Phosphorylation of Crk Adaptor Protein by Cdc42-Activated Pak2 and Identification of Phosphorylation Sites

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ABSTRACT

The p21-activated protein kinase Pak2 is activated in response to a variety of stresses. Pak2 is activated by binding of Cdc42(GTP) followed by autophosphorylation or by caspase3 cleavage. Pak2 modifies various targeted substrates. In this study Crk, an adaptor protein, was phosphorylated by Pak2 using (γ-32P)ATP. Phosphorylation of Crk was analyzed by SDS-polyacrylamide gel electrophoresis and phosphorimaging. Results from scintillation counting showed 0.6 mol/mol of 32P incorporated into Crk. Phosphoaminoacid analysis showed that Crk was phosphorylated on serine, not threonine. To identify the sites of phosphorylation, phosphorylated Crk was digested by the proteases Glu-C or trypsin and subjected to two-dimensional phosphopeptide mapping. The maps yielded two phosphopeptides, which suggested two phosphopeptides contained the same phosphorylation site or two distinctive phosphorylation sites on Crk. Potential phosphorylation sites in two sequences of Crk are located near the SH2 and SH3 domains.

FACULTY MENTORS

Jolinda Traugh
Department of Biochemistry
Jisun Lee worked as a dishwasher in my laboratory for several years, and also prepared electrophoresis buffer and solutions for staining and destaining proteins. She became interested in our research on the protein kinase Pak2, and received a campus Nova Award last summer to enable her to work in the laboratory full time. She has mastered a number of techniques to analyze phosphorylated proteins and identify the sites phosphorylated by Pak2. Jisun is a delight to work with. Her interest and motivation in writing this paper attests to her rapid growth and outstanding potential as a scientist.

Jin-Hun Jung
Department of Biochemistry
We have been studying a protein kinase, Pak2, which is activated under a variety of stress conditions, such as DNA-damaging and hyperosmolarity. Jisun Lee has been working on phosphorylation of a proto-oncoprotein Crk by Pak2. She has made a significant contribution to identification of the site of phosphorylation on Crk, which can lead us to study a functional relationship of the two proteins in cells. She has successfully developed the research skills and related knowledge on her project. It has been a great pleasure to work with her.
PHOSPHORYLATION OF CRK ADAPTOR PROTEIN BY CDC42-ACTIVATED PAK2 AND IDENTIFICATION OF PHOSPHORYLATION SITES

Jisun Lee

Introduction

The protein Crk, chicken tumor virus no. 10 regulator of kinase, is a key adapter protein that functions in several signal transduction pathways. Crk has an important role as a linkage between tyrosine kinases and small G proteins, and leads to the regulation of cell growth, motility, apoptosis, and transcription (1). Also, as an oncoprotein, Crk is responsible for malignant features of cancers (1). Crk is composed of two isoforms, CrkI and CrkII. The activity of CrkI has been studied more than of CrkII. CrkII has three domains, Src homology 2 (SH2), N-terminal 3 (SH3n), and C-terminal 3 (SH3c) domains (2). CrkII has a phosphorylation site on Tyr221 between N-terminal and C-terminal domains of SH3 (3) as indicated in Fig. 1. This phosphorylated tyrosine provides an intramolecular binding interaction with the SH2 domain of CrkII (3, 4). In this study, CrkII is phosphorylated by Pak2 and possible phosphorylation sites are identified.

Pak2, p21-activated kinase 2, is activated in response to various cell stresses, such as DNA damaging agents or ionizing radiation (5). Pak2 is activated either by binding of the small G protein Cdc42 or by cleavage with caspase 3, followed by autophosphorylation (5, 6). There are 7 serine and 1 threonine sites that are identified as autophosphorylation sites for Pak2 (6) as shown in Fig. 2. The sequence on substrates that allows recognition and phosphorylation by Pak2 is represented as (K/R)RXS (7). The basic amino acids lysine or arginine at the -3 position and arginine at -2 position and any type of amino acid at -1 position on the substrate, allow phosphorylation by Pak2 (7). Consequently, the features of this sequence can be applied to identify possible phosphorylation sites on Crk for Pak2.

In this research, the phosphorylation of CrkII by Cdc42-activated Pak2 was studied to examine whether the Crk is a good substrate for Pak2 and analyzed the level of Crk phosphorylation by Pak2. The characteristics of the determinants for phosphorylation by Pak2 were applied and analyzed by phosphopeptide mapping and possible sites were identified. By studying phosphorylation of Crk with Pak2, basic links between Pak2 and Crk can be achieved. Furthermore, the regulation of Crk’s critical functions in regulation of cell growth and apoptosis by Pak2 can be studied in further research for therapeutic treatment of human cancers.

Results

GST-Crk was phosphorylated by GST-Pak2 in Vitro

Pak2, Crk, and Cdc42 were identified based upon their molecular weights as shown in Coomassie Blue staining in Fig. 3 (top panel). To observe the phosphorylation of Crk by Cdc42-activated Pak2, phosphorimaging was used. During the time course, there was a significant increase in phosphorylation of Crk by Cdc42-activated Pak2 in Fig. 3 (bottom panel). Autophosphorylation of Pak2 that was activated by Cdc42 was shown in the phosphorimaging as well.

Figure 1. Schematic structure of Crk

Figure 2. Phosphorylation sites of Pak2. Seven phosphorylation sites of serine and one phosphorylation site of threonine and a caspase cleavage site is indicates within the structure of Pak2.

Figure 3. Phosphorylation of Crk by Pak2. Top panel: Crk (10 ug) was incubated with active Pak2 (1 ug) over time, analyzed by SDS-PAGE, and stained with Comassie blue. Bottom panel: radiolabeled Crk was detected by phosphorimaging.
To examine the effects of the phosphorylation of Crk, the protein bands of Crk were subjected to scintillation counting. Each phosphorylated Crk band was excised from the polyacrylamide gel and quantified for $^{32}$P incorporation. The amount of $^{32}$P was adjusted to the actual amount of Crk protein due to the fact that there was a slight difference between the amounts of proteins in the gel. Fig. 4 shows that phosphate incorporation was nearly linear over time. The maximum phosphorylation obtained at 80 min reached a level of 0.6 pmol of $^{32}$P incorporated per pmol of Crk. A similar amount of $^{32}$P was observed at 100 min.

2-D Mapping of Crk for analysis of the phosphorylation site

To study the sites of phosphorylation of Crk, Crk was analyzed by phosphopeptide mapping. Two proteinases, Glu-C and trypsin were used in the evaluation. The protein bands in the gel were cleaved by trypsin or Glu-C to generate phosphopeptides. Glu-C cleaved Crk after glutamic acid, and trypsin cleaved after arginine and lysine. In the first dimension electrophoresis the peptides migrated by size, and in second dimension chromatography the peptides migrated by charge and size. Fig. 5 displayed the products of the phosphopeptide mapping. In these two dimensional maps, two main spots with similar sizes were detected at similar places in both maps, which suggested similar sizes of sequences with similar charges. Closely positioned phosphopeptides with similar charges and sizes, could be interpreted as one phosphopeptide that contains two phosphorylation sites or two different phosphopeptides that have their own phosphorylation site.

Figure 4. Gamma-$^{32}$P incorporation into Crk (pmol/pmol). The molar ratio of $^{32}$P and Crk was obtained from the data in Fig. 3 and plotted over time.

![Gamma-$^{32}$P incorporation into Crk](image1)

Figure 5. Two-dimensional phosphopeptide mapping of Crk phosphorylated by Pak2. Phosphorimages of phosphopeptide maps of Crk. Right: peptide digested with trypsin. Left: peptide digested with Glu-C. The origins are indicated with an arrow.
Discussion

CrkII was phosphorylated by Pak2 that was activated by the binding of Cdc42 followed by autophosphorylation. The phosphorylation and scintillation counting of $^{32}$P showed Crk as an efficient substrate for Cdc42-activated Pak2, with 0.6 pmol/pmol of $^{32}$P incorporation into Crk after 80 min of reaction time. To determine the sites of phosphorylation on Crk, the peptides corresponding with the spots on the 2-D maps were compared regarding their positions in the sequence of Crk. In Fig. 6, the sequences underlined with black lines indicate Glu-C digested peptides and the sequences underlined with green lines indicate trypsin digested peptides. The peptides in the boxes are candidate phosphorylation sites determined by the (K/R)RXS sequence containing the determinants for potential substrates phosphorylated by Pak2. Due to the fact that the spots in each of the phosphopeptide maps were close to each other, exact numbers of the sites of phosphorylation cannot be identified. Glu-C cleaved at glutamic acid, and trypsin cleaved at lysine and arginine. According to similar sizes of phosphopeptides cleaved in both 2-D maps, and the (K/R)RXS phosphorylation sequence, the underlined sequences are only sequences that contain similar sizes and charges for each phosphopeptide. Thus identification of phosphorylation sites via mass spectrometry would be essential for further study on Crk.

Materials and Methods

Expression and Purification of Proteins

Crk and Cdc42 were transformed in *Escherichia coli* individually and Pak2 was expressed in TNSB-4 insect cells. The proteins were purified by glutathione affinity with glutathione-Sepharose 4B beads. GST was the tag used to purify these proteins. GST-Pak2, GST-Cdc42, and GST-Crk were removed from the glutathione beads in 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0). Each protein and three different concentrations of bovine serum albumin (BSA) were subjected to SDS-Polyacrylamide gel electrophoresis (PAGE). The gel was stained and the densities of stained protein bands were quantified via ImageJ for their concentrations.

Phosphorylation assay

Phosphorylation of Crk with Pak2 was carried out in a total volume of 26 ul and incubated at 30°C for 1 min to 100 min. Crk (1 µg) was phosphorylated by Pak2 (0.1 µg) that was activated by Cdc42 (1 µg) and autophosphorylated in a mixture of 50 mM Tris-HCl (pH 7.4), 50 mM NaCl, and 0.18 mM GTP_S. The reaction was radiolabeled with ($\gamma$-$^{32}$P)ATP (500 cpm/pmol) in a volume of 26 ul containing 20 nM Tris-HCl (pH 7.4), 10 mM MgCl$_2$, 30 mM 2-mercaptoethanol, and 0.2 mM ATP. Each reaction was terminated by adding...
SDS-sample buffer. The reactions were subjected to SDS-Polyacrylamide gel electrophoresis (PAGE) on a 10% polyacrylamide gel. The gel was stained with Coomassie Blue, de-strained, and dried followed phosphorimaging. The dried gel bands were then quantified and counted for incorporation of $^{32}$P into Crk by scintillation counting.

**Phosphopeptide Mapping**

Two phosphorylated Crk protein bands that exhibited most high levels of phosphorylation were selected for phosphopeptide mappings. The gel band incubated for 80 min was digested with trypsin, and the other gel band incubated for 60 min was digested with Glu-C. The amount of the endoproteinases used a ratio of 1:20 of protease:protein. The trypsin-treated gel was incubated at 35°C and Glu-C-treated gel was incubated at 26°C overnight. Following lyophilization of phosphopeptides, they were subjected to two-dimensional phosphopeptide mapping. The phosphopeptide mapping was composed of two stages. The first dimension of the mapping was electrophoresis in buffer containing butanol and glacial acetic acid (pH 3.1) for 2 hours at a voltage of 600, and the second dimension of the mapping was chromatography in buffer containing butanol, pyridine, and glacial acetic acid for 6 hours. The 2-D maps were visualized by phosphorimaging.

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**References**


