Probing Protein/Protein Interactions with Mass Spectrometry and Isotopic Labeling: Analysis of the p21/Cdk2 Complex

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An important first step in understanding protein/protein recognition is the identification of the amino acids at the protein/protein interface. A variety of approaches are used to study these interfaces, ranging from random mutagenesis and chemical cross-linking to high-resolution structure determination. Protease mapping is an established method for probing the primary structure of proteins[1,2] and has traditionally been performed through the use of chromatography and/or gel electrophoresis techniques in combination with Edman degradation NH_2-terminal sequencing.[3] Proteolytic cleavage can also provide indirect information about the domain structure of proteins, but the method is not usually applied to protein/protein complexes due to the limitations of analytical methods to resolve and identify the multiple fragments that are produced. However, matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is well-suited[4] to the analysis of complex mixtures of biomolecules, offering high sensitivity, resolving power and accuracy, and thus the prospect of studying higher order protein structure when combined with protease mapping.[5] The method has been demonstrated previously for the analysis of a protein/DNA complex,[5] and we have extended it to the more difficult problem of protein/protein complexes. To facilitate assignment of the peptide fragments to the individual proteins, uniform isotopic labeling of one protein with ^{15}N is used.

We report the application of MALDI mass spectrometry to proteolytic analysis of a complex of two proteins, allowing ready identification of the exact sites of proteolytic cleavage and providing information on protein/protein interactions. MALDI analysis was performed on digests of the kinase inhibitory domain of the cell cycle regulatory protein, $p21^{Waf1/Cip1/Sdi1}$ [6,7] (p21-B), in the free solution state and in the 1:1 complex of p21-B and cyclin-dependent kinase 2 (Cdk2). This kinase inhibitory domain is unusual in that it exhibits none of the hallmarks of a folded protein as determined on the basis of CD spectropolarimetry and NMR spectroscopy[8] while still inhibiting cyclin A/Cdk2 at sub-nanomolar concentrations.[8] The p21-B primary amino acid sequence contains 12 potential trypsin cleavage sites, at positions 9, 16, 19, 20, 32, 46, 48, 67, 69, 75, 83, and 84 (numbering refers to the full-length p21 amino acid sequence). MALDI mass spectra of the trypsin digests of free p21-B (see ref 9) reveal peaks corresponding to tryptic cleavage at all possible sites (a partial spectrum is shown in Figure 1, panel a). While some sites are preferred relative to others, all sites are readily accessible to trypsin, consistent with the view that p21-B does not assume a compact structure that would protect some sites from proteolytic cleavage.[8]

p21-B binds to Cdk2 alone ($K_d \sim 10\ \mu M$) and to the cyclin A/Cdk2 complex ($K_d < 1$ nM)[8] through a region corresponding to amino acids 17−77.[10−16] Due to the dramatically higher affinity of p21-B for the cyclin A/Cdk2 complex versus Cdk2 alone, we believe that there are interactions between p21-B and *both* the cyclin A and Cdk2 components of the ternary complex. In order to identify those amino acids involved in the physical interactions between p21-B and Cdk2, we have mapped the p21-B/Cdk2 interface using trypsin as a general probe of amino acid accessibility. MALDI mass spectra for trypsin digests of p21-B in the presence of a slight molar excess of Cdk2 (17 μM p21-B, 20 μM Cdk2[17]) show that several p21-B fragments that were produced in the absence of Cdk2 disappear in the presence of Cdk2 (compare panels a and b, Figure 1); specifically, amino acids 46, 48, and 67 are protected from trypsin in the Cdk2 complex. Interestingly, cleavage at amino acid 32 is enhanced in the presence of Cdk2.

Interpretation of the mass spectra for the 1:1 p21-B/Cdk2 complex is complicated by the appearance of peaks due to tryptic cleavage of both p21-B and Cdk2. Since the goal of this work was to identify p21-B peaks that disappear in the presence Cdk2, the appearance of additional peaks from Cdk2 is a significant complication. We have eliminated this complication by performing a duplicate set of experiments using ^{15}N-labeled p21-B within the p21-B/Cdk2 complex. MH^+ ions for ^{15}N-p21-B are shifted to higher mass by an increment of ΔM Daltons, with ΔM approx. equaling the number of nitrogen atoms in the protein fragment, while those from Cdk2 are not shifted. Comparison of the spectra for p21-B/Cdk2 complex samples prepared with unlabeled (Figure 1, panel b) and ^{15}N-labeled p21-B (Figure 1, panel c) allows unambiguous differentiation of p21-B and Cdk2 fragments and identification of protected amino acids for p21-B within the complex (Figure 2, compare top and bottom panels) clearly revealing a segment of 22 amino acids, and possibly extending to 36 amino acids, of p21-B that is protected from trypsin cleavage. Changes in the susceptibility of p21-B to trypsin cleavage due to interaction with Cdk2 can arise from several physical effects of Cdk2 on p21-B: changes in p21-B amino acid accessibility, conformation, and/or polypeptide chain flexibility. These can originate from either direct effects on interfacial residues or indirect effects at remote sites, due, e.g., to conformational changes on binding. Our method does not differentiate between the various direct and/or indirect mechanisms. However, the localized and

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(17) Trypsin digests of the p21-B/Cdk2 complex were performed as described for free p21-B. Duplicate digests were prepared using either unlabeled or ^{15}N-labeled p21-B. ^{15}N-labeled p21-B and Cdk2 were prepared as previously described (ref 8 and references contained therein).

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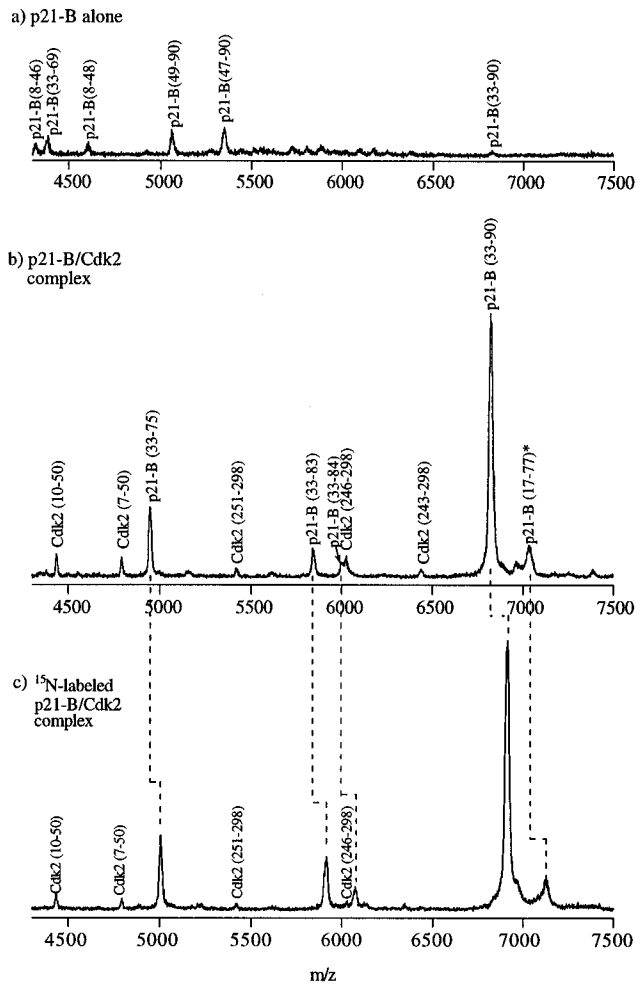

Figure 1. Partial MALDI mass spectra for trypsin cleavage of p21-B (panel a) and the p21-B/Cdk2 complex (panels b and c) obtained with a Perseptive Voyager Elite MALDI mass spectrometer. The complex was prepared with natural isotopic abundance p21-B (panel c) and ^{15}N-labeled p21-B (panel d). The dashed lines indicate peaks that are shifted in panel c with respect to panel b and correspond to p21-B proteolytic fragments. Peaks are labeled to show the protein fragment (p21-B or Cdk2) to which they correspond, giving the amino acid positions of the NH_2- and COOH-termini of the fragment.

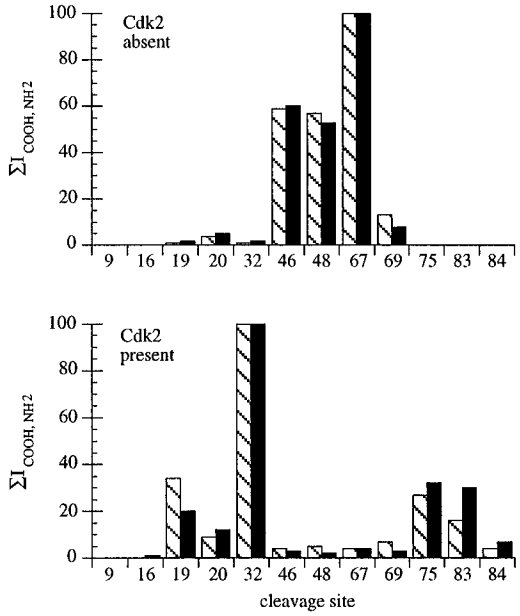

Figure 2. Protected regions of p21-B within the p21-B/Cdk2 complex. Trypsin accessibility at a particular cleavage site (with cleavage between the numbered site and the following site), expressed as the sum of MALDI mass spectral peak intensities for all fragments with COOH- and NH_2-termini corresponding to scission at that particular site ($\Sigma I_{COOH,NH2}$), versus position within the primary amino acid sequence (numbered with respect to the full-length p21 sequence). $\Sigma I_{COOH,NH2}$ values derived from each spectrum are internally normalized such that the largest value corresponds to 100%. Vertical scales for the top and bottom panels cannot be quantitatively compared. Results are shown for natural isotopic abundance (hashed bars) and ^{15}N-labeled (solid bars) p21-B in the absence of Cdk2 (top) and in the presence of Cdk2 (bottom).

contiguous nature of the highly protected region of p21-B identified here strongly suggests that this region constitutes the Cdk2 binding site on p21-B. In support of this conclusion, the results of mutagenesis experiments[18] show that mutation of charged residues at positions 44, 46, 47, 48, 52, 56, 60, and 62 to alanine reduces the affinity of full-length p21 for Cdk2. In the future, we will utilize proteases with different cleavage specificities to probe the amino acids between the extreme protected and unprotected sites and thereby provide a higher-resolution map of this binding site.

Our experiments were performed without the need for mutagenesis and simply depended on the preparation of ^{15}N-labeled protein using routine procedures. The combined use of isotopic labeling, proteolysis, and MALDI mass spectrometry offers a general approach to detailed mapping of protein/protein interfaces and will allow, in the future, the identification of well-defined protein/protein complexes for high-resolution structural analysis using NMR spectroscopy and X-ray crystallography, as has recently been reported for the $dTAF_{42}/dTAF_{62}$ complex using electrospray mass spectrometry techniques.[19] In addition, our isotope labeling/proteolysis/MALDI methodology can be combined with other well-established techniques, such as chemical cross-linking, to provide more detailed and accurate analyses of protein/protein interaction sites.

The method we report surpasses previous non-mass spectrometric approaches due to the ability to resolve protein fragments at the level of single amino acid differences and due to the ability to provide exact determination of both the NH_2-terminus and COOH-terminus of proteolytic fragments. Further, we have utilized MALDI which is more tolerant of heterogeneous protein mixtures and salts than electrospray ionization. Our approach is readily generalized to other protein/protein complexes for which isotopic labeling is possible and may allow, in the future, rapid access to highly accurate "maps" of protein/protein interfaces. One limitation is that only protein fragments of less than ~20 kDa can currently be analyzed with suitable accuracy using MALDI mass spectrometry, precluding analysis of protein complexes that yield only fragments larger than 20 kDa. In these cases, however, through judicious choice of protease and adjustment of experimental conditions, proteolysis may be controlled to yield fragments in the molecular weight range appropriate for detailed mass analysis. The studies reported here address interactions between a highly flexible protein (p21-B) and a compact, globular protein (Cdk2); the methodology, however, is equally well-suited for the study of other proteins, flexible or globular, for which complex formation is accompanied by changes in amino acid accessibility, conformation, and/or flexibility.

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