A Strategy for the Rapid Identification of Phosphorylation Sites in the Phosphoproteome*

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Edman phosphate (³²P) release sequencing provides a high sensitivity means of identifying phosphorylation sites in proteins that complements mass spectrometry techniques. We have developed a bioinformatic assessment tool, the cleavage of radiolabeled protein (CRP) program, which enables experimental identification of phosphorylation sites via ³²P labeling and Edman degradation of cleaved proteins obtained at femtomole levels. By observing the Edman cycle(s) in which radioactivity is found, candidate phosphorylation sites are identified by determining which residues occur at the observed number of cycles downstream from a peptide cleavage site. In cases where more than one residue could be responsible for the observed radioactivity, additional experiments with cleavage reagents having alternative specificities may resolve the ambiguity. Given a protein sequence and a cleavage site, CRP performs these experiments in silico, identifying resolved sites based on user-supplied experimental data, as well as suggesting combinations of reagents for additional analyses. Analysis of the PhosphoBase protein sequence database suggests that CRP data from two cleavage experiments can be used to identify unambiguously 60% of known phosphorylation sites. Data from additional cleavage experiments may increase the overall coverage to 70% of known sites. By comparing theoretical data obtained from the CRP program with ³²P release data obtained from an Edman sequencer, a known phosphorylation site was identified unambiguously and correctly. In addition, our results show that in vivo phosphorylation sites can be determined routinely by differential proteolysis analysis and Edman cycling with less than 1 fmol of protein and 1000 cpm. Molecular & Cellular Proteomics 1:314–322, 2002.

Proteomic technologies have transformed the manner in which proteins and their contributions to cellular function are viewed (for review see Refs. 1–3). The completed human genome map represents an invaluable tool that presents a comprehensive account of the structure and sequence of human genes. However, it offers limited insight regarding the various post-translational modifications, such as phosphorylation, that occur on proteins in the cell. Contemporary proteomic analysis uses two-dimensional gel electrophoresis to separate cellular proteins and mass spectrometry to identify the proteins in these gels. The identification of a protein can usually be achieved at the femtomole level with the employment of tandem mass spectrometry to decode the primary amino acid sequences. Numerous advances in proteomics tools, including advances in high sensitivity protein staining and two-dimensional electrophoresis techniques, refinements in ampholytic technology, and the advent of accurate, sensitive, and affordable mass spectrometers including matrix-assisted laser desorption ionization mass spectrometers and quadrupole tandem mass spectrometers has allowed a shift to high throughput analysis of large numbers of candidate proteins. Recent functional proteomic analyses are now being employed specifically to describe the architecture of signal transduction pathways at the level of the individual kinase or phosphatase (4, 5); however, significant barriers still limit the ability to identify individual phosphoproteins and their sites of phosphorylation within a proteome analysis.

Phosphoproteins are often a small fraction of the individual protein concentration and present at low copy number in cells. Prediction of the phosphorylation status of proteins from sequence patterns or more sophisticated neural network motifs has limited sensitivity and greatly lacks specificity (6). Protein phosphorylation must therefore be observed directly. We have developed a bioinformatic assessment tool, CRP,¹ that enables access via ³²P labeling and Edman sequencing to concentrations of phosphorylation sites that are below the femtomole level.

EXPERIMENTAL PROCEDURES

Materials—Mouse submaxillary gland endoproteinase Arg-C and Staphylococcus aureus V8 protease (endoproteinase Glu-C) were from Sigma. Achromobacter lyticus endoproteinase Lys-C was from

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¹ The abbreviations used are: CRP, cleavage of radiolabeled protein; PVM, modified polyvinylidene difluoride membrane; PRP, platelet rich plasma; HPLC, high pressure liquid chromatography.
Phosphorylated residues were located by determining the cycles in which $^{32}$P was released when samples were subjected to sequential Edman degradation under conditions that optimized recovery of $^{32}$P (10).

Phosphorylated Hsp27 was immunoprecipitated from the in vivo $^{32}$P-labeled platelet lysate. The lysate was pre-cleared with protein G-agarose beads (1 h at 5 °C). Platelet lysate was incubated overnight with 10 μg of monoclonal mouse anti-Hsp27 followed by harvest with protein G-agarose. Immunoprecipitated proteins were eluted from protein G with 50 μg glycine, pH 2.0. The pH of the eluate was quickly neutralized with 1 μl phosphate buffer, pH 8. The protein samples were incubated overnight at 37 °C with the protease of choice; the resulting peptides were processed for Edman sequencing as described above.

CRP Program World Wide Web Interface—The acquired $^{32}$P release data were interpreted using the CRP program, accessible at fasta.bioch.virginia.edu/crp/. The program allows for the input of protein sequence data in the same format as a normal BLAST query, either a raw sequence or a FASTA-formatted sequence or via a unique sequence identifier (see www.ncbi.nlm.nih.gov/blast/html/search.html for details). As an example, the sequence for myelin basic protein, a commonly used protein kinase substrate protein, was processed by the CRP program (Fig. 1A). CRP-generated theoretical cleavage data were obtained by selecting the specific carboxyl-terminal amino acid at which to cut, in this case, at Arg residues. CRP displays results of the in silico cleavage as a table of the Edman cycles in which radioactivity might be observed, listing each associated potential site (Fig. 1B). The percent coverage, or the cumulative number of observable potential phosphorylation sites, is also provided. With myelin basic protein and proteinase with endoproteinase Arg-C, 21 cycles are required to ensure 100% coverage of 35 possible phosphorylation sites. The program highlights sites that agree with known phosphorylation target consensus sequences and provides a link to the EXPASy PROSITE database (11), where information pertaining to the consensus site is available.

If radioactivity was observed in a cycle containing multiple potential phosphorylation sites, the determination becomes ambiguous; by selecting all cycles (identifiable) in which radioactivity was actually observed (including any unambiguous, as well as the ambiguous cycles), CRP provides a second table (Fig. 1C), detailing the new cycle positions of the potential phosphorylation residues when the original protein is cut at different cleavage sites. These data permit the selection of proteases for second, and if necessary, third cleavages that could yield unambiguous data.

RESULTS

Theoretical Potential for the CRP-based Analysis of Phosphoproteins—We analyzed the ability of the CRP technique to determine the phosphorylation site(s) of proteins in silico, using either the SwissProt (11) or PhosphoBase (12) protein sequence databases. We selected an arbitrary Edman cycle cutoff limit of 25 cycles; any residues appearing after 25 cycles were considered unresolved by the experiment. Residues that appeared within 25 cycles but were found in a cycle containing other residues were also considered unresolved. Therefore, within a single cleavage experiment, any residue found alone within a cycle below the cutoff was considered resolved; i.e. if radioactivity were observed in that cycle, it would identify unambiguously that residue as phosphorylated.

The left panel of Fig. 2 demonstrates the percent coverage
Cleavage of Radiolabeled Protein Program

A

Cleaved Radioactivity of Phosphopeptides

Enter an accession number or other unique identifier: 

Enter your protein sequence in fasta format:

```plaintext
maeqkrpqeqhrhpkylslattermdharhgfipklhrtdtgdldsggrffgddkdrcakprkgpgevwp
wtkpgrslpsharqeqpglonmykdshhparkahtahgrlqpkabgtrtdpnnwffknvrt
prttppqeqkqgrqlalsrfewaegqgrqfegygradsykeahkfgkvdaqytlkslkfkl
ggderagspmarr
```

Cleave at which residue? [ ] R [ ] Arg

Submit Query  Clear  Reset

B

Histogram of expected 'hot spots'

| Cycle # | Potential Phosphorylation Sites | # in Cycle | Coverage |
|---------|---------------------------------|------------|----------|
| 1       | S068  S076  T093  T107  T125  S190 | 6          | 178      |
| 2       | S088  T036  S057  S142  S159  S188 | 6          | 344      |
| 3       | S003  S013  S137  S192            | 4          | 107      |
| 4       | Y096  Y161                          | 2          | 511      |
| 5       | Y015  S072  S129  S139  Y154      | 5          | 658      |
| 6       | S098  S163                          | 2          | 711      |
| 7       | S041                          | 1          | 744      |
| 8       | T018                          | 1          | 774      |
| 9       | Y084                          | 1          | 804      |
| 10      | S020                          | 1          | 824      |
| 11      | T021  S103                      | 2          | 881      |
| 12      | S087                          | 1          | 911      |
| 13      |                                |            |          |
| 14      |                                |            |          |
| 15      |                                |            |          |
| 16      | T122                          | 1          | 944      |
| 17      |                                |            |          |
| 18      |                                |            |          |
| 19      | T176                          | 1          | 974      |
| 20      |                                |            |          |
| 21      | S178                          | 1          | 1000     |

Process selected cycles → Submit Query

1 11 21 21 41
MAEQKRQPQHRHPKYLSLATTERMDHARHGFIPLHRTDTGDESCGGRFFGD KDRCAKPRKGPG
WTKPGRSLPSHARQEQPGLOMYKDSHPARKAHTAHGRLMQPKABGTRTDPPNWFKNVRT
PRTTPPQEQKQGRQLALSRFEWAGQGRQFEGYGRADSYKEAHKFGKVDQAQYTLKSLKFKL
GGDERAGSPMARR

Fig. 1
**Potential Phosphorylation Residues’ Cycle Position**
*When Cut at Specific Cleavage Sites*

| Cleavage Site | S08 | S09 | S011 | S057 | S058 | S066 | T093 | T125 | S142 | S159 | S188 | S190 |
|---------------|-----|-----|------|------|------|------|------|------|------|------|------|------|
| A             | 14  | 15  | 21   | 23   | 24   | 26   | 43   | 61   | 81   | 103  | 109  | 110  |
| B             | 12  | 16  | 26   | 27   | 28   | 30   | 41   | 61   | 81   | 103  | 109  | 110  |
| C             | 14  | 15  | 21   | 23   | 24   | 26   | 43   | 61   | 81   | 103  | 109  | 110  |
| D             | 12  | 16  | 26   | 27   | 28   | 30   | 41   | 61   | 81   | 103  | 109  | 110  |

**Cleavage Residues: F**

| Potential P-Sites | S142 |
|-------------------|------|
| Cycles            | 1    |

**Cleavage Residues: R**

| Potential P-Sites | S08  |
|-------------------|------|
| S08               | 7    |
| S09               | 8    |
| S125              | 9    |
| S188              | 11   |
| S057              | 12   |
| S066              | 13   |
| T093              | 17   |
| S142              | 18   |
| S159              | 19   |
| S188              | 20   |
| S190              | 21   |

**Cleavage Residues: K**

| Potential P-Sites | S08  |
|-------------------|------|
| S08               | 3    |
| T093              | 4    |
| S159              | 5    |
| S188              | 6    |
| S190              | 7    |

**Cleavage Residues: W**

| Potential P-Sites | S08  |
|-------------------|------|
| S08               | 9    |
| S09               | 14   |
| S125              | 16   |
| S188              | 21   |
| S057              | 26   |
| S066              | 29   |
| T093              | 31   |
| S142              | 32   |

**Fig. 1.** The CRP program interface. A, initial data entry screen allowing accession number or protein sequence inputs. The sequence of myelin basic protein is shown in the protein sequence input box, and cleavage with endoproteinase Arg-C has been selected. By submitting the query, a histogram of expected hot spots is presented (B). The theoretical data displayed include the following: Cycle #, corresponds to the Edman sequence cycle number; Potential Phosphorylation Sites, any Ser, Thr, or Tyr residues present in the protein; # in Cycle, the number of phosphorylatable residues present in a particular cycle number; Coverage, the cumulative percentage of phosphorylatable residues included in a particular number of Edman sequencing cycles or cycle numbers. By selecting (->) one or more particular cycle number(s) and clicking on “Submit Query,” a new screen showing a table of the cycle position of the potential phosphorylation residues when cut at specific cleavage sites is obtained (C). These data show all the phosphorylation sites within the selected cycle number(s) along the top of the table and all amino acids along the side of the table. By orienting a particular residue with a particular cleavage site, the cycle number in which 32P release should occur is obtained. For ease of use, the potential phosphorylation sites for ascending cycle numbers for each cleavage site are also presented (D).
from theoretically obtained data using each of five cleavage agents that target methionine (M), tryptophan (W), phenylalanine (F), lysine (K), or arginine (R). Coverage is measured for all potential phosphorylation sites in SwissProt (SP) and PhosphoBase (PB), as well as only those sites described by PhosphoBase annotations (PB*). In SwissProt, around 14% of Ser, Thr, or Tyr sites are resolvable by a single cleavage experiment. Approximately 20% of the known phosphorylation sites from PhosphoBase are resolvable. The relative performance of the various cleavage agents reflects the frequencies of their cleavage sites. Sites that occur more frequently in a protein will lead to a larger number of shorter fragments, increasing the coverage achievable within 25 cycles, but also increasing the chance of continued ambiguity. Sites that occur less frequently will lead to a smaller number of shorter fragments, increasing the coverage achievable within 25 cycles, but also increasing the chance of continued ambiguity. However, most of the remaining sites remain unresolved because of stretches of sequence longer than 25 residues that cannot be cleaved, leaving the phosphorylation sites above the 25-cycle threshold. Increasing the cycle threshold to 40, for instance, increases dramatically coverage at all levels of measurement (achieving nearly 90% coverage with some triple cleavage experiments).

For the sake of simplicity, these theoretical experiments assume that only one residue in the protein is phosphorylated. If radioactivity is found in more than one cycle, then additional experiments would produce more candidate residues, increasing the possibility of continued ambiguity. However, a lower limit on coverage may be easily calculated as simply the sum of residues resolvable by each single cleavage; for a triple cleavage experiment, the coverage could be as low as 30%. This represents the worst-case scenario; all of the potential sites of a protein are phosphorylated.

To increase the probability of uniquely identifying phosphorylation sites, a phosphoamino acid analysis can be completed on an aliquot of the phosphoprotein prior to Edman cycle analysis to determine whether the phosphorylation site is a phospho-Ser, -Thr, or -Tyr. By limiting the total number of residues under consideration, this information reduces dramatically the complexity of the CRP results and further resolves ambiguity, increasing the theoretical coverage to nearly 100% in most triple cleavage experiments (data not shown).

Application of CRP Analysis in the Identification of Phosphorylation Sites—To test our strategy, we took advantage of the in vitro phosphorylation of telokin documented previously (7). Telokin is a small acidic protein (17 kDa) with a serine/threonine-rich amino terminus and contains substrate recognition sequences for a variety of kinases, including cAMP-dependent protein kinase. Using conventional methods, we have identified previously a single site of in vitro phosphorylation on telokin by cAMP-dependent protein kinase as Ser-13 (7).

As shown in Fig. 3A, when peptides from an endoproteinase Lys-C digest were applied to the Edman sequencer, $^{32}$P release was observed in cycle 2; when peptides from a cyanogen bromide digest were applied to the Edman sequencer, $^{32}$P release was observed in cycle 10. The theoretical results for an endoproteinase Lys-C digest of telokin are displayed in Fig. 3B. In theory, $^{32}$P release in cycle 2 could be the result of
Identification of Thrombin-sensitive Phosphorylation Sites on Hsp27 in Vivo—We used human platelets as a model system to test the CRP method of phosphorylation site identification on in vivo target proteins. The cellular ATP stores of platelets can be spiked easily by $[^{32}P]$orthophosphate isotope labeling, and there is a large and rapidly growing body of information on the different phosphoproteins that emerge within platelets from agonist stimulation (13, 14). A number of proteins exhibit increased phosphorylation in response to thrombin stimulation (Fig. 4). Fourteen phosphoproteins were selected for mixed-peptide sequencing, and one of those proteins, Hsp27, was selected as a candidate phosphoprotein for CRP analysis.

We immunoprecipitated Hsp27 from $[^{32}P]$-labeled platelets treated with thrombin. All of the potential phosphorylation sites and the theoretical results of an endoprotease Arg-C digest of Hsp27 were identified using the CRP program (Fig. 5A). This analysis showed that 25 rounds of Edman sequencing were sufficient to cover 87% of all serine, threonine, and
tyrosine residues present in Hsp27. Twenty-five rounds of Edman degradation chemistry were carried out on the 32P-labeled peptides obtained from the endoproteinase Arg-C digest of the immunoprecipitated Hsp27. This analysis yielded radioactivity only in cycle 3. Inspection of the analysis shown in Fig. 5 shows that six potential phosphorylation sites would produce a signal in cycle 3. Further analysis of these sites by the CRP program algorithm indicated that digestion at Glu

FIG. 4. Identification of phosphoproteins in human platelets by mixed peptide sequencing. Platelets from 1.0 ml of human blood were 32P-labeled for 90 min and then treated with thrombin (0.2 units) in the presence of calyculin A (10 μM) for 1 min. Preliminary experiments determined that calyculin treatment alone did not enhance significantly the level of phosphorylation. Phosphoproteins were characterized on two-dimensional SDS-PAGE gels. The separated proteins were transferred to PVM, stained with Amido Black, and autoradiographed. 32P-Labeled proteins that corresponded to stained proteins were cut from the PVM, treated with CnBr solution, and sequenced by mixed peptide sequencing. Radioactive values (cpm) were theoretical values based on the primary sequence of the identified proteins and were determined using the EXPASY program (expasy.hcuge.ch/ch2d/pi_tool.html). 1, myosin light chain 20, pl 5.09, 19.8 kDa; 2, Rho guanine nucleotide dissociation inhibitor (Rho GDI), pl 5.1, 23.0 kDa; 3, neurogranin, pl 6.53, 7.4 kDa; 4, RAP1A, pl 6.39, 20.9 kDa; 5, tubulin, pl 4.75, 49.7 kDa; 6, Pleckstrin, pl 8.32, 40.1 kDa; 7, GPla-II, pl 5.3, 84.6 kDa; 8, Rho-GAP, pl 5.91, 105 kDa; 9, ESTA05935; 10, HSP27, pl 5.98, 22.7 kDa; 11, c-Raf oncogenic fusion protein, pl 5.15, 47.0 kDa; 12, amphiphysin homologue, pl 4.95, 64.0 kDa; 13, HSP90, pl 4.94, 84.6 kDa; 14, pleckstrin fragments.

FIG. 5. Identification of thrombin-sensitive phosphorylation sites on Hsp27 by CRP analysis. A, theoretical results were obtained from the CRP program for an endoproteinase Arg-C digest of Hsp27. B, Hsp27 was immunoprecipitated from 32P-labeled platelets treated with thrombin and calyculin A for 5 min. Peptides (~1000 cpm) obtained from endoproteinase Arg-C digestion were cross-linked to Immobilon P (Perspective Biosystems), and the membrane was placed in an Applied Biosystems Procise 494cLc automated sequenator. Twenty-five cycles of Edman degradation chemistry were carried out, and the released phenylthiohydantoin (PTH) amino acids were collected, and their radioactivity was determined by Cerenkov counting. C, CRP program display of the new cycle positions of the potential phosphorylation residues when Hsp27 is cut with different proteases. D, the results of 32P release from peptides (~2500 cpm) obtained from an endoproteinase Glu-C digest of immunoprecipitated Hsp27 are presented.
followed by Edman cycle analysis would identify uniquely the position of the thrombin-stimulated in vivo phosphorylation site(s). Indeed, Edman degradation chemistry carried out on the peptides obtained from the endoproteinase Glu-C digest yielded radioactivity in cycles 12, 14, and 18. Thus, Ser-15, Ser-78, and Ser-82 were identified unambiguously as major in vivo thrombin-sensitive phosphorylation sites in Hsp27.

**DISCUSSION**

Traditionally, protein phosphorylation sites are located by enzymatic cleavage of a $^{32}$P-radiolabeled phosphoprotein into peptides followed by HPLC C18 reverse phase chromatography or two-dimensional thin layer chromatography to isolate and separate the $^{32}$P phosphopeptide. The sequence of the phosphopeptide is then obtained by Edman sequencing. The need for a large amount of starting material (more than pmol amounts of protein) and the length of time to completion has made this procedure prohibitive to high throughput studies of the phosphoproteome. We have developed a strategy that permits identification of phosphorylation sites from in vivo or in vitro $^{32}$P-labeled proteins of known sequence at the sub-femtomolar level.

From our initial assessment of the thrombin-stimulated platelet phosphoproteome (Fig. 4), we selected Hsp27 as a phosphoprotein for CRP analysis. In human platelets, increased phosphorylation of Hsp27 through the p38/mitogen-activated protein kinase-activated protein kinase 2 pathway in response to thrombin treatment has been observed previously (15). Hsp27 phosphorylation is associated with platelet aggregation and regulation of microfilament organization. Activation of p38/mitogen-activated protein kinase-activated protein kinase 2 pathway after thrombin stimulation leads to a marked shift from the 27-kDa unphosphorylated form to at least three major phosphorylated forms. The phosphorylation sites on Hsp27 (16, 17) have been mapped previously using conventional methods, i.e. proteolytic digestion and fractionation of the peptides by reverse phase HPLC followed by Edman sequence analysis. The sites phosphorylated by mitogen-activated protein kinase-activated protein kinase 2 after in vivo thrombin treatment were identified as Ser-15, -78, and -82 (16, 17). The present study confirms Hsp27 as a target of phosphorylation during thrombin stimulation but more importantly demonstrates the ability of the CRP analysis to ascertain multiple sites of phosphorylation on a target phosphoprotein isolated from an in vivo source.

Some limitations to the CRP methodology do exist; phosphorylation sites directly adjacent to Lys or Arg may not be identified because of steric occlusion of the protease, the 3° structure of the protease may prevent proteolysis at every site hence giving rise to missed cleavages, and ragged cuts at frequently occurring Lys-Lys or Arg-Lys motifs may lead to ambiguous results. However, with experience and careful consideration of the amino acid sequence of the phosphoprotein, these limitations are circumventable. We have had much success using the CRP methodology for the identification of in vitro phosphorylation sites. Although telokin is at the lower end of degree of difficulty, we have successfully used the methodology to determine multiple phosphorylation sites on proteins with molecular masses of up to 130 kDa. With minimal starting product (i.e. <10 fmol of protein) we have identified multiple sites of phosphorylation on the protein kinase C phosphatase inhibitor protein, CPI-17 (18), the myosin targeting subunit (MYPT1) of smooth muscle myosin phosphatase, 2 and the transcriptional co-activator cAMP-response element-binding protein-binding protein (CBP/p300). 3 To increase the probability of identifying phosphorylation sites, a phosphoamino acid analysis can be completed on an aliquot of the phosphoprotein prior to Edman cycle analysis to determine whether the phosphorylation site is a phospho-Ser, -Thr, and/or -Tyr. By limiting the total number of residues under consideration, this information reduces dramatically the complexity of the CRP results and resolves further assignment ambiguities, increasing the theoretical coverage to nearly 100% in most triple cleavage experiments. The CRP analysis methodology complements existing mass spectrometry techniques (19–21) for phosphorylation site identification, because it presents an alternative method of identification in situations where peptides are unable to be resolved by mass spectrometry.

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