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In Vitro Inhibition of Ras–Raf Association by Short Peptides

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Seven amino acid peptides were tested as *in vitro* inhibitors of oncogenic Ras–Raf association. The sequences of these peptides were derived from the H-Ras effector region (amino acids 25 to 51) and the Ras binding domain of Raf-1 (amino acids 64 to 105). Eleven out of the twenty-one Ras 7-mers tested inhibited formation of the Ras–Raf complex by at least 20% at 100 μM. The most potent of these inhibitory peptides contained the effector residues 32 to 37 or 40 to 45. Of the Raf-1 peptides tested, only the 94-ECCAVFR-100 and 95-CCAVFRL-101 peptides were significant inhibitors of Ras–Raf binding. The 95-101 Raf peptide had an IC_{50} value of 7 μM and also inhibited Ras–RalGDS binding. Analysis of the 95–101 peptide showed that its inhibitory activity required at least one cysteine followed by several hydrophobic residues. Our results demonstrate the feasibility of using small molecules as inhibitors of Ras protein–protein interactions.

Molecular genetic analysis of a majority of human malignancies has demonstrated that abnormal regulation of the Ras signaling pathway is a common element. The mammalian *ras* genes (Ha-*ras*, Ki-*ras* and N-*ras*) encode homologous 21 kilodalton, membrane associated proteins [1]. The Ras proteins are small molecular weight G-proteins, becoming active upon GTP binding, and inactive following hydrolysis of the GTP [2]. Activation of specific growth factor receptors promotes the exchange of GTP for GDP on Ras [3]. Oncogenically activated Ras proteins have either impaired GTPases or enhanced nucleotide exchange rates that increase the proportion of Ras-GTP complex in the cell [4]. The Ras proteins control a key point in signal transduction between growth factor receptors and the Raf-dependent MAP kinase signaling cascade [5].

Once bound to GTP, a small surface region of Ras, commonly known as the effector loop, or switch I, changes conformation [6]. The effector loop consists of amino acids 32 to 40, and directly mediates the association of Ras with it's target proteins. The effector loop is flanked on both sides by residues which do not change confirmation but contribute to the specificity of the binding interaction with different target proteins. This effector region of Ras-GTP binds to an amino-terminal domain of the Raf serine/threonine kinase known as the Ras binding domain or RBD [5]. The 100 amino acid RBD of Raf-1 has been well defined by both genetic and biophysical studies [7, 8]. In this laboratory, we have previously used alanine scanning mutagenesis and peptide contact epitope scanning to pinpoint specific sites of interaction between Ras and Raf-1 [9]. Alanine scanning mutagenesis suggested that Raf residues 64-67 and 80-103 were important for association with Ras-GTP. Individual residues Q66, R67, K84, K87, R89 and D91 were further identified as the most critical individual points of contact with the Ras protein. A peptide containing Raf-1 residues 91-105 was found to potently interfere with Ras-Raf association. We also reported that fifteen amino acid peptides overlapping Ras residues 25 to 51 could also interfere with the association of Ras and Raf-1 *in vitro*. The results of our study were confirmed by the solution of a co-crystal consisting of Raf-1 residues 55 to 131 complexed to a fragment of the Ras-related protein Rap1A [8]. From this structure it was inferred that an extended beta sheet is formed between the binding surfaces of Ras and the Raf-1 RBD which is held together in part by interactions.

Aside from identifying potential binding sites in the Raf protein, our previous study suggested that the Ras-Raf-1 protein interaction could be blocked with moderately sized peptides. An important question remained whether inhibitory peptides could be shortened in length and remain effective in preventing Ras-Raf interactions. In this study we examined the effects of truncation and modification of both Ras effector region

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and Raf-1 RBD inhibitory peptides on their ability to interfere with the Ras-Raf binding interaction. We report that both the 15-mer Ras and Raf-1 based peptides could be shortened to at least seven amino acids and still effectively block Ras-Raf association. Furthermore, the potency of these small peptides could be increased by modification. These results provide support for the further development of small molecular weight inhibitors of the Ras-Raf protein-protein interaction as anti-proliferative therapeutics.

METHODS

Peptide synthesis. Peptides were synthesized by Chiron Mimotopes Peptide Systems using the Multipin peptide synthesis procedure. All peptides were acetylated and ended with a C-terminal amide. Purity was estimated at approximately 90% for the peptide set. Peptides were resuspended in degassed, room temperature dimethylformamide by brief sonication, at a concentration of 4 mM. Peptide solutions were stored at −80° C under nitrogen.

Inhibition of Ras-Raf-1 RBD association by peptides. An ELISA method was used to detect the binding of Ras[L61]-GTP to the Raf-1 RBD. The method used was identical to that already published for Ras and p120RasGAP except that GST-Raf[1-149] was substituted for GST-GAP [10]. The ability of each peptide to interfere with the association of Raf and Ras was measured by adding increasing concentrations of each peptide in dimethylformamide (DMF) to the assay at the time of Ras-GTP addition, keeping the final DMF concentration at 5%. The assay was found to be unaffected by the presence of 10% DMF.

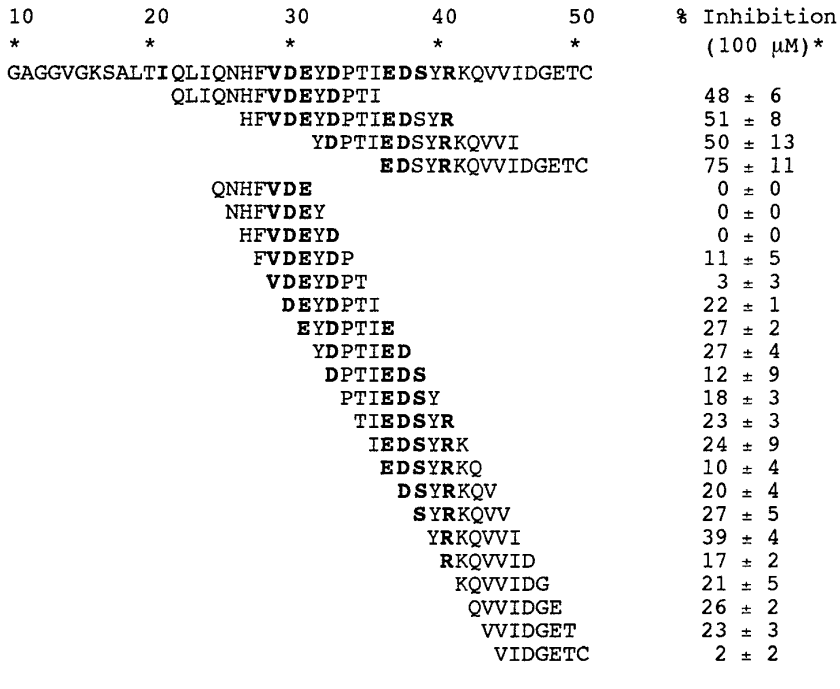
Protein purification and guanine nucleotide loading of H-Ras[L61]. H-Ras[L61] was expressed in *E. coli* and purified using Mono Q anion-exchange chromatography and size exclusion chromatography using Sephadex G-75 as previously described [11]. Ras was bound to radioactively labeled GTP using a protocol described elsewhere [11]. Raf [1-149]-GST was expressed in *E. coli* strain RR1laci^q as described [9]. Proteins were stored frozen at −80° C.

RESULTS

Seven-amino acid peptides derived from the effector region can interfere with Ras-Raf association. We previously demonstrated that overlapping, 15-amino acid peptides spanning residues 25 to 51 of H-Ras could inhibit formation of a Ras-Raf complex *in vitro.* A series of overlapping seven amino acid-long peptides were synthesized spanning the 25 to 51 effector region of Ras and tested as inhibitors of Ras/Raf binding (Figure 1). Eleven out of the twenty-one Ras 7-mers tested inhibited formation of the Ras-Raf complex by at least 20% at a concentration of 100 μM. The most potent inhibitory peptides contained amino acids 32 to 37 or 40 to 45. Only the 40-46 Ras peptide approximated the potency of the 15 amino acid Ras peptides in preventing Ras-Raf association (39% inhibition at 100 μM).

Seven-amino acid peptides derived from the Raf-1 RBD can potently interfere with Ras-Raf association. A series of seven amino acid-long peptides were synthesized spanning regions of Raf-1 believed to be involved in Ras binding (64 to 70, 83 to 89 and 91 to 105) and tested as inhibitors of Ras/Raf binding (Figure 2). The 64-70 peptide was completely noncompetitive, similar to the 61-75 peptide we previously reported. The 83-89 peptide weakly inhibited Ras-Raf association as was previously observed with the fifteen amino acid 80-94 peptide. We attempted to increase the competitive ability of the 83-89 peptide by decreasing the hydrophobicity of the peptide. Residues 83, 86 and 88 were variously substituted with alanine, aspartic acid or lysine. The most hydrophilic peptide, in which M83 was replaced with aspartic acid and V88 was substituted with arginine, showed a four-fold increase in the inhibition of Ras-Raf binding. Out of all the Raf-based 7-mers, the 95-101 peptide most potently inhibited Ras-Raf association. This result is consistent with our previous mutagenic analysis and peptide inhibitor studies which demonstrated the importance of Raf-1 residues 91-103 in Ras binding. The 95-101 and larger 91-105 peptides had similar IC_{50} values for blocking Ras-Raf association showing that residues 95-101 are the active region of the larger peptide. The 95-101 peptide was also observed to interfere with Ras/RalGDS associations (Figure 3) confirming the specificity of the peptide for binding to the Ras protein. The 95-101 peptide had little to no effect on Ras-GTP exchange at the IC_{50} concentration (data not shown).

Substitution analysis of the 95-101 Raf peptide. We wished to identify essential and dispensable amino acids within the sequence of the 95-101 peptide inhibitor. Each position was modified with a conservative amino acid change as shown below in Figure 4 and the resulting peptides tested as inhibitors of Ras/Raf binding. The potency of the 95-101 peptide was reduced three to four-fold when one or both cysteines were changed to serine. Changing both cysteines to serine reduced inhibition by 10-fold, confirming the importance of these two residues. The addition of up to 8 mM DTT to the assay had no effect on inhibition by the 95-101 peptide (data not shown) suggesting that disulfide bond formation is not part of the mechanism of action. Substitution of V98 with threonine, F99 with tyrosine and R100 with lysine had little effect on the ability of the peptide to interfere with Ras-Raf binding. Substituting L101 with alanine significantly decreased the ability of the peptide to inhibit. Transposition of C91 to the C-terminus of the peptide resulted in a loss of activity similar to the substitution of either cysteine with serine. Introducing N-methylated amino acids into the core of the 95-101 peptide resulted in a complete loss of activity, which was likely due either to decreased solubility (which was not detected) or loss of rotational freedom within the peptide preventing an active binding conformation. The potency of the 95-101 peptide was improved somewhat by changing alanine 97 to an isoleucine. While this change did not effect the IC_{50}, the maximum level of inhibition was attained at lower concentrations of the peptide (data not shown).



* Data for 15-mer peptides from previous study at 200 μM.

FIG. 1. Ras effector region 7-mers inhibit Ras-Raf association. Seven amino acid peptides based upon the amino acid sequence of the Ras effector region were tested for their ability to inhibit Ras[L61]-GTP and Raf-1 RBD complex formation. Residues in bold are known to interact directly with the Raf-1 RBD [8].

DISCUSSION

We have examined the ability of small, seven amino acid peptides to interfere with the specific association of Ras-GTP with the Raf-1 RBD. Specifically, this study was undertaken to test the ability of relatively small macromolecules to effectively block protein-protein interactions between oncogenic Ras and its primary effector molecule Raf-1. The successful application of this technique would justify the development of therapeutic small molecule inhibitors of Ras designed to interfere with target binding. The sequences of these peptides were based upon regions of both Ras and Raf-1 previously demonstrated to be involved in binding. Clearly

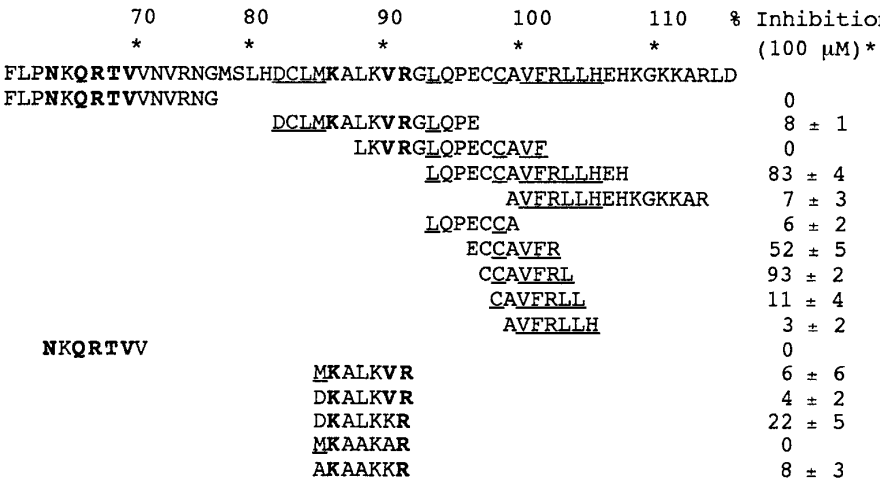


FIG. 2. Raf-1 RBD 7-mers inhibit Ras-Raf association. Seven amino acid peptides based upon the amino acid sequence of the Raf-1 RBD were tested for their ability to inhibit Ras[L61]-GTP and Raf-1 RBD complex formation. Residues in bold interact with Ras-GTP [8]. Underlined residues are also important for stable association between Ras and Raf [9].

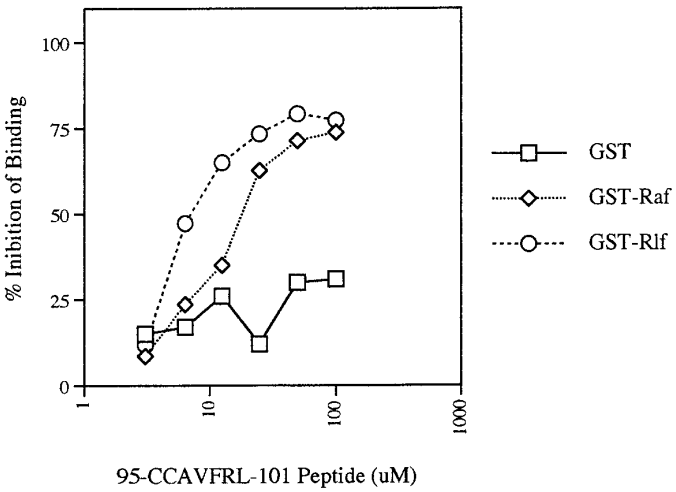


FIG. 3. The Raf-1 95-CCAVFRL-101 peptide interferes with the association of Ras with both Raf-1 and Rlf. The 95-CCAVFRL-101 peptide was tested at increasing concentrations for the ability to inhibit complex formation between Ras[L61]-GTP and either the Raf-1 or Rlf RBDs.

seven amino acid peptides derived from the protein binding sites of both Ras and Raf-1 were capable of inhibiting the stable association of Ras-GTP with the Raf-1 RBD, demonstrating that relatively small molecules can disrupt the formation of the Ras-Raf signaling complex.

The seven amino acid Ras-based peptides progressively overlapped the effector region and flanking residues. Inhibition was seen with 7-mers containing sequences derived from the conformation-dependent loop (residues 32-40) as well as C-terminal to the loop. The most potent inhibitory Ras peptide contained primarily residues which flanked the effector loop (residues 40-46). Only the 40-46 peptide was approximately as potent as the original 15 amino acid Ras peptide inhibitors. These results are supportive of the importance of an extended region of Ras in mediating target protein binding.

In contrast to the Ras-based peptides, 7-mer peptides derived from the Raf-1 RBD were mostly non-inhibitory. As we previously reported, peptides derived from the two regions of the Raf-1 RBD shown to be involved in Ras binding by X-ray crystallography were poor inhibitors (residues 64-70 and 83-89). Residues 83-89 are part of an amphipathic alpha helix which positions several basic residues for electrostatic interactions with Ras. We hoped that reducing the hydrophobic component of this peptide by substituting charged amino acids into non-binding positions would increase the ability of the peptide to inhibit Ras-Raf association. In fact substitution of aspartic acid for M83 and lysine for V88 did increase the effectiveness of the peptide, but not to a degree considered practical for therapeutic inhibition. The most effective Raf peptide was the 95-101 peptide which inhibited the association of Ras and Raf at a low μM concentration. Although the role of these amino acids in binding to Ras-GTP is not yet clear, peptides containing this sequence are potent inhibitors. That this peptide binds specifically to Ras is supported by our observations that the 95-101 seven-mer also interferes with Ras-RalGDS complex formation without promoting nucleotide exchange or hydrolysis.

The active residues in the 95-101 inhibitor peptide were characterized by synthesizing additional peptides in which each residue was substituted with a conservative amino acid. From this analysis we conclude that the presence of two adjacent cysteines followed by hydrophobic amino acids are important for the low μM inhibition of Ras-Raf binding. In fact, increasing the hydrophobicity at position 97 improved the inhibitory properties of the peptide. Positions 98, 99 and 100 could tolerate conservative changes without reducing potency. N-methyl substituted amino acids were placed into positions 96, 97 and 98 and were observed to completely inactivate the inhibitory properties of the peptide. The presence of the N-methyl group prevents free rotation around the peptide bond, suggesting that the 95-101 seven-mer requires a specific conformation for its activity.

The results of this study suggest that small molecules can function as specific inhibitors of Ras-Raf association and therefore Raf activation. The properties of the Raf-1 95-101 peptide suggest that a reasonably small non-peptidyl derivative could be synthesized which would bind to Ras and prevent interactions with downstream target proteins such as Raf and RalGDS. Unfortunately, the 95-101 peptide did not remain in solution following dilution into tissue culture medium, preventing analysis of its properties as an *in vivo* inhibitor of Ras function (data not shown). However, our results demonstrate that that the affinity of Ras-GTP

Peptide Sequence	IC50 (μM)
91-LQPECCAVFRLLHEH-105	10
CCAVFRL	7
SCAVFRL	23
CSAVFRL	30
SSAVFRL	75
CCIVFRL	5
CCATFRL	7
CCAVYRL	10
CCAVFKL	10
CCAVFRA	21
CAVFRLC	28
CC(N-methyl-A)VFRL	nd
CCA(N-methyl-V)FRL	nd
CCAV(N-methyl-F)RL	nd

FIG. 4. Substitution analysis of the Raf-1 95-CCAVFRL-101 peptide inhibitor. Variants of the 95-CCAVFRL-101 peptide were synthesized containing amino acid substitutions at different positions. The IC_{50} value for inhibiting Ras[L61]-GTP and Raf-1 RBD association was determined for each peptide.

for Raf and RalGDS is sufficiently low to prevent stable protein associations with a molecule at least as small as a seven amino acid peptide.

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