Use of Capillary Electrophoresis and Endogenous Fluorescent Substrate To Monitor Intracellular Activation of Protein Kinase A

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Use of Capillary Electrophoresis and Endogenous Fluorescent Substrate To Monitor Intracellular Activation of Protein Kinase A

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Here we demonstrate for the first time the use of an endogenous multiphosphorylatable substrate for monitoring the intracellular activation of kinase with capillary electrophoresis. First, we devised a novel PCR-based strategy for controlled generation of short multirepeat DNA sequences and applied this method to generate a green fluorescence protein (GFP)-tagged protein substrate containing eight phosphorylation sites for protein kinase A (PKA). The protein substrate was transiently expressed in C2C12 rat myoblast cells, and intracellular PKA was then activated by adding [8]-bromo-cyclic AMP to the cell culture medium. Phosphorylated product and nonphosphorylated substrate present in the crude cell extract were separated by capillary zone electrophoresis and detected with laser-induced fluorescence of the GFP tag. The identities of two electrophoretic peaks were confirmed by both phosphorylation of the substrate and dephosphorylation of the product in vitro. The proposed method was applied to monitoring the activation of PKA in single myoblast cells. It advantageously allowed us to avoid microinjection of the substrate, the procedure that is both hard to perform and excessively invasive when applied to small mammalian cells.

Protein kinases constitute a large family of enzymes catalyzing the process of protein phosphorylation.[1] Kinases are the key players in the transduction of cellular signals that regulate the majority of critical cellular activities. Abnormal activities of multiple proteins kinases result from, and contribute to, a number of pathological processes, including carcinogenesis.[2−4]

Due to the genomic instability,[5] tumor tissues are highly heterogeneous—they are characterized by significant cell-to-cell variations in both their morphology and their biochemistry. To understand the molecular mechanisms of tumorigenesis, the biochemistry of cancerous cells has to be studied at the single-cell level. Although classical flow and image cytometry allow for a variety of single-cell analyses, they have very limited capabilities with respect to measuring enzyme activities. To be used in monitoring enzymatic activities, flow and image cytometry require fluorogenic substrates, which are not available for the majority of regulatory enzymes.

Enzyme activities in single cells can be alternatively studied with capillary electrophoresis (CE) that separates the substrate from the product of an enzymatic reaction. CE does not require fluorogenic substrates but rather utilizes fluorescently labeled substrates that can be readily synthesized for a vast variety of enzymes. Recently, CE was used to correlate the activities of glycosyltransferases with the cell cycle in cancer cells.[6] This technique has also been used to assay the activation of individual kinases within single *Xenopus* eggs.[7] In these experiments, an exogenous fluorescent substrate was introduced into relatively large egg cells by microinjection.

Where plasma membrane is permeable to the substrate, substrate delivery to the cells can be simply achieved by including the exogenous substrate in the culture media. Use of the exogenous substrate, however, becomes difficult if the plasma membrane is not permeable to the substrate, the cells are too small, or both, to apply microinjection (microinjection becomes technologically challenging and excessively invasive when applied to small mammalian somatic cells). In this work, we demonstrate for the first time the use of CE and an endogenous green fluorescence protein (GFP)-tagged substrate to assay intracellular enzymatic activities. This method was applied to monitoring the activation of protein kinase A (PKA) in single C2C12 cells. To enhance the separation of phosphorylated products from substrate by CE, we generated a multiphosphorylatable substrate for PKA containing eight phosphorylatable serine residues. We developed a novel PCR-based method for synthesis of short repetitive sequences and applied this novel technique to create the GFP-tagged substrate. Our novel method for synthesis of short repetitive peptides is believed to be an invaluable tool for molecular biology, and in the authors' opinion, this work is regarded as one of many of its applications.

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EXPERIMENTAL SECTION

Generation of the Multiphosphorylatable Substrate. Two complementary 36-base-long oligonucleotides of the following sequence, C AGG AGG AGG AGC ATC AAC AGG AGG AGG AGC ATC AA, were custom-synthesized by Genosys (Oakville, ON, Canada). The sequence consisted of two 18-base pair repeats. Upon ligation of the final multirepeat sequence with the vector, the sequence was brought into a desirable genetic frame coding for multiple repeats of six amino acids, RRRSIN, that were recognized as a phosphorylation site by PKA. The two 36-base-long oligonucleotides were used as substrates in a PCR reaction to generate sequences with a larger number of 18-base pair repeats. The PCR conditions were optimized to promote the hybridization of 18 base pairs of the two complementary sequences, so that an 18-base pair single-stranded overhang was available for polymerization at both 5′ and 3′ ends of the hybrid. Thus, the substrates acted as both the template and the primer.

PCR reactions were carried out on an Eppendorff thermocycler using 600 ng of the substrate and 2.5 units of *pfu* polymerase (Stratagene, Cedar Creek, TX), 3 mM concentrations of each dNTP and 12 mM $MgCl_2$ (all from Sigma-Aldrich) in a 50-μL reaction volume. The amplification procedure comprised of 1-min denaturation at 94 °C followed by 2-min annealing at 65 °C (core T_m + 10 °C). Extension was carried out at 72 °C for 5 min. The reaction was repeated for 35 cycles.

PCR products were resolved on 1% technical grade agarose (Biorad, Mississauga, ON, Canada) and gel purified using a Qiaquick Gel purification kit (Qiagen, Mississauga, ON, Canada). Plasmid DNA purification was carried out using a GenElute plasmid purification kit (Sigma-Aldrich).

DH5a competent cells were purchased from Invitrogen (Burlington, ON, Canada) and used for the propagation of plasmid DNA. Bacterial growth medium consisted of homemade Luria broth and selection for positive transformants was carried out in the presence of 40−50 μg/mL kanamycin (Sigma-Aldrich).

Purified PCR products had blunt ends and were directly cloned into the *Sma*I cut site of the pEGFP-C1 vector (Clontech, Palo Alto, CA). The pEGFP-C1 vector was a gift of Dr. A. P. Bedard (Department of Biology, York University). *Sma*I was purchased from Sigma-Aldrich, and enzymatic digestion of the pEGFP vector was carried out using 5 μL of *Sma*I enzyme in the presence of 1× digestion buffer. One μL of T4 DNA ligase (400 units/μL) (BioLabs, Pickering, ON, Canada) was used for blunt-end ligation. Five nanograms of the linearized vector was used in conjunction with 1 μg of PCR products. The ligation reaction was carried out overnight at 4 °C. Fifty microliters of competent DH5a cells (Invitrogen) was transformed by ligation products, using 50 μL of the ligation mixture. Competent cells were heat-shocked for 3 min at 42 °C and grown overnight at 37 °C, in the presence of 50 μg/mL kanamycin after a 2-h incubation in the absence of kanamycin, to allow for expression of the antibiotic resistance gene.

Plasmid DNA minipreparations were made from the overnight culture, using GenElute plasmid purification kits (Sigma-Aldrich). Purified DNA plasmids were then digested with 10 units of *Sma*I for 2 h to linearize the ligated pEGFP vectors. After the inactivation of *Sma*I (65 °C for 10 min), the mixture was transformed back into DH5a cells as described above and the cells were plated in the presence of 40 μg/mL kanamycin. Selected single colonies were then inoculated overnight in Luria broth, and plasmid minipreparations were made from the overnight cultures, using the GenElute kit. Positive clones carrying the recombinant insert were identified, using a restriction digestion assay. Positive clones carrying recombinant insert lacked the *Sma*I cut site, whereas the self-ligated vectors contained the cut site. Plasmid DNA preparations were digested with *Sma*I, and products were resolved on a 1% agarose gel. Positive clones (i.e., circular plasmids) were then subjected to sequencing.

Sequencing was carried out on an ABI Prism DNA sequencer (Applied Biosystems, Foster City, CA) in the Core Molecular Biology Facility at York University. A primer for sequencing comprised of a unique 22-mer sequence 5′CATGGTCCTGCTGGAGTTCGTC3′ (Genosys).

Gene Transfection and Substrate Expression in Cell Culture. Transfection of C2C12 cells was carried out using Lipofectamine (Invitrogen), according to the manufacturer's protocol. Culture media consisted of DMEM in 12% FBS (Invitrogen). Cells were grown in a Revco CO_2 incubator at 37 °C in the 5% CO_2 atmosphere to 70% confluency and transfected with 5 μg of plasmid DNA, as suggested by the manufacturer of Lipofectamine. Cells were harvested by trypsinization 17 h past transfection.

Preparation of Crude Cell Extract. Trypsin-harvested cells were subjected to centrifugation at 1000*g* for 5 min. The pellet was washed 5 times with phosphate-buffered saline solution (PBS), resuspended in 2 mL of PBS, and subjected to sonication. The pellet was kept on ice during the sonication procedure. Sonication cycles consisted of six bursts each for 10 s with 30-s cooling intervals between the bursts. Lysis cycles were monitored by microscopy to ensure the complete cell lysis.

Phosphorylation and Dephosphorylation Reactions. Ten units of PKA catalytic subunit (Sigma-Aldrich) were incubated at 37 °C with 10 μL of cell extract and 1 μL of 200 μM ATP, 500 mM Tris, and 100 mM $MgCl_2$ for 30 min. cAMP-induced phosphorylation was carried out by incubating cells or the cell extract at 37 °C in the presence of 0.5 mM membrane permeable [8]-Br-cAMP (Sigma-Aldrich) for 60 min. The dephosphorylation procedure included incubation of the phosphorylated extract with 10 units of CIP alkaline phosphatase (BioLabs) at 30 °C.

Capillary Electrophoresis. Instrumentation for CE is described elsewhere.[8] A 10 mM Borax solution at pH 9.2 was used as a run buffer. Separation was carried out in a positive polarity mode (positive electrode at the injection end) with an electric field of 400 V/cm. Individual cells were injected into the capillary as described earlier.[8] On-column cell lysis was carried out by a plug of 5 mM sodium dodecyl sulfate (SDS) in 10 mM Borax, pH 9.2, injected after the injection of a cell. The components of the buffer were bought from Sigma-Aldrich.

RESULTS AND DISCUSSION

Endogenous Substrate with a Single Phosphorylation Site. In 1999, Yang et al. generated a GFP-tagged substrate for PKA by replacing the last three codons of GFP with a sequence encoding six amino acids, RRRSII, that represents a single-consensus phosphorylation target of PKA.[9] These authors demonstrated

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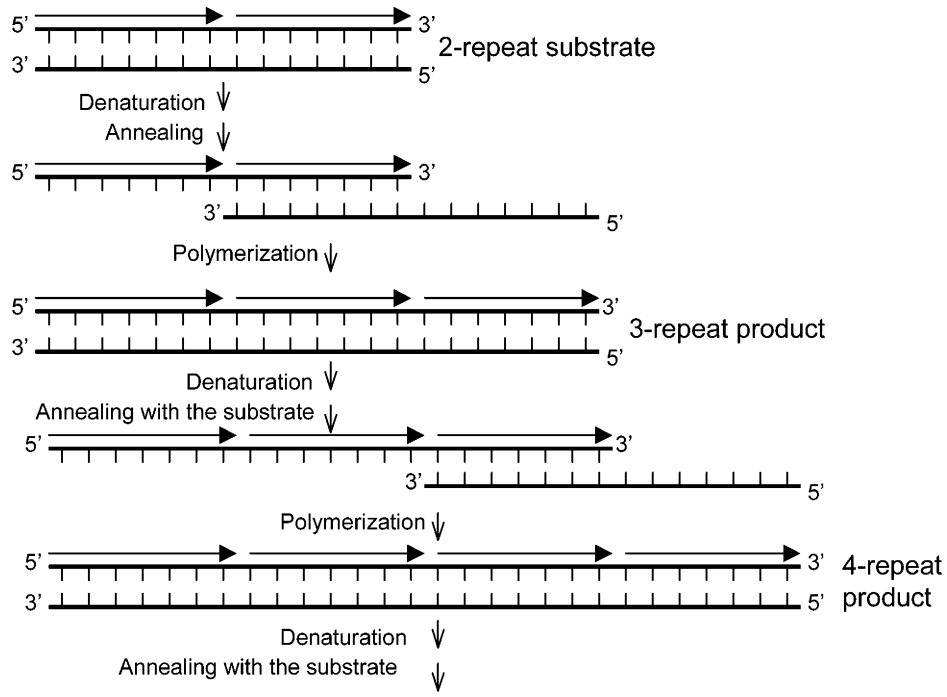

Figure 1. Schematic representation of the controlled PCR production of short multirepeat sequences. When the PCR reaction is carried out in excess of the 2-repeat substrate, the multirepeat products react predominantly with the 2-repeat substrate. This prevents the uncontrolled formation of long multirepeat sequences through the annealing of multirepeat products between themselves.

phosphorylation of the RRRSII-GFP substrate by PKA in vitro and showed that this enzyme did not phosphorylate GFP itself.

In our first experiments, we attempted to use the above-mentioned endogenous substrate to monitor the activation of PKA with CE. We expressed the RRRSII-GFP gene, generously provided by Dr. K. Shokat (University of California, Los Angeles), in BL21 *Escherichia coli* cells and produced the cell extract containing the substrate. The substrate in the extract was phosphorylated by treatment with exogenous catalytic subunit of PKA. However, we failed to find the capillary zone electrophoresis (CZE) conditions for baseline separation of the nonphosphorylated substrate from its phosphorylated product. The addition of a single phosphate group into a modified GFP protein did not sufficiently change its electrophoretic mobility to achieve the separation. To increase the phosphorylation-induced mobility shift, we designed an endogenous substrate with eight phosphorylation sites, $(RRRSIN)_8$-GFP.

Method for Controlled Generation of Short Multirepeat DNA Sequences. To generate the $(RRRSIN)_8$-GFP substrate, the DNA sequence encoding $(RRRSIN)_8$ had to be first constructed. The oligonucleotide sequence encoding the $(RRRSIN)_8$ peptide consists of 144 bases. Chemical synthesis of an oligonucleotide of this length is challenging; therefore, we searched for other means of generating this short multirepeat sequence. We use the term "short multirepeat sequence" for sequences with the number of repeats in the range of 2−10.

Molecular biology approaches typically used for generation of short multirepeat DNA sequences include laborious rounds of cloning and enzyme digestion and thus were considered not practical.[10] We optimized a simple PCR-based method that allows for controlled production of short multirepeat sequences from a 2-repeat substrate. The method is a modified version of the procedure suggested by Hemat and McEntee for the production of long multirepeat sequences (>1000 repeats).[11] The essence of this method is schematically depicted in Figure 1. Two complementary sequences of a 2-repeat substrate are chemically synthesized and used as both template and primer in a PCR reaction. In contrast to the procedure suggested by Hemat and McEntee, in our method, the PCR conditions are optimized to maintain the substrate in excess to the products during the entire course of the PCR reaction. This can be achieved by increasing the initial concentration of the substrate in the reaction mixture or by adding in the substrate between the PCR cycles. Under such conditions, the higher molecular weight products (3-repeat, 4-repeat, etc.) react predominantly with the substrate and not between themselves. This promotes a linear elongation of the products, in contrast to exponential elongation under the Hemat and McEntee conditions. To facilitate this reaction, the temperature regime of PCR is optimized to allow for 1-repeat hybrids to form. One-repeat hybrids with the 2-repeat substrate give rise to products that are 1 repeat longer than the elongated multimer. Two-repeat hybrids convert the 2-repeat substrate into an exact replica of the multimer involved in hybridization. As a result, the reaction proceeds in a well-controlled fashion producing predominantly short multirepeat sequences. The PCR-based method was used to generate the 8-repeat product as described in the Experimental Section.

Our method for the controlled generation of short multirepeat sequences on its own is a valuable addition to molecular biology

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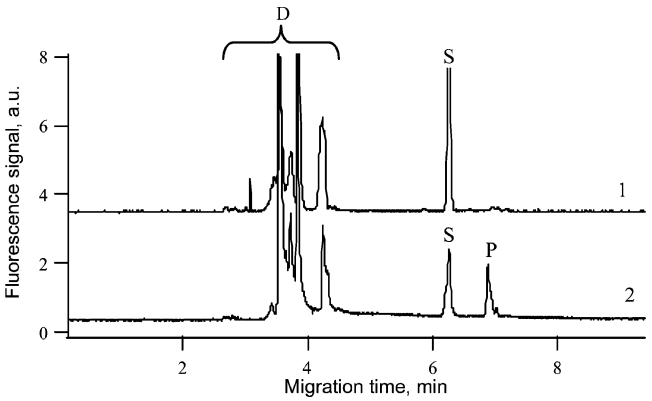

Figure 2. CZE analysis of the endogenous GFP-tagged multiphosphorylatable substrate and its phosphorylated product. Phosphorylation was carried out in the crude cell extract by the addition of exogenous catalytic subunit of PKA. Line 1 corresponds to the untreated extract while line 2 corresponds to the PKA-treated extract. Peaks S and P correspond to the substrate and its phosphorylated product, respectively. CZE was carried out in 10 mM Borax buffer, pH 9.2, at 400 V/cm.

tools. It will find applications in the following: (i) the production of repetitive endogenous peptide substrates, (ii) the construction of expression reporter vectors containing a limited number of tandem regulatory boxes, and (iii) the determination of the minimum size of a repetitive sequence conferring a function to a protein, e.g., the pathogenic length of polyglutamic tract in Huntington disease.

Endogenous Substrate with Eight Phosphorylation Sites. We used the method described above and standard molecular biology techniques to generate the $(RRRSIN)_8$-GFP gene with eight serine residues phosphorylatable by PKA. The gene was transiently expressed in C2C12 cells. The cells were harvested 17 h past transfection and washed, and a crude cellular extract containing $(RRRSIN)_8$-GFP was prepared. The CZE analysis of the extract revealed a series of peaks, D, with short migration times and a single peak S with long migration time (Figure 2). To reveal which peak corresponded to the substrate, the extract was treated with the exogenous catalytic subunit of PKA. Such a treatment did not change peaks D, which allowed us to ascribe them to nonphosphorylatable products of substrate degradation (precautions were taken to exclude exogenous trypsin, used to harvest the cells, as a cause of substrate proteolysis).

In contrast, the treatment with PKA profoundly influenced peak S. The amplitude of peak S progressively decreased with increasing time of extract incubation with PKA. The decrease of peak S was accompanied by the appearance a new peak, P, whose amplitude grew with increasing incubation time. These observations indicated that peak S corresponded to the $(RRRSIN)_8$-GFP substrate, while peak P corresponded to its phosphorylation product. Similar results were obtained when [8]-Br-cAMP was added to the extract without adding the exogenous PKA. Cyclic AMP activated endogenous cellular PKA present in the crude extract, and this resulted in the conversion of the substrate into its phosphorylated product. The phosphorylated product was also present in the extract prepared from C2C12 cells incubated with the membrane-permeable [8]-Br-cAMP agent, suggesting that the amount of endogenous intracellular PKA is sufficient to bring about intracellular phosphorylation of the GFP substrate. In this

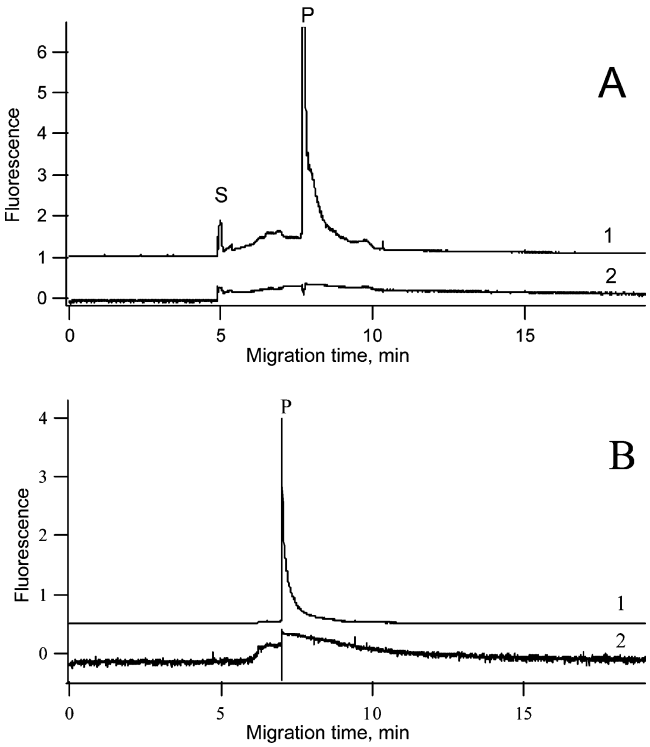

Figure 3. Analyses of PKA-catalyzed phosphorylation of the endogenous GFP-tagged multiphosphorylatable substrate in single C2C12 cells. Panel A shows the representative electropherogram from a cell, which was not treated with [8]-Br-cAMP; panel B shows the electropherogram from a cell that was treated with [8]-Br-cAMP. In both panels, traces 1 illustrate fluorescence intensity (au) while traces 2 correspond to fluorescence anisotropy (absolute units). CZE was carried out in 10 mM Borax buffer, pH 9.2, at 400 V/cm.

experiment, the cells were thoroughly washed from [8]-Br-cAMP prior to lysis, to ensure that it did not contaminate the extract. The identities of the two peaks, S and P, were also confirmed by treating the phosphorylated product with CIP alkaline phosphatase. Upon such treatment, the peak corresponding to the phosphorylated product, peak P, disappeared while the peak of the nonphosphorylated substrate, peak S, increased. The above observations suggest that $(RRRSIN)_8$-GFP is an endogenous substrate of PKA and that its phosphorylation product can be well separated from the substrate by CZE.

Monitoring of Induced PKA Activity in Single Cells. To assay for the activation of the endogenous PKA upon treatment with [8]-Br-cAMP within single C2C12 cells, the GFP-tagged substrate was expressed in these cells by transient transfection, and cells were harvested 17 h after the transfection. Single C2C12 cells were injected into the capillary and lysed by a subsequently injected plug of the run buffer supplemented with 5 mM SDS. The electropherograms of single cells differed from those of the cell extract. The cells that were not treated with c-AMP generated two peaks with significantly different intensities but virtually identical values of fluorescence anisotropy (Figure 3A). The equality of the peaks' anisotropy suggested that neither one of the peaks was the product of GFP degradation, which would, otherwise, be expected to have a lower anisotropy value. The peak with a shorter migration time was ascribed to the substrate (S) while the peak with longer migration time was ascribed to the phosphorylated product (P). This interpretation was confirmed

by the analysis of c-AMP-treated cells; peak S completely disappeared in the treated cells (Figure 3B). In total, 40 single cells of two types (c-AMP treated and nontreated) were analyzed, and two similar types of profiles were obtained from all of them.

The difference between the extract experiments and the single-cell experiment was quite profound. In single cells, most of the substrate was phosphorylated even without exogenous activation of endogenous PKA. This indicates that the endogenous PKA and cAMP are sufficient for phosphorylation of the *most* of the expressed substrate. The induction of PKA activity with the exogenous [8]-Br-cAMP resulted in complete conversion of the substrate to the phosphorylated product. In the cell extract, in contrast, the phosphorylated product was not observed, unless the PKA activity was induced with an exogenous agent. We think that this difference was caused by a metabolic bias associated with cell extract preparation.[12] In intact cells, the substrate may be accessible to PKA and inaccessible to phosphatases, due to differential compartmentalization. As a result, the substrate can be slowly phosphorylated by the endogenous PKA/cAMP, even in the absence of external activation of PKA. In single-cell analyses, the cell is subjected to separation immediately after its lysis, preventing dephosphorylation of the phosphorylated product. In the cell extract, in contrast, all intracellular compartments are destroyed and the phosphorylated product formed by endogenous PKA and cAMP could be targeted by intracellular phosphatases and rapidly dephosphorylated. Furthermore, the cell extract preparation procedure could potentially decrease the kinase activity while not significantly decreasing the phosphatase activity. This would also lead to dephosphorylation of the substrate in the cell extract.

To conclude, we demonstrated for the first time the use of an endogenous GFP-tagged multiphosphorylatable substrate and CE for monitoring of kinase activation in single cells. The endogenous substrate advantageously allowed us to avoid microinjection, the procedure that is both hard to perform and excessively invasive when applied to small mammalian cells. The demonstrated approach can be used to study other enzymes involved in posttranslational modifications of the proteins, thus widening the prospective of single-cell proteomics.

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