"Peptide Walking" Is a Novel Method for Mapping Functional Domains in Proteins

ITS APPLICATION TO THE Ras-DEPENDENT ACTIVATION OF NADPH OXIDASE*

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Activation of the superoxide generating NADPH oxidase of phagocytes involves the assembly of a multimeric complex and is dependent on the participation of the small molecular weight GTP-binding protein Ras (1 or 2). This model system was used for mapping functional domains in the primary sequence of Rac1, based on assessing the inhibitory effect of 90 individual overlapping pentadecapeptides, spanning the entire length of Rac1, on NADPH oxidase activation in two types of cell-free assay. Five functional domains were identified, each consisting of a cluster of contiguous residues shared by members of five groups of overlapping inhibitory peptides. Four of the five domains are exposed on the molecular surface of Rac1 and were not identified previously by mutational analysis; the fifth corresponds to a polybasic motif near the carboxyl terminus, confirming earlier reports. Screening the entire linear sequence of a protein with a battery of overlapping peptides for interference with its ability to interact with upstream or downstream molecules should be of wide applicability as a reliable, fast, and economical method for mapping of functionally relevant domains.

Oxygen-derived radicals are essential components in the microbial armory of phagocytic cells. The primordial oxygen radical, superoxide (O$_2^-$), is generated by the one-electron reduction of molecular oxygen catalyzed by a membrane-contained heterodimeric flavocytochrome (cytochrome b$_{559}$) utilizing cytosolic NADPH as the electron donor (reviewed in Refs. 1 and 2). The catalytic activity of cytochrome b$_{559}$ is initiated by its interaction with at least two cytosolic components, p47$^\text{phox}$ and p67$^\text{phox}$, which translocate to the membrane, leading to the assembly of what is known as the NADPH oxidase complex (reviewed in Ref. 3). NADPH oxidase activation can be mimicked in vitro by a cell-free system, consisting, in its most elementary form, of phagocyte membranes and cytosol (4, 5) and in its more sophisticated versions, of purified or recombinant components (6, 7), supplemented with a critical concentration of an anionic amphiphile, such as arachidonic acid or the sodium or lithium salts of dodecylsulfonic acid.

Activation of NADPH oxidase was found to involve a GTP-binding protein (8, 9) and this was identified as the Rho-like GTPase Rac1 (10) or Rac2 (11). Rac is found in the phagocyte cytosol as a heterodimer with the regulatory protein GDP dissociation inhibitor for Rho (Rho GDI) (10, 12). The central role of Rac in the activation of NADPH oxidase complex suggests that this well defined system could serve as a suitable model for the understanding of Rac function in particular and that of proteins of the Rho subfamily, in general. So far, two approaches have been used for this purpose. In the first, interaction of Rac with individual oxidase components was investigated, leading to the finding that Rac1, in the GTP-bound form, binds to p67$^\text{phox}$ (13, 14). The second approach was based on studying the effect of mutations performed in the putative effector region of Rac (based on the Ras model) on the ability of Rac to support NADPH oxidase activation in the cell-free system. This methodology led to the identification of residues 26, 27, 28, 30, 33, 35, 36, 38, 40, and 45 in Rac1 as being essential for the expression of oxidase activating activity (13, 15–17).

Here we describe a novel methodology for the identification of functional domains in the primary structure of proteins. This is based on testing a large set of overlapping synthetic peptides, spanning the entire amino acid sequence of the protein, for an inhibitory effect on an enzymatic reaction in which the relevant protein is an obligatory participant. We have applied this approach to the analysis of Rac1, with the purpose of identifying domains essential for sustaining activation of NADPH oxidase. Overlapping peptides were used in the past, almost exclusively, for mapping antibody-defined (18) and T cell-defined (19) linear epitopes in protein antigens.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—Ninety overlapping pentadecapeptides, spanning the entire length of the Rac1 sequence, starting at the first 15 residues at the amino terminus, were synthesized by the multipin synthesis method (20) by Chiron Mimotope Peptide Systems, Clayton, Australia. Peptides overlap by 13 residues, with the exception of peptides 89 and 90, which overlap by 14 residues. All peptides were acetylated at the NH$_2$ terminus and ended with a COOH-terminal amide. Purity was approximately 70%. The peptides were dissolved in a mixture (v/v) of 75 parts dimethyl sulfoxide and 25 parts water, to a concentration of 2 mM, divided into small aliquots and stored frozen at −75 °C.

Preparation of NADPH Oxidase Components—Subcellular fractions were obtained from guinea pig peritoneal macrophages, as described in Ref. 21. Membranes were solubilized in n-octyl-$\beta$-D-glucopyranoside and processed as described in Ref. 22. A cytosolic fraction enriched in p47$^\text{phox}$ and p67$^\text{phox}$ was prepared as described in Ref. 23. Highly purified Rac1-Rho GDI dimer was prepared from macrophage cytosol, as recently described (12, 24). Recombinant human Rac1 was isolated by purification of a glutathione S-transferase fusion protein from Escherichia coli transformed with Rac1-cDNA subcloned into the bacterial expression vector pGEX2T (25). Purified recombinant Rac1 was loaded with guanosine 5'-O-(thio)triphosphate (GTP$\gamma$S), as described in Ref. 10.

Cell-free NADPH Oxidase Assay—Cell-free O$_2^-$ production assays were performed at 25 °C in 96-well plates in final volumes of 170–174 μl. Reaction mixtures consisted of (in the order of addition): 10 μl of solubilized membrane (resulting in a final concentration of cytochrome b$_{559}$ of 5 mM); 10 μl of cytosol or a mixture of 10 μl of a cytosolic fraction...
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Enriched in p47phox and p67phox (corresponding to final concentrations of 1.5 and 0.6 mM, respectively) and 4 μl of highly purified Rac1-Rho GDI dimer (4.1 nm Rac1) or 2 μl of recombinant GTPγS-bound Rac1 (130 mM), and 140 μl of assay buffer (2), containing 200 μM cytochrome c and 330 μM lithium dodecyl sulfate, as the activator. The reaction mixtures were incubated for 90 s and catalysis initiated by the addition of 10 μl of NADPH oxidase activities, in the absence of Rac1, were less than 5 mol of O₂.

Fig. 2. Peptides prevent NADPH oxidase activation but do not interfere with catalytic activity. Peptide 46 (residues 91–105) was tested in a reaction mixture consisting of solubilized membrane, a cytosolic fraction enriched in p47phox and p67phox, and purified Rac1-Rho GDI dimers. Results are expressed as maximum rates of O₂-reduction, at 550 nm, in a kinetic assay consisting of 30 measurements at 10-s intervals performed in a Ceres 900 HDi microplate reader (Bio-Tek Instruments), using KC Junior software. Results are derived from 4–9 experiments.

RESULTS AND DISCUSSION

Each of the 90 individual peptides was tested, at a concentration of 20 μM, for the ability to inhibit O₂-reduction in two types of cell-free NADPH oxidase activation assays. The first assay consisted of solubilized macrophage membranes combined with cytosol, whereas the second contained solubilized macrophage membranes combined with a cytosolic fraction enriched in p47phox and p67phox and highly purified cytosolic Rac1-Rho GDI. As apparent in Fig. 1, several groups of peptides caused significant and reproducible inhibition of NADPH oxidase activation. There was very good correlation between the ability of specific peptides to inhibit O₂-reduction by mixtures of membranes and total cytosol (Fig. 1A) and their effect in a cell-free system consisting of membranes and purified cytosolic components (Fig. 1B). Substitution of purified Rac1-Rho GDI dimer by recombinant Rac1, in the GTPγS-bound form, yielded identical results (data not shown). With one exception, all inhibitory peptides corresponded to residues located in the carboxy-terminal two-thirds of the Rac1 molecule. The inhibitory peptides were clustered in five groups: a, peptides 34–37 (spanning residues 67–87); b, peptides 46–53 (spanning residues 91–119); c, peptides 60–62 (spanning residues 119–137); d, peptides 77–83 (spanning residues 153–179); and e, peptides 86–90 (spanning residues 171–192). In addition to these clusters, three isolated peptides were found to be inhibitory: peptide 7 (residues 13–27) was weakly inhibitory, whereas peptides 43 (residues 85–99) and 55 (residues 109–123) caused significant inhibition.

There were significant differences in the inhibitory potencies of peptides belonging to the various groups. Thus, peptides in groups a and c were the least active (IC₅₀ exceeding 40 μM); peptides in groups b and d were more active (IC₅₀ in the 20–40 μM range), whereas peptides in group e were the most potent (IC₅₀ in the 1 μM range). There were no differences in the IC₅₀ values of peptides when tested in cell-free assays containing purified Rac1-Rho GDI dimer (posttranslationally processed Rac1, in the GDP-bound form; Ref. 26) or recombinant monomeric Rac1 (unprocessed, converted to the GTPγS-bound form). Peptides were inhibitory only when added simultaneously with all components of the cell-free reaction (Fig. 2). No inhibition was observed when peptides were added 1 min after the initiation of activation, indicating that Rac1 peptides interfere with NADPH oxidase activation (assembly) and not with its catalytic function.

Since NADPH oxidase assembly involves the interaction of Rac1 with at least one other oxidase component (13, 14) and probably with other proteins (14, 27–29), inhibition of NADPH oxidase activation by Rac1 peptides offers a suitable model for the mapping of domains in Rac1 involved in protein–protein interactions. The most straightforward interpretation of our findings is that inhibitory peptides mimic domains in the intact Rac1 molecule and, consequently, compete with Rac1 for binding to another component of NADPH oxidase. However, it cannot, at present, be excluded that peptides interfere with some form of intramolecular interaction within Rac1, necessary for NADPH oxidase activation.
Analysis of the five groups of inhibitory peptides revealed that all members of one group shared a minimal sequence domain or part of it. The precise boundaries of the domains could not be determined with absolute certitude by the present set of peptides and will require finer analysis, using peptides with a higher degree of overlap as well as truncated peptides.

A remarkable feature of the grouping of inhibitory peptides was that some clusters could be divided into two subclusters of contiguous active peptides, divided by a group of one or more peptides with lesser or no inhibitory activity. This situation was most clearly expressed in peptide clusters a and d, as illustrated in Fig. 3. It can be seen that in both clusters, the expression of inhibitory activity was maximal when the putative domain was exposed at or close to the center of the peptide (peptides 79–81) and reappeared when exposed at the amino-terminal end (peptide 82).

![Table](https://example.com/table.png)

**Fig. 3.** Relationship between the ability of overlapping Rac1 peptides to inhibit NADPH oxidase activation and the location of the putative functional domain in the peptide sequences.

Results are derived from experiments in which peptides were added to reaction mixtures consisting of solubilized membrane and cytosol (see Fig. 1A). A, maximal inhibition was obtained when the putative domain HHCPN was exposed at the carboxyl-terminal end of the peptide (peptide 47). Activity was absent when the domain was at the center of the peptide (peptide 50) and reappeared when exposed at the amino-terminal end (peptide 52). B, maximal inhibition was obtained when the putative domain RGLKTVF was exposed at the carboxyl-terminal end of the peptide (peptide 78). Activity was absent when the domain was at or close to the center of the peptide (peptides 79–81) and reappeared when exposed at the amino-terminal end (peptide 82).

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**Fig. 4.** Putative functional domains in Rac1 required for activation of NADPH oxidase. A, the five domains revealed by inhibition of NADPH oxidase by clusters of overlapping peptides are indicated by the bold underlined single-letter amino acid codes in the linear sequence of Rac1. B, probable secondary structure elements corresponding to the five domains in Rac1, based on sequence alignment with Ras (34).

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structural elements, analogous to domains a and b in Rac1, were found to be required for stimulation of endocytosis by Rab5 (42) and correspond to two effector-activating clusters of residues in the α-subunit of the trimeric GTP-binding protein Ga (43). To the best of our knowledge, there are no reports on functional significance, in other GTPases, of residues analogous to domains c and d in Rac1, as defined here.

We propose peptide walking as a general method for the identification of domains in the primary sequence of proteins interacting with either downstream effectors or upstream activators. The relative ease with which a large number of overlapping peptides can be prepared by the multipin synthesis method, with various lengths and extent of overlap, and either free or modified ends, makes this method especially attractive. Peptide walking appears to offer excellent reproducibility, opportunities for automation and is faster and more economical than site-directed mutagenesis or the generation of truncated or chimeric proteins. A number of techniques can be used for assessing the effect of peptides, in addition to inhibition of the protein’s activity, as presently described. These include inhibition by the peptides of specific protein to protein binding and measuring binding of the peptides to potential targets or modulators of the protein being mapped. Experiments using the latter technique for the identification of the NADPH oxidase component(s) recognized by Rac1 and of the domains in Rac1 involved in recognition are in progress.

Peptide walking is, probably, not an effective approach to the detection of discontinuous (assembled) domains in proteins. Additional restraints might be imposed by the variable ability of peptides to assume a conformation similar to that of the corresponding region in the intact molecule. These limitations could explain the inability of peptide walking to identify the presumed effector domain of Rac1 (residues 26–48). For optimal results, therefore, peptide walking should be applied as one component of a wider mapping strategy, to include mutagenesis and the use of truncated and chimeric proteins.

Addendum—While this manuscript was being prepared we became aware of the report by Barnard et al. (44), describing the use of a limited number of overlapping peptides, corresponding to the Ras binding domain of c-Raf-1, for defining sites of interaction between Raf and Ras.

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