forceps inhibited the farnesylation of Ras by binding to its carboxyl terminus, they might not inhibit the processing of other proteins by FTase. To test the specificity of MF3, we therefore used four CaaX-containing peptides, derived from Lamin B, K-Ras B and H-Ras, as well as a chimera of the H-Ras and K-Ras sequences ('H-Ras + K-Ras'; Figure 3), which were biotinylated at their amino termini, as FTase substrates in the presence of varying concentrations of MF3. Satisfyingly, molecular forceps MF3 showed different effects on the farnesylation of these substrates by yeast GST–FTase. Although MF3 has no influence on the modification of the Lamin B peptide at concentrations up to 1 mM, it shows a weak effect on the K-Ras B peptide, and a stronger impact on the H-Ras/K-Ras B chimera. MF3 strongly impedes the farnesylation of biotinylated H-Ras peptide with an IC_{50} of about 100 μM, which is the same as we observed when using Ras protein as the substrate (Figure 3). Preliminary experiments with purified FTase from bovine brain [41] showed a similar pattern. (The mammalian FTase was a generous gift from P. Casey.) These results indicate the inhibition of farnesylation is sequence dependent and not dependent on the presence of the Ras protein structure.

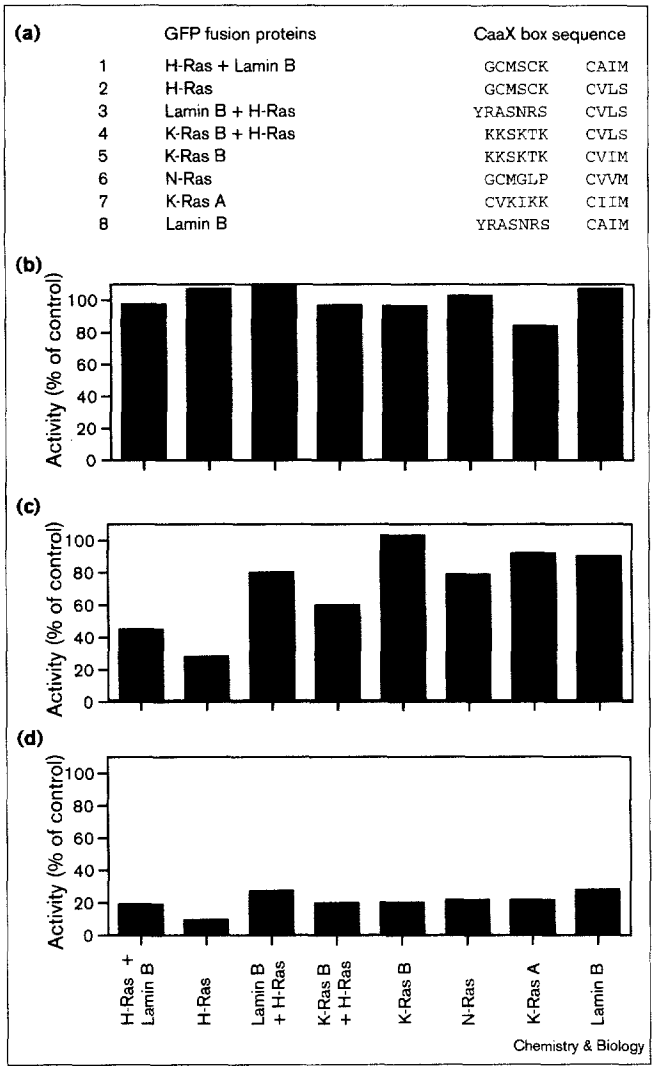
During the course of preparing the biotinylated peptides for our sequence-specificity studies, we noticed that the solubility of some biotinylated peptides in aqueous media was poor. We therefore constructed His-tagged green fluorescent protein (GFP) with more than a dozen carboxy-terminal epitopes and tested these in farnesylation assays. As shown in Figure 4, the negative molecular forceps, MF8, shows no inhibition on the farnesylation with any CaaX fusion protein (Figure 4b). A commercial FTase inhibitor, the farnesylpyrophosphate analog FPT-II [42], shows broad inhibition of the activity of FTase on different CaaX fusion proteins, although the strongest impact seems to be on the farnesylation of H-Ras (Figure 4d). MF3 shows again the strong preference for the GFP–H-Ras–CaaX protein (Figure 4c).

Our studies show that the forceps can recognize more than the carboxy-terminal tetrapeptide of farnesylation substrates. This is not surprising given that our original screening utilized a specific octapeptide epitope.

MF3 does not bind to FTase

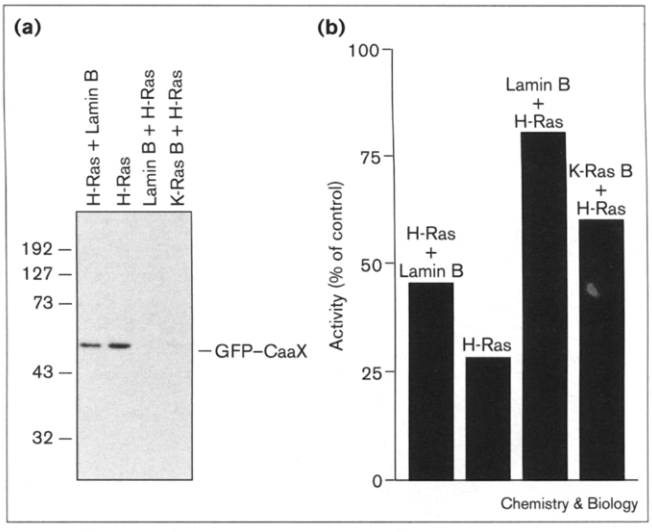
To confirm further that the inhibition of farnesylation of GFP–CaaX proteins is due to the binding of the molecular

Figure 4



Generation of His-tagged GFP–CaaX fusion proteins as substrates of FTase and the effect of molecular forceps on their farnesylation activity. His-tagged GFP–CaaX fusion proteins were generated and used as substrates for the FTase assay in the presence of 250 μM of molecular forceps MF8 or MF3 or 2.5 μM of the farnesylpyrophosphate analog, FPTII. **(a)** List of His-tagged GFP–CaaX fusion proteins and their carboxy-terminal sequence. **(b)** FTase assay in the presence of negative molecular forceps MF8. **(c)** FTase assay in the presence of molecular forceps MF3. **(d)** FTase assay in the presence of the FTase inhibitor FPTII.

Figure 5



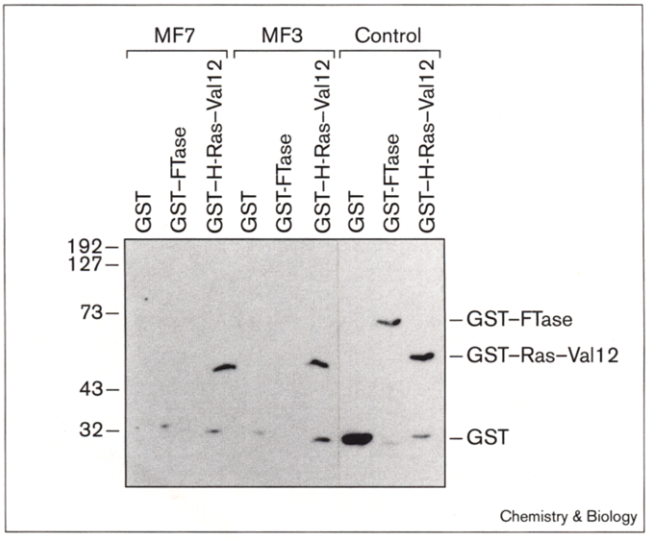
MF3 binds strongly to the carboxy-terminal sequence of H-Ras. The binding strength of MF3 to various GFP–CaaX fusion proteins correlates with the inhibitory activity in the farnesylation assays. **(a)** Biotinylated MF3 was incubated with various His-tagged GFP–CaaX fusion proteins, and the bound proteins were precipitated with avidin beads, analyzed by SDS–PAGE, and detected by anti-$(His)_6$ antibody. **(b)** FTase inhibition assay of different His-tagged GFP–CaaX fusion proteins with MF3. The concentration of MF3 used in the assay was 250 μM. Biotinylated and nonbiotinylated MF3 show the same inhibition.

forceps to their CaaX sequence and not to binding of FTase, we performed *in vitro* binding assays by incubating different GFP–CaaX fusion proteins with biotinylated MF3. We incorporated a biotin label to enable precipitation of protein–forceps complexes. These biotin labels were incorporated by modifying the carboxy-terminal part of the forceps with βAla–Glu–Lys(Biotin)–βAla-OH. The proteins were incubated in solution with the biotinylated forceps and the complexes were precipitated with avidin sepharose beads. After elution of the bound proteins from the beads and gel electrophoresis, we detected the proteins using western blotting with the suitable antibodies.

As demonstrated and discussed earlier, MF3 affects the farnesylation of H-Ras most, followed in extent by H-Ras/Lamin B, and K-Ras/H-Ras, and so forth, as shown in Figure 5b. We found that the *in vitro* binding strength of MF3 to the GFP–CaaX proteins correlates with its inhibitory activity. As shown in Figure 5a, the molecular forceps MF3 bind strongly to the CaaX sequence of H-Ras, and distinguish the different CaaX sequences, a pattern that is reflected in the inhibitory effect on the farnesylation of these sequences. To test the binding of MF3 to FTase, biotinylated MF3 was incubated with GST–H-Ras–Val12 and GST–FTase. Although MF3 clearly interacts with GST–H-Ras–Val12 at a concentration of 250 μM, we did not observe any binding to yeast FTase (Figure 6). These results suggest that, although MF3 can bind to Ras, it fails to bind FTase, implying that MF3 does not act as an enzyme inhibitor, but that its activity is caused by its interaction with the substrate.

In recent years, the use of combinatorial libraries has opened novel ways to generate artificial receptors. Our studies started from three assumptions: first, we should be able to find forceps that selectively recognize peptides in

Figure 6



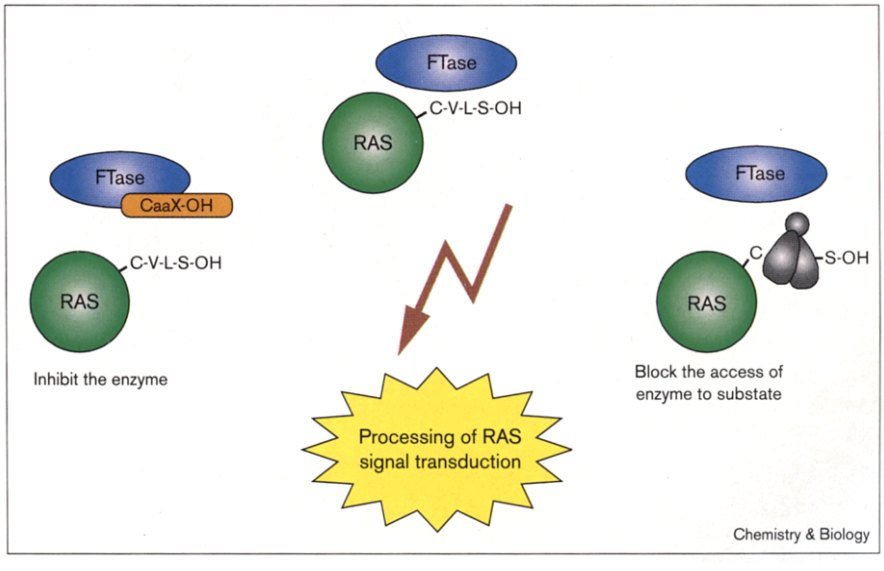
MF3's effect on the farnesylation of H-Ras is due to its binding of the carboxy-terminal domain and not binding to FTase. GST, GST–FTase and GST–H-Ras–Val12 were incubated with 250 μM of biotinylated MF7 or MF3. Proteins bound to the biotinylated molecular forceps were precipated with avidin beads, analyzed by SDS–PAGE and detected with primary anti-GST antibody and secondary horseradish peroxidase conjugated anti-rabbit antibody. MF7 is one molecular forceps that was found to bind Ras but does not inhibit its farnesylation.

aqueous solution; second, these forceps should recognize the peptide as an epitope embedded in a protein; finally, epitope-binding forceps should be capable of modulating the biological function of the target proteins. Using a receptor design that we had established before, we generated a library of molecular forceps and screened them against the farnesylation site of the Ras protein. Our results prove the validity of our assumptions. Although the actual numbers of molecules tested might be too low for a final assessment of the most efficient screening strategy, we clearly demonstrate that artificial receptor molecules from libraries can specifically bind the epitopes of proteins and are able to modulate biochemical processes associated with these proteins.

In our present application we have used the carboxy-terminal CaaX sequence of Ras. It is unlikely that this is the only peptide epitope that will yield to this type of approach. Biological systems have evolved to recognize simple amino-acid motifs, such as the RGD loop involved in fibronectin recognition, the MGCXXS sequence involved in myristilation, and SH2 and SH3 recognition peptides, to mention but a few. There are many applications that can be conceived for artificial receptor molecules that bind such epitopes. Besides the potential therapeutic application of these molecules, they can, with the appropriate modifications, be useful tools for the purification or diagnostic detection of proteins, in western blots or in a physiological environment.

Although our studies give the first indication that such applications may be possible, our molecular forceps and the strategies to identify them are far from offering a complete and elaborated system. There are many obstacles that we face on the way to the various applications. For example, the affinity of our forceps is low and requires their use in high concentrations. Beyond working towards higher affinities, the specificity of the interactions will have to be improved. In the course of such improvements, we will also face the challenge to generate structures that

Figure 7



Artificial synthetic small molecules can behave like an antibody to bind to the targeted epitope of protein specifically. MF3, the positive molecular forceps selected from our encoded peptidic combinatorial library, can specifically bind to the carboxyl terminus of Ras and prevent its farnesylation. It recognizes the epitope of a particular protein and therefore inhibits the farnesylation of Ras more selectively than inhibitors that interact with the enzyme.

can easily penetrate cells. To date we do not know whether the classical modifications employed by medicinal chemists will allow us to create molecular forceps that can pass the cell membrane or whether we will need a completely different receptor design. Nevertheless, we believe that our results give proof of the viability of the concept and can be the basis for studies that strive to generate new forceps with improved properties.

Significance

In recent years, combinatorial libraries have become a major tool in drug discovery and drug development. Furthermore, encoded combinatorial libraries have been used to disclose ligands for well-designed synthetic macrocyclic host molecules and to elucidate their specificities for peptide sequences. These studies led, via receptors with more flexibility, to simple host molecules without elaborate design that are accessible to combinatorial synthesis. We have generated libraries of molecular forceps, receptor molecules of branched peptidic structure, and screened them for molecules that bind to the carboxy-terminal farnesylation site of the Ras protein.

Ras proteins play a central role in signal transduction and the mutated forms of Ras are commonly found in human tumors. They undergo a complex post-translational processing that is crucial for their functioning. The inhibition of the farnesylation of the carboxyl terminus, the first step in the processing sequence, has been shown to be a very effective way to revert the transformed phenotype of cells containing mutated Ras proteins.

We show that molecular forceps that bind to the carboxyl terminus of Ras can also impede the farnesylation of Ras by FTase. Our findings give proof for a new concept, as we interfere with the enzymatic process not by inhibiting the enzyme but by masking the substrate epitope with a small molecule (Figure 7). Many other epitopes are subject to biochemical processing and could be targeted in the same fashion, including glycosylation sites, myristilation motifs or even phosphorylation sites. Our strategy might allow the selective binding of such proteins and modulation of their activities.

Material and methods

Generation of the molecular forceps libraries

The encoded forceps library was generated using the split-mix strategy [30,31]. Tentagel-NH_2 15 g; (RAPP Polymere, Tübingen, Germany, 90 μm bead diameter, 0.29 mmole/g of NH_2) was used as solid support and the amino acids were attached using standard Fmoc/Boc (fluorenylmethoxycarbonyl/butyloxycarbonyl)-peptide solid phase mediated by HOBt/DIC (hydroxybenzotriazole/diisopropylcarbodiimide) [43]. The completeness of the couplings was monitored with Bromophenol Blue [44]. The catechol tags [32] were attached as carboxylic acids using HOBt/DIC using approximately 1% of the available amino groups for each tag. The structures of the linking core (L1–L3) are shown in Figure 1. A combination of 15 different amino-acids (glycine and the D/L enantiomeres of valine, phenylalanine, serine, glutamine, glutamic acid, lysine and proline) was used for the structural diversity. After four split-mix coupling cycles, we generated a library of $3 \times 15^4 = 151{,}875$ members. The library was deprotected by treatment with 20% piperidine/DMF and 95% TFA / 2.5% water / 2.5% thioanisole and stored at 4°C.

Peptide synthesis and resynthesis of positive molecular forceps

Selected molecular forceps were resynthesized on Tentagel resin or on Wang resin (Novabiochem) for molecular forceps used in solution studies. For the solution-binding studies biotin was incorporated to allow for the handling of the forceps. This was achieved by derivatizing the carboxy-terminal carboxylic acid of the molecular forceps with βAla–Glu(OtBu)–Lys(Biotin)–βAla-OH. Short linear peptides required in the solution studies were also prepared on Wang resin. All these batch syntheses were performed on a RAININ Symphony multiple peptide synthesizer using standard Fmoc/Boc-peptide chemistry with HBTU-mediated couplings. The products were purified using HPLC using a WATERS Delta Prep 3000 with 15 μ C-18-column (25 mm × 300 mm) and a linear gradient of 0.1% TFA/water to 0.1% TFA/70% CH_3CN/water and were characterized by MALDI–TOF mass spectrometry and peptide sequencing.

Expression and purification of proteins

The DNA constructs coding for the proteins were cloned into the appropriate expression vectors and expressed in *E. coli*. GST and GST-H-Ras-Val12, and GST-yeast FTase [40] were purified according to the manufacturer's protocols through glutathione-agarose columns (Pharmacia BioTech). His-tagged GFP [45] and its fusion proteins with the various CaaX peptides were purified by affinity chromatography through nickel-loaded sepharose [46]. The proteins had the octapeptide HHHH-HHGG fused to the amino terminus of GFP and the peptide sequences listed in Figure 4 fused to the carboxy-terminal amino acid of GFP.

Screening of the molecular forceps libraries with His-tagged Ras peptide

The peptide HHHHHH-GSMSCKCVLS was incubated with library beads in 50 mM Tris at pH 8.0 room temperature overnight. The bead suspension was then equilibrated with a solution of copper(II) sulfate and washed to remove excess of His-tagged ligands and copper ions. After addition of dilute solutions of benzidine and hydrogen peroxide, beads interacting with the His-tagged ligands develop a brown color which increases over time [39]. The brown/dark beads were picked under a dissecting microscope (30-fold magnification) and decoded using GC analysis [32].

Screening of the molecular forceps libraries with fluorescein-labeled Ras protein

Purified GST and GST-H-Ras-Val12 proteins were labeled at their sulfhydryl groups with fluorescein-5-maleimide following the manufacturer's guidelines (Pierce Chemical Company) using a buffer containing 20 mM sodium phosphate pH 7.0, 150 mM NaCl, and 5 mM EDTA. About 7000 forceps library beads were first incubated with FM–GST (after blocking with binding buffer containing 20 mM sodium phosphate pH 7.0, 500 mM NaCl, 0.1% Tween-20, 0.02% NaN_3, and 5 mg/ml bovine serum albumin (BSA)), and green fluorescent beads (~350 beads, ~5% of total) were removed. The remaining beads were incubated with FM-GST-H-Ras–Val12, washed with the binding buffer three times, and examined under the microscope. The positive beads were picked up (65 of ~6600 beads, 0.9% of total), washed and decoded using GC analysis [32].

Reconfirming the binding of forceps molecules to the Ras protein

The positive forceps molecules were synthesized on the Tentagel beads. The beads were washed several times with water and incubated with the binding buffer containing bovine serum albumin. Then the beads were equilibrated with FM-GST-H-Ras-Val12 for 2 h. After washing with the binding buffer three times, they were examined under the dissecting microscope.

FTase inhibition assay – filter assay for proteins

To assay the farnesylation of protein substrates, we employed a precipitation assay. The FTase assay was carried out according to the protocol described by Casey and coworkers [41] with the following modifications. 5 μg of H-Ras or GFP–CaaX fusion proteins and the test compounds were mixed in 40 μl of transferase buffer (final buffer concentration: 50 mM Tris–HCl, pH 7.7, 20 mM KCl, 5 mM $MgCl_2$, 5 μM $ZnCl_2$, 2 mM DTT and 0.5 mM Zwittergent 3–14) and preincubated on ice for 10 min. Enzyme solution (10 μl; containing 0.1 μg of yeast GST-FTase [41]) and 0.1 μCi [^{3}H]-FPP/FPP (1:2) were added and incubated at 37°C for 30 min. The reaction was stopped by addition of 250 μl of 4% SDS. After 20 min at room temperature, 250 μl of 30% trichloroacetic acid (TCA) were added, vortexed and incubated at room temperature for 30 min. The mixture was filtered through a membrane (Schleicher & Schuell AE91, 25 mM, 0.8 μ). The membrane was washed three times with 1 ml of 4% SDS and 6% TCA, followed by 1 ml of 6% TCA (twice). The extent of incorporated radioactivity on the membrane was quantified by scintillation counting.

FTase inhibition assay – scintillation proximity assay for peptides

To assay the farnesylation of biotinylated peptides, we employed a scintillation proximity assay. The scintillation proximity assay of FTase was carried out according to the manufacturer's protocol (Amersham Life Science) using biotinylated CaaX peptides as substrates [47]. The biotinylated peptides (10 pmol) were farnesylated for 20 min in the assay buffer (50 mM Hepes, 30 mM $MgCl_2$, 20 mM KCl, 5 mM DTT, 0.01% Triton X-100, pH 7.5) with 0.1 μCi [^{3}H]-FPP and 0.1 μg of yeast GST-FTase [41]. The peptides were then precipitated with 'STOP/bead reagent' and the incorporated radioactivity on the beads was quantified by scintillation counting.

In vitro *binding assay between the forceps molecules and GST-fusion proteins*

GST, GST–FTase, GST–H-Ras-Val12 or GFP–CaaX fusion proteins (6 μg) were mixed with biotinylated molecular forceps (final concentration 250 μM) in the binding buffer containing 50 mM Tris, pH 7.5, 100 mM NaCl, 4 mg/ml ovalbumin, 1 mg/ml gelatin in a final volume of 40 μl, and incubated on ice for 2 h. Then 100 μl of avidin-sepharose beads which had been equilibrated with the binding buffer were added and incubated for 30 min with constant shaking. The beads were washed with the binding buffer three times, and finally resuspended in 80 μl of SDS sample buffer. The supernatant was loaded onto a 10% SDS–polyacrylamide gel, and the GST bearing proteins were detected using western blotting using primary anti-GST antibody and horseradish peroxidase-conjugated secondary anti-rabbit antibody. The GFP–CaaX fusion proteins were detected using primary anti-$(His)_6$-antibody and horseradish peroxidase-conjugated secondary anti-rabbit antibody.

Acknowledgements

We want to thank W. Clark Still (Columbia University) for the fruitful discussions and his ongoing support. We appreciate the gift of the pRSET-GFP plasmid by Roger Tsien and Andrew Cubitt (UCSD) and thank Fuyu Tamanoi (UCLA) for providing us with the yeast FTase pGEX-DPR1 and pBC-RAM2 constructs. We acknowledge the gift of purified FTase from bovine brain by Patrick Casey and John Mohoney (Duke University Medical Center). We are especially thankful to Ryuji Kobayashi, Nora E. Poppito, and Kimberly A. Wanat from the Cold Spring Harbor Laboratory Protein Facility for their diligent help in sequencing peptides. The research reported here was supported by NCI-grant OIG-CA39829 (MHW), NCI Young Investigator Development Funds NIH 5P30CA45508-09 (HPN), NSF-grants MCB-9728062 and CHE-9709042 (HPN), and Cold Spring Harbor Laboratory Association Interim Research Funds (HPN). D.L.D. and R.L. thank the NIH for NCI Training Grants.

References

1. Thompson, L.A. & Ellman, J.A. (1996). Synthesis and application of small molecule libraries. *Chem. Rev.* **96**, 555-600.
2. Terrett, N.K., Gardner, M., Gordon, D.W., Kobylecki, R.J. & Steele, J. (1995). Combinatorial synthesis – the design of compound libraries and their application to drug discovery. *Tetrahedron* **51**, 8135-8173.
3. Gallop, M.A., Barrett, R.W., Dower, W.J., Fodor, S.P.A. & Gordon, E.M. (1994). Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries. *J. Med. Chem.* **37**, 1233-1251.
4. Gordon, E.M., Barrett, R.W., Dower, W.J., Fodor, S.P.A. & Gallop, M.A. (1994). Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions. *J. Med. Chem.* **37**, 1385-1401.
5. Lowe, G. (1995). Combinatorial chemistry. *Chem. Soc. Rev.* 309-317.
6. Balkenhohl. F., von dem Bussche-Hünnefeld, C., Lansky, A. & Zechel, C. (1996). Combinatorial synthesis of small organic molecules. *Angew. Chem. Int. Ed. Engl.* **35**, 2288-2337.
7. Lam, K.S., Lebl, M. & Krchňák, V. (1997). The "one-bead-one-compound" library method. *Chem. Rev.* **97**, 411-448.
8. Still, W.C. (1996). Discovery of sequence-selective peptide binding by synthetic receptors using encoded combinatorial libraries. *Accounts Chem. Res.* **29**, 155-163.
9. Gennari, C., Nestler, H.P., Piarulli, U. & Salom, B. (1997). Combinatorial libraries: studies in molecular recognition and the quest for new catalysts. *Liebigs Ann./Recueil* 637-647.
10. Borman, S. (1994). Combinatorial chemistry used to probe binding. *Chem. Eng. News* **72**, 37-38.
11. de Miguel, Y.R. & Sanders, J.K.M. (1998). Generation and screening of synthetic receptor libraries. *Curr. Opin. Chem. Biol.* **2**, 417-421.
12. Nestler, H.P. & Liu, R. (1998). Combinatorial libraries: studies in molecular recognition. *Combin. Chem. High Throughput Screen.* **1**, 133-146.
13. Boyce, R., Li, G., Nestler, H.P., Suenaga, T. & Still, W.C. (1994). Peptidosteroidal receptors for opoid peptides. sequence-selective binding using a synthetic receptor library. *J. Am. Chem. Soc.* **116**, 7955-7956.
14. Gennari, C., Nestler, H.P., Salom, B. & Still, W.C. (1995). Synthetic receptors based on vinylogous sulfonyl peptides. *Angew. Chem. Int. Ed. Engl.* **34**, 1765-1768.
15. Nestler, H.P. (1996). Sequence-selective, non-macrocyclic, two-armed receptors for peptides. *Mol. Diversity* **2**, 35-40.
16. Barbacid, M. (1987). *Ras* genes. *Ann. Rev. Biochem.* **56**, 779-827.
17. Lowy, D.R. & Willumsen, B.M. (1993). Function and regulation of *Ras*. *Ann. Rev. Biochem.* **62**, 851-891.
18. Clarke, S., Vogel, J.P., Deschenes, R.J. & Stock, J. (1988). Post-translational modification of the Ha-*ras* oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc. Natl Acad. Sci. USA* **85**, 4643-4647.
19. Gutierrez, L., Magee, A.I., Marshall, C.J. & Hancock, J.F. (1989). Post-translational processing of *p21ras* is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J.* **8**, 1093-1098.
20. Powers, S., *et al*,. & Wigler, M.H. (1986). *RAM*, a gene of yeast required for a functional modification of *RAS* proteins and for production of mating pheromone a-factor. *Cell* **47**, 413-422.
21. Hancock, J.F., Magee, A.I., Childs, J.E. & Marshall, C.J. (1989). All *ras* proteins are polyisoprenylated but only some are palmitoylated. *Cell* **57**, 1167-1177.
22. Kato, K., Cox, A.D., Hisaka, M.M., Graham, S.M., Buss, J.E. & Der, C.J. (1992). Isoprenoid addition to Ras protein Is the critical modification for Its membrane association and transforming activity. *Proc. Natl Acad. Sci. USA* **89**, 6403-6407.
23. Kohl, N.E., *et al*., & Gibbs, J.B. (1993). Selective inhibition of *Ras*-dependent transformation by a farnesyl transferase inhibitor. *Science* **260**, 1934-1937.
24. James, G.L., *et al*., & Marsters, J.C., Jr. (1993). Benzodiazepine peptidomimetics: potent inhibitors of Ras farnesylation in animal cells. *Science* **260**, 1937-1942.
25. Nigam, M., Seong, C.-M., Qian, Y., Hamilton, A.D. & Sebti, S.M. (1993). Potent inhibition of human tumor $p21^{ras}$ farnesyltransferase by A^1A^2-lacking $p21^{ras}$ CA^1A^2X peptidomimetics. *J. Biol. Chem.* **268**, 20695-20698.
26. Wallace, A., *et al*., & Pessi, A. (1996). Selection of potent inhibitors of farnesyl-protein transferase from a synthetic tetrapeptide combinatorial library. *J. Biol. Chem.* **271**, 31306-31311.
27. Kohl, N.E., *et al*., & Oliff, A.I. (1994). Peptidyl farnesyltransferase inhibitors block the growth of *Ras*-dependent tumors in nude mice. *Proc. Natl Acad. Sci. USA* **91**, 9141-9145.
28. Miyake, M., Mizutani, S., Koide, H. & Kaziro, Y. (1996). Unfarnesylated transforming Ras mutant inhibits Ras-signaling pathway by forming a stable Ras/Raf complex in the cytosol. *FEBS Lett.* **378**, 15-18.

29. Cheng, Y., Suenaga, T. & Still, W.C. (1996). Sequence-selective peptide binding with a peptido-A,B-*trans*-steroidal receptor selected from an Encoded combinatorial receptor library. *J. Am. Chem. Soc.* **118**, 1813-1814.
30. Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M. & Knapp, R.J. (1991). A new type of synthetic peptide library for identifying ligand-binding activity. *Nature* **354**, 82-86.
31. Furka, Á., Sebestyén, F., Asgedom, M. & Dibô, G. (1991). General method for rapid synthesis of multicomponent peptide mixtures. *Int. J. Pept. Protein Res.* **37**, 487-493.
32. Nestler, H.P., Bartlett, P.A. & Still, W.C. (1994). A general method for molecular tagging of encoded combinatorial chemistry libraries. *J. Org. Chem.* **59**, 4723-4724.
33. Ohlmeyer, M.H.J., *et al.*, & Still, W.C. (1993). Complex synthetic chemical libraries indexed with molecular tags. *Proc. Natl Acad. Sci. USA* **90**, 10922-10926.
34. Chen, J.K. & Schreiber, S.L. (1995). Combinatorial synthesis and multidimensional NMR spectroscopy: an approach to understanding protein-ligand interactions. *Angew. Chem. Int. Ed. Engl.* **34**, 953-969.
35. Chen, J.K., Lane, W.S., Brauer, A.W., Tanaka, A. & Schreiber, S.L. (1993). Biased combinatorial libraries: novel ligands for the SH3 domain of phosphatidylinositol 3-kinase. *J. Am. Chem. Soc.* **115**, 12591-12592.
36. Borchardt, A. & Still, W.C. (1994). Synthetic receptor binding elucidated with an encoded combinatorial library. *J. Am. Chem. Soc.* **116**, 373-374.
37. Wennemers, H., Yoon, S.S. & Still, W.C. (1995). Cyclooligomeric receptors based on trimesic acid and 1,2-diamines. minimal structure for sequence-selective peptide binding. *J. Org. Chem.* **60**, 1108-1109.
38. Wennemers, H. & Still, W.C. (1994). Peptide complexation in water. sequence-selective binding with simple dye molecules. *Tetrahedron Lett.* **35**, 6413-6416.
39. Liu, R., Sherlock, R. & Nestler, H.P. (1998). Using the copper-catalyzed benzidine blue reaction for the detection of binding of hexahistidine-tagged proteins and peptides to combinatorial library beads. In *Innovation and Perspectives in Solid Phase Synthesis 1998.* (Epton, R. ed) Mayflower Scientific Ltd., Kingswinford, UK.
40. Tamanoi, F. & Mitsuzawa, H. (1995). Use of yeast for identification of farnesyltransferase inhibitors and for generation of mutant farnesyl transferases. *Methods Enzymol.* **255**, 82-91.
41. Moomaw, J.F., Zhang, F.L. & Casey, P.J. (1995). Isolation of protein prenyltransferases from bovine brain and baculovirus expression system. *Methods Enzymol.* **250**, 12-21.
42. Garcia, A.M., Rowell, C., Ackermann, K., Kowalczyk, J.J. & Lewis, M.D. (1993). Peptidomimetic inhibitors of Ras farnesylation and function in whole cells. *J. Biol. Chem.* **268**, 18415-18418.
43. Stewart, J.M. & Young, J.D. (1984). *Solid phase peptide synthesis.* Pierce Chemical Company, Rockford, IL.
44. Krchňák, V., Vágner, J., Safár, P. & Lebl, M. (1988). Noninvasive continuous monitoring of solid-phase peptide synthesis by acid-base indicator. *Collect. Czech. Chem. Commun.* **53**, 2542-2548.
45. Cubitt, A.B., Heim, R., Adams, S.R., Boyd, A.E., Gross, L.A. & Tsien, R.Y. (1995). Understanding, improving, and using green fluorescent proteins. *Trends Biochem. Sci.* **20**, 448-455.
46. Feng, G. & Winkler, M.E. (1995). Single-step purification of His^6-MutH, His^6-MutL, and His^6-MutS repair proteins of *Escherichia coli K12*. *BioTechniques* **19**, 956-965.
47. Reiss, Y., Goldstein, J.L., Seabra, M.C., Casey, P.J. & Brown, M.S. (1990). Inhibition of purified $p21^{ras}$ farnesyl:protein transferase by Cys-AAX tetrapeptides. *Cell* **62**, 81-88.

Because *Chemistry & Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published via the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cmb – for further information, see the explanation on the contents pages.