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Chemical Cytometry for Monitoring Metabolism of a Ras-Mimicking Substrate in Single Cells

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Background: Chemical cytometry is an emerging technology that analyzes chemical contents of single cells by means of capillary electrophoresis or capillary chromatography. It has a potential to become an indispensable tool in analyses of heterogeneous cell populations such as those in tumors. Ras oncogenes are found in 30% of human cancers. To become fully functional products, oncogenic Ras proteins require at least three posttranslational modifications: farnesylation, endoproteolysis, and carboxyl-methylation. Therefore, enzymes that catalyze the three reactions, farnesyltransferase (FTase), endoprotease (EPase), and methyltransferase (MTase), are considered highly attractive therapeutic targets. In this work, we used chemical cytometry to study the metabolism of a pentapeptide substrate that can mimic Ras proteins with respect to their posttranslational modifications in solution.
Methods: Mouse mammary gland tumor cells (4T1) and mouse embryo fibroblasts (NIH3T3) were incubated with a fluorescently labeled pentapeptide substrate, 2′,7′-difluorofluorescein-5-carboxyl-Gly-Cys-Val-Ilu-Ala. Cells were washed from the substrate and resuspended in phosphate buffered saline. Uptake of the substrate by the cells was monitored by laser scanning confocal microscopy. Single cells were injected into the capillary, lysed, and subjected to capillary electrophoresis. Fluorescent metabolic products were detected by laser-induced fluorescence and compared with products obtained by the conversion of the substrate by FTase, EPase, and MTase in solution. Co-sampling of single cells with the in-vitro products was used for such comparison.
Results: Confocal microscopy data showed that the substrate permeated the plasma membrane and clustered in the cytoplasm. Further capillary electrophoresis and chemical cytometry analyses showed that the substrate was converted into three fluorescently labeled products, two of which were secreted in the culture medium and one remained in the cells. The intracellular product was present at approximately 100,000 molecules per cell. The three metabolic products of the substrate were found to be different from the products of its processing by FTase, EPase, and MTase in solution.
Conclusions: This is the first report of chemical cytometry in the context of Ras-signaling studies. The chemical cytometry method used in this work will find applications in the development of suitable peptide substrates for monitoring enzyme activities in single cells. © 2004 Wiley-Liss, Inc.

Key terms: single-cell capillary electrophoresis; laser-induced fluorescence detection; Ras posttranslational modifications; farnesylation; endoproteolysis; carboxyl-methylation; fluorescently labeled substrate; cancer; mammalian cells; mouse mammary gland tumor cells; mouse embryo fibroblasts

Tissues are comprised of heterogeneous cell populations that consist of cells that differ in biological function, morphology, and chemistry. Heterogeneous cell populations are generated during many fundamental biological processes. Among them are physiologic processes, such as embryogenesis and tissue regeneration, and pathologic processes, such as neurodegeneration and carcinogenesis. To elucidate the molecular mechanisms of these processes, we need to study the chemical differences between individual cells within heterogeneous cell populations formed during these processes. The complexity of those molecular mechanisms requires a multicomponent chemical analysis of single cells. Classic biochemical and cytometric techniques are not applicable to multicomponent chemical analysis of single cells. Classic biochemistry requires large biochemical homogenates prepared from millions of cells, whereas classic cytometry (image cytom-

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etry and flow cytometry) permits the simultaneous assay of only a limited number of chemical species determined by the number of spectral channels available (rarely more than three). Alternatively, the multicomponent analysis of single cells can be fulfilled using chemical cytometry. Chemical cytometry uses microseparation techniques, such as capillary electrophoresis and capillary chromatography, to study the chemical contents of single cells. For a broad overview of the new technique, we refer the reader to a recent review on chemical cytometry and its applications (1). One of the forms of chemical cytometry is metabolic cytometry, which monitors a cascade of biosynthetic and biodegradation products generated in a single cell (2). Metabolic cytometry can be used to assess multiple enzymatic activities involved in metabolic cascades in single cells. In addition to providing information on the metabolic heterogeneity of cell populations, metabolic cytometry is more accurate than bulk analyses. It avoids biases associated with substrate and product degradation during sample processing (3). Metabolic cytometry was applied to study metabolism of oligosaccharides in single cells (2). It was also applied to monitoring kinase activities in single cells (4,5). In this work we used metabolic cytometry to study the metabolism of a pentapeptide substrate that can mimic Ras proteins with regard to their posttranslational modifications.

Ras proteins are guanosine triphosphate binding proteins that play a pivotal role in the control of many cellular processes, including normal and transformed cell growth and cell differentiation. Mutated forms of Ras are found in many human cancers, with the highest incidences in pancreatic (90%) and colon (50%) adenocarcinomas (6,7). To become functional, Ras proteins have to localize in the plasma membrane, which requires that Ras undergo at least three sequential posttranslational modifications: farnesylation, endoproteolysis, and carboxyl-methylation, catalyzed by farnesyltransferase (FTase), endoprotease (EPase), and methyltransferase (MTase), respectively (8). All three reactions occur at the carboxy terminus of Ras in a region called a Ca_1a_2X motif, where C is cysteine, a_1 and a_2 are usually small aliphatic amino acids, and X is one of the following amino acids: methionine, serine, glutamine, or alanine (Fig. 1). During farnesylation, a highly hydrophobic farnesyl group is transferred from farnesyl diphosphate to the cysteine residue of the Ca_1a_2X motif. Endoproteolysis targets farnesylated Ras and results in the cleavage of the a_1a_2X tripeptide. Finally, the terminal cysteine group is carboxymethylated.

FTase, EPase, and MTase are specific only to the Ca_1a_2X motif of Ras; therefore, short peptides with this motif at the carboxy terminus can mimic Ras with respect to the three enzymatic reactions. Such peptides can be used to measure activities of the three enzymes. We recently demonstrated that a fluorescently labeled pentapeptide as a substrate can be sequentially converted into three products by FTase, EPase, and MTase (Fig. 1) (9). In the same work, we also developed a very sensitive method that allows simultaneous quantities of the substrate and the products. Here we used laser-scanning confocal microscopy and chemical cytometry to study permeation and the metabolism of the substrate in single cells.

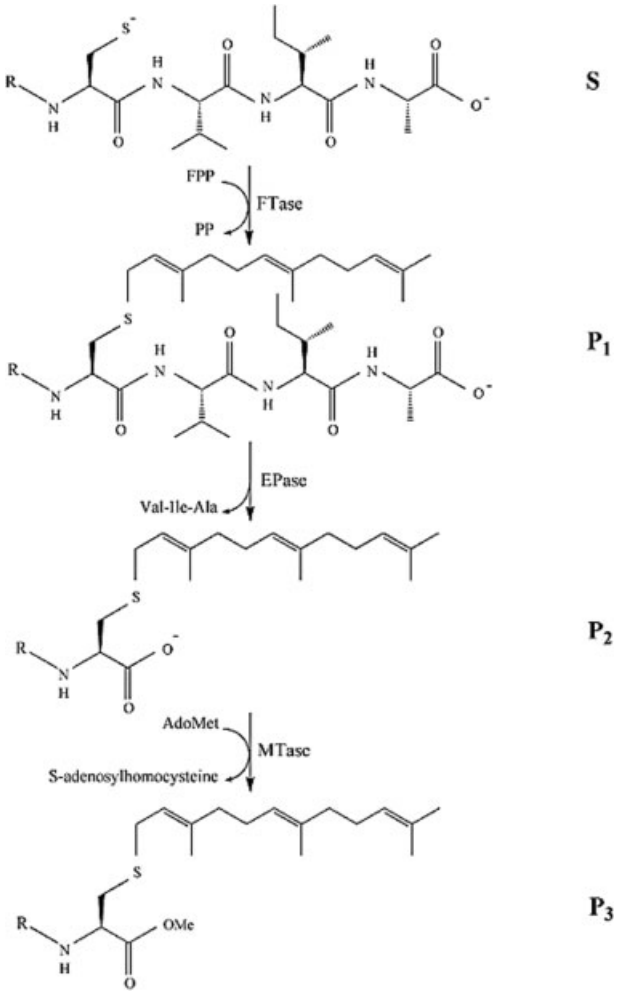

FIG. 1. A cascade of three enzymatic reactions that catalyze a common pathway of (i) posttranslational modifications of Ras-proteins and (ii) sequential conversion of S into products P_1 to P_3. The three reactions are catalyzed by FTase, EPase and MTase, respectively. R is Ras protein without the Ca_1a_2X motif or S without the Ca_1a_2X motif. AdoMet, S-adenosyl-L-methionine; FPP, farnesyl diphosphate; OMe, methoxy group; PP, diphosphate.

MATERIALS AND METHODS

Materials

Yeast *Saccharomyces cerevisiae* FTase, farnesyl diphosphate, S-adenosyl-L-methyl-methionine, polyvinyl alcohol, all buffer components, and sodium dodecylsulfate (SDS) were purchased from Sigma-Aldrich (Oakville, ON, Canada). The recombinant yeast *Saccharomyces cerevisiae* (strain JDY101) EPase (yRCE1p) and 2′,7′-difluorofluorescein-5-carboxyl-Gly-Cys-Val-Ile-Ala (S) were provided by Dr. C. Dale Poulter (University of Utah). A crude yeast membrane preparation from *Saccharomyces cerevisiae* (strain AH 109) was used as a source of MTase. Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Me-

dium (DMEM), fetal bovine serum, antibiotics mixture, and 0.25% trypsin were purchased from Invitrogen Canada Inc. (Burlington, ON, Canada).

Three products, P_1, P_2, and P_3, of sequential farnesylation, endoproteolysis and carboxyl-methylation of S, respectively, were obtained as described previously (9). These products were used as internal standards in chemical cytometry to examine whether intracellular metabolites of S were identical to P_1 to P_3.

Cell Cultures

Cells lines 4T1 (mouse mammary gland tumor cells) and NIH3T3 (mouse embryo fibroblasts) were used in this study. Cells were grown to 80%–90% confluence in DMEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in 5% CO_2 atmosphere.

For confocal microscopy and chemical cytometry, cells were incubated in serum-free DMEM with different concentrations of S (100 nM, 0.5 μM, and 1 μM) for 24 and 48 h. After incubation, cells were washed three times with PBS to remove residual substrate. Cells were then harvested, washed five times with PBS, and resuspended in PBS or in serum-free DMEM.

Chemical Cytometry

Our chemical cytometry analyses were based on capillary electrophoresis (CE) of single cells with laser-induced fluorescence (LIF) detection. Analyses were performed by using a modified version of the base instrument described in detail elsewhere (10). The modified apparatus used new models of the capillary holder and micromanipulator (Scitomix, Concord, ON, Canada) to allow for more reliable and convenient procedures of cell injection and CE separation.

Cell injection was carried out under an inverted IX-70 Olympus microscope (Carsen Group, Markham, ON, Canada). Twenty microliters of cell suspension was deposited on a microscope slide coated with polyvinyl alcohol to decrease cell adhesion to the surface (11), and cells were allowed to settle down. A fused silica capillary of 48 cm × 20 μm inner diameter × 150 μm outer diameter (Polymicro, Phoenix, AZ, USA) was prefilled with the SDS-containing electrophoresis run buffer: 25 mM sodium tetraborate at pH 8.3 supplemented with 25 mM SDS. This run buffer facilitates cell lysis and separation of analyzed components in CE (9). The capillary was held vertically over the microscope slide by means of a multifunctional capillary holder (Scitomix), which was mounted on a long-travel three-dimensional micromanipulator (Scitomix). The base of the manipulator was fixed to the microscope body. The capillary was located in the center of the field of view of the microscope, with the tip of the capillary at a distance of 10 to 20 μm from the surface. A cell chosen for analysis was superimposed with the orifice of the capillary by moving a microscope stage and drawn into the capillary by a suction pulse of 9.1 kPa for 1 s. While in the capillary, the cell was lysed in less than 1 min by SDS, which was a component of the run buffer (see above). The capillary was then immersed in the run buffer, and a high voltage of 400 V/cm was applied across the capillary (positive electrode at the injection end and grounded electrode at the distal end). Fluorescently labeled species (S and its products) were separated and detected with the LIF detector located at the distal end of the capillary. Fluorescence was excited by a 488-nm line of an argon-ion laser (Melles Griot, Nepean, ON, Canada) and filtered from stray and scattered laser light with a band pass filter centered at 520 nm (Omega Optical, Brattleboro, VT, USA). The capillary was rinsed with the run buffer solution for 2 min before each run. At the end of each run, the capillary was rinsed with 100 mM HCl and 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min.

Some control experiments required regular CE analyses of liquid samples. Such analyses were carried out with the same instrument as chemical cytometric experiments and used the same experimental settings.

Confocal Microscopy

The confocal fluorescence microscopic images in darkness and in transmitted light for the cells of both types (4T1 and NIH3T3), chosen at random, were obtained with an Olympus FluoView 300 confocal laser-scanning microscopic system (Carsen Group). A 5-mW argon ion laser (Melles Griot) at 488 nm was used as the source of fluorescence excitation. Three-dimensional fluorescence images of cells were reconstructed by superimposing optical sections obtained with a 0.2-μm step size between sections.

RESULTS

In our previous work, we used a fluorescently labeled pentapeptide (S) to monitor the activities of three enzymes (PFTase, EPase, and MTase) in solution (9). The goal of this project was to study the metabolism of S in cells.

Membrane Permeability

Plasma membrane permeability for S was studied by confocal laser-scanning microscopy. Twenty cells of each type, 4T1 and NIH3T3, were imaged after 1 day and 2 days of incubation in serum-free culture media with different concentrations of S at 37°C in 5% CO_2 atmosphere. Figure 2 shows a typical fluorescent image of a middle section of an NIH3T3 cell (1 μM S, 24-h incubation). Similar images were obtained for 4T1 cells. Fluorescence appeared to be clustered in the cytoplasm. The intensity of fluorescence increased with the concentration of S in incubation media and with incubation time. Incubations longer than 48 hours did not change fluorescence intensity. If the cell culture media did not contain S, no intracellular fluorescence was observed.

Negative Controls

Two types of cell media, serum-free DMEM and PBS, were used in chemical cytometry. Approximately 70 pl of medium was co-sampled with every cell and could have

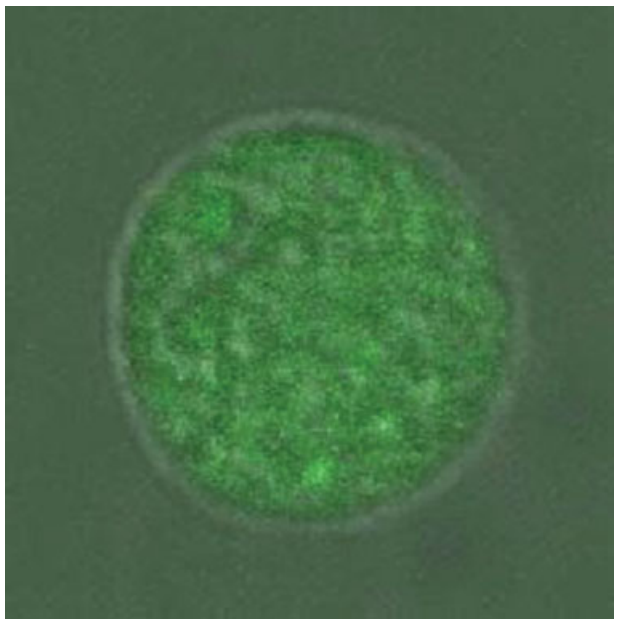

FIG. 2. Fluorescent confocal image of an NIH3T3 cell that depicts an intracellular distribution of S and products of its intracellular metabolism. Cells were incubated in serum-free DMEM supplemented with 1 μM S at 37°C for 24 h, washed with PBS, and resuspended in PBS before imaging. Image represents the middle optical section of the cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

introduced bias if the medium contained any fluorescent component. To understand the contribution of the medium, we analyzed the media solutions without cells by CE-LIF. PBS did not generate any fluorescent signal (Fig. 3, trace 1), whereas electropherograms of serum-free DMEM contained two peaks (Fig. 3, trace 2).

In addition, we performed chemical cytometry analyses of cells incubated in the S-free media to examine whether cells had any native fluorescence in the region of 520 nm. When cells were incubated in serum-free DMEM without S, only peaks of DMEM were observed, suggesting that cellular components do not introduce any background fluorescence (Fig. 3, trace 3). The two peaks in trace 3 are identical to those of pure serum-free DMEM and originate from coinjection of serum-free DMEM during cell sampling into the capillary.

To study the stability of S, we incubated S in serum-free DMEM without cells for 24 h, diluted it 30 times to avoid overloading the detector, and then subjected it to CE-LIF. The resulting electropherogram contained one major peak that corresponded to S and a minor peak from serum-free DMEM (Fig. 3, trace 4). The concentration of S did not change significantly during incubation. It is interesting that the first peak of serum-free DMEM (with a 6-min migration time) decreased by more than 30 times when serum-free DMEM was incubated with S, suggesting that the corresponding fluorescent component decomposed during incubation. In contrast to the first peak, the second peak (with a 7.5-min migration time) decreased by less than 30 times. The nature of these peaks and the mechanism of its decomposition are beyond the scopes of this work and are not discussed further.

Products of Intracellular Conversion of S

The products of S formed in cells could be retained in the cell or secreted outside. We first examined cell media for the presence of secreted products. Cells were incubated in S-containing serum-free DMEM for 24 h and serum-free DMEM was then analyzed for new fluorescent products. Two new peaks, A and B, which corresponded to unknown products of S, were found in the cell-containing medium (Fig. 4, peaks A and B). The amount of component A, calculated from electropherograms, ranged from 7% to 15% of the amount of S, and that of component B ranged from 2% to 4% of the amount of S. Results were identical for S-containing culture media incubated with 4T1 and NIH3T3 cells.

We then studied the products of intracellular conversion of S that were retained by cells. After incubation in S-containing serum-free DMEM, cells were washed and resuspended in serum-free DMEM or in PBS. Then single cells were sampled for chemical cytometry. Single-cell electropherograms showed no products A and B, suggesting that these products are secreted into the medium relatively quickly during the washing procedure. This conclusion was confirmed by our failure to detect A and B in the S-free cell media, in which the cells were incubated after washing them from the S-containing media. Electropherograms showed a peak corresponding to intact S and an additional peak C corresponding to another product of intracellular conversion of S (Fig. 5). Qualitatively similar results were obtained for NIH3T3 and 4T1 cells. To ex-

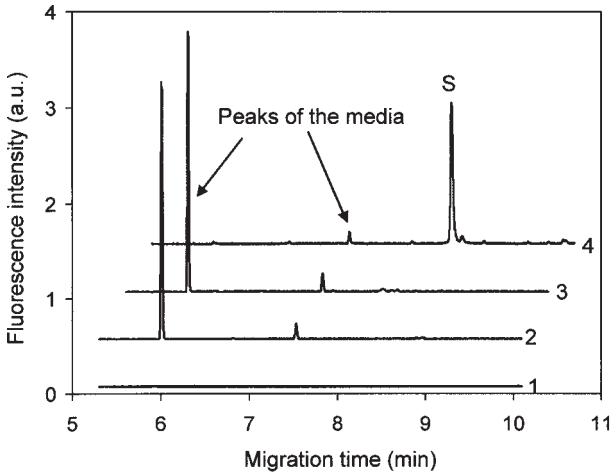

FIG. 3. CE-LIF electropherograms of PBS (trace 1), serum-free DMEM (trace 2), a 4T1 cell that was injected into the capillary with a plug of serum-free DMEM (trace 3), and serum-free DMEM supplemented with 1 μM S that was incubated at 37°C for 24 h without cells (trace 4). The sample for trace 4 was diluted 30 times to avoid overloading the detector. Traces 3 and 4 are offset along the horizontal axis by 0.3 and 0.6 min, respectively. Traces 2, 3, and 4 are offset along the vertical axis by 0.5, 1.0, and 1.5 units, respectively.

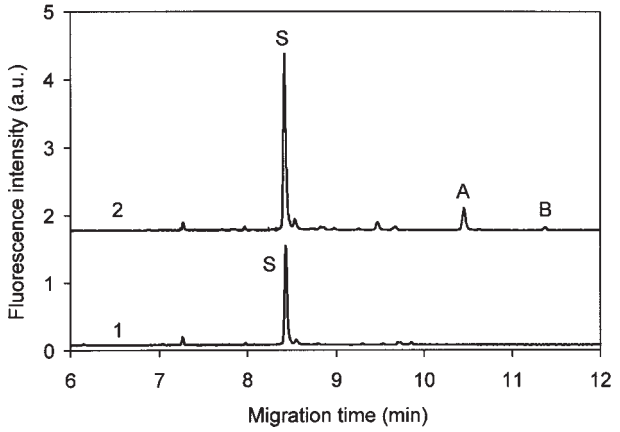

FIG. 4. Secretion of two products, A and B, of intracellular metabolism of S into the cell media. Trace 2 shows the CE-LIF electropherogram of serum-free DMEM, supplemented with 1 μM S, and incubated with 4T1 cells at 37°C for 24 h. Electropherogram 1 corresponds to a control experiment, in which the same medium was incubated under identical conditions but without cells. Both samples were diluted 20 times with serum-free DMEM to avoid overloading the detector. Trace 2 is offset along the vertical axis by 1.7 units.

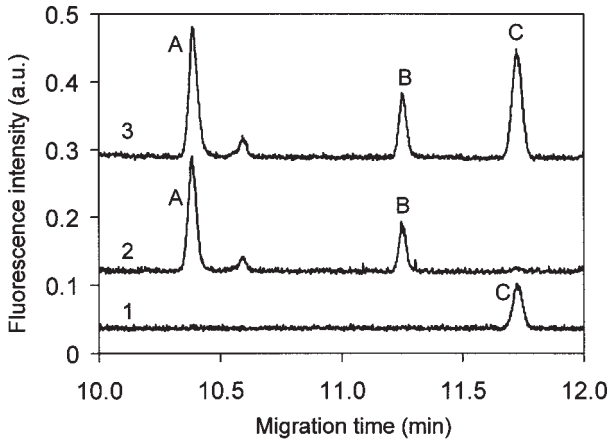

FIG. 6. Proof that product C differs from A and B. The three traces show CE-LIF electropherograms obtained from (i) a 4T1 cell that was incubated in serum-free DMEM supplemented with 1 μM S at 37°C for 24 h, washed from the medium by PBS, and resuspended in PBS before sampling into the capillary (trace 1); (ii) serum-free DMEM supplemented with 1 μM S after incubation with 4T1 cells at 37°C for 24 h (trace 2); and (iii) co-sampling of the first two (trace 3). The medium used for traces 2 and 3 was diluted 20 times with serum-free DMEM before sampling in CE to avoid overloading the detector.

amine whether or not two types of cells differed in the amount of product C, we determined relative amounts of C with respect to S. Relative amounts were obtained by dividing the area of peak C by that of peak S from the same electropherogram. Although this parameter varied considerably from cell to cell within the same cell type, the two types of cells showed statistically significant difference in this parameter. Average values of the relative amount of C were 1.35 for NIH3T3 (n = 14) and 1.1 for 4T1cells (n = 31). Thirty-one percent of 4T1 cells and 50% of NIH3T3 cells had the relative amount of C greater than 1.

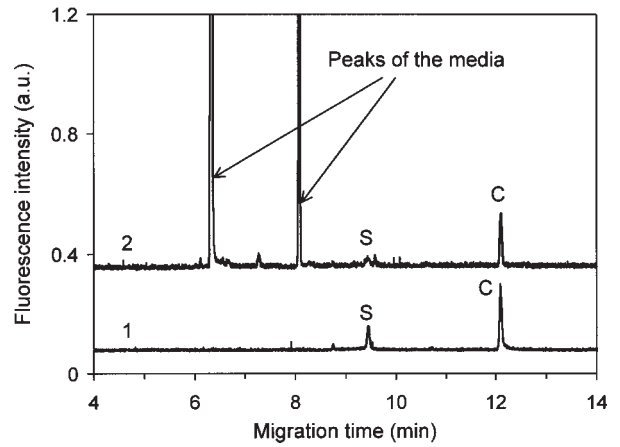

FIG. 5. CE-LIF electropherograms of single 4T1 cells injected into the capillary with plugs of PBS (trace 1) and serum-free DMEM (trace 2). Before CE-LIF analysis, cells were (i) incubated in serum-free DMEM supplemented with 1 μM S at 37°C for 24 h, (ii) washed with PBS (trace 1) or serum-free DMEM (trace 2), and (iii) resuspended in PBS (trace 1) or serum-free DMEM (trace 2). Peak C corresponds to an intracellular metabolite of S. Trace 2 is offset along the vertical axis by 0.3 units.

There is a noticeable difference of the migration times of peak S in Figures 3 to 5. This difference is due to different velocities of the electro-osmotic flow. The velocity of electro-osmotic flow is highly sensitive to a number of parameters including (a) temperature, (b) pH and ionic strength of the run buffer, and (c) modifications of capillary walls by the sample components. Slight variation in these parameters can lead to noticeable shifts of migrations times from run to run.

Thus, chemical cytometry showed only a single product of intracellular conversion of S retained in cells. The migration time of this product in CE differed considerably from those of S and fluorescent components of serum-free DMEM, providing favorable conditions for reliable quantitation of the product.

Identification of Products A, B, and C

Due to very low amounts of products A, B, and C, their identification by structure-analysis methods, such as mass spectrometry or nuclear magnetic resonance, was impossible. The only method available to us to identify A, B, and, C was indirect: the comparison of their migration times in CE with those of authentic standards in coinjection experiments. Coinjection experiments are designed so that the species in question is sampled simultaneously with a series of authentic standards. If the peak of the examined species is not completely merged with any of the peaks of the standards, this species is different from all the standards. Alternatively, if the examined species merges completely with any of the authentic standards, there is a high probability that this species is identical to the standard. It should be noted that only structural analysis could provide the ultimate proof of the identity.

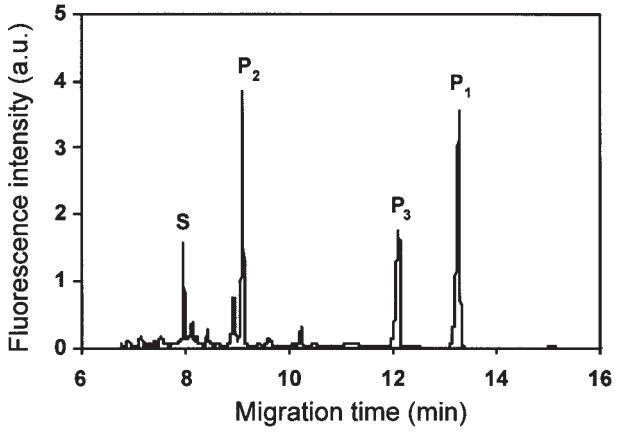

FIG. 7. CE-LIF separation of S and three products of its sequential farnesylation (P_1), endoproteolysis (P_2), and carboxyl-methylation (P_3). Reactions were conducted in solution under conditions described in detail previously (9).

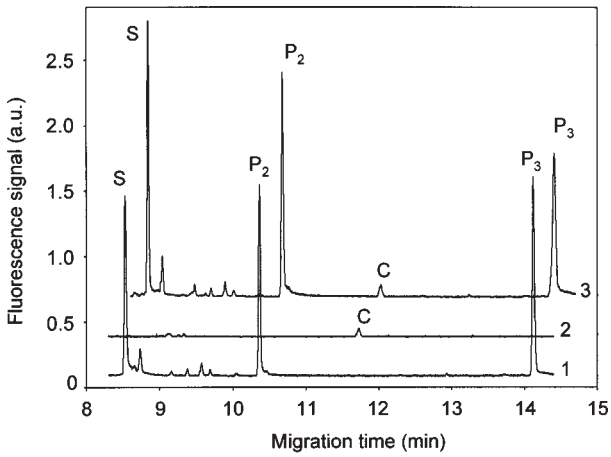

FIG. 9. Proof that product C differs from P_2 and P_3. Three traces are CE-LIF electropherograms obtained from a mixture of P_2 and P_3 (trace 1), a 4T1 cell similar to those in Figure 6 (trace 2), and coinjection of the first two (trace 3). Trace 3 is offset along the horizontal axis by 0.3 min. Traces 2 and 3 are offset along the vertical axis by 0.3 and 0.6 units, respectively.

A and B were found in culture media and C was found inside cells; therefore, we operated with electropherograms that contained A and B separately from C (Figs. 4 and 5). Although the migration time of C differed considerably from those of A and B in separate electropherograms (Fig. 6, traces 1 and 2), there was a finite probability that C was identical to A or B. To exclude this option, we conducted a co-sampling experiment in which a single cell (source of C) was injection into the capillary together with a cell culture medium (source of A and B). The resulting electropherogram contained three peaks, confirming that A, B, and C corresponded to three different products of S (Fig. 6, trace 3).

Next, we compared migration times of peaks A, B, and C with those of the authentic standards of their potential products. S was initially designed as a substrate for monitoring activities of PFTase, EPase, and MTase in solutions (9). Therefore, we examined whether or not A, B, and C corresponded to products P_1, P_2, and P_3 of sequential farnesylation, endoproteolysis, and carboxyl-methylation of S. Figure 7 shows the result of CE-LIF separation of S and P_1, P_2, and P_3. Our initial comparison of migration times of products A and B obtained in one experiment with those of products P_1, P_2, and P_3 obtained in another experiment showed that the migration time of B was significantly different from those of P_1, P_2, and P_3. Thus, product B is different from any of the three standards. Migration times of A and P_2 were within the limits of experimental error (Fig. 8, traces 1 and 2). This could indicate that peak A corresponded to product P_2. To examine this hypothesis, we performed a co-sampling experiment of the cell culture medium that contained A and a solution that contained P_2. The electropherogram contained two distinct peaks, indicating that product A is also different from the three standards (Fig. 8, trace 3).

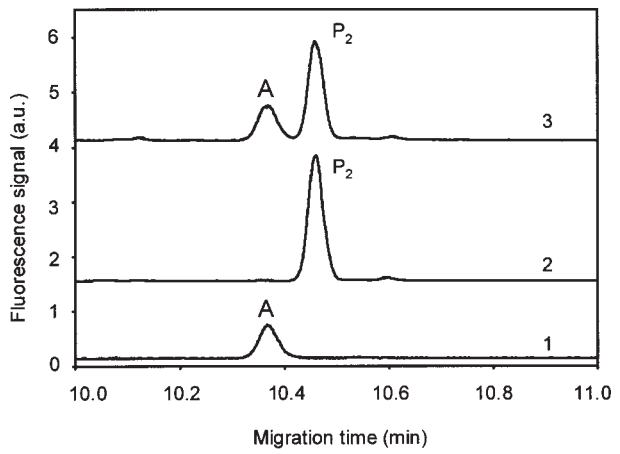

FIG. 8. Proof that product A differs from P_2. Trace 1 is the CE-LIF electropherogram obtained from the serum-free DMEM cell culture medium similar to that in Figure 6. Trace 2 is the CE-LIF electropherogram of P_2. Trace 3 is the CE electropherogram from coinjection of the first two. Traces 2 and 3 are offset along the vertical axis by 1.3 and 4 units, respectively.

We then examined the identity of C. Analysis of separate electropherograms showed that the migration time of C was intermediate between those of P_2 and P_3 (Fig. 9, traces 1 and 2). To prove that C was undoubtedly different from P_2 and P_3, we conducted a co-sampling experiment in which a single cell (source of C) was injected into the capillary with a mixture of P_2 and P_3. The resulting electropherogram contained three peaks confirming that C is different from P_1 and P_2. The migration time of P_1 was longer that that of P_3 (Fig. 7); therefore, C also is different from P_1. Thus, A, B, and C did not correspond to P_1, P_2, and P_3.

DISCUSSION

Due to genomic instability, cell populations within tumors are highly heterogeneous in their biochemistry and

this heterogeneity is informative for cancer diagnosis and prognosis. Many products of oncogenes are regulated by posttranslational modifications, such as phosphorylation, glycosylation, and lipidation. Monitoring the heterogeneity in the activity of enzymes that catalyze posttranslational modifications requires measuring the amounts of substrates and products at the single-cell level. Flow and image cytometries require fluorogenic substrates (compounds that become fluorescent only when undergoing enzymatic conversion) for such measurements. Fluorogenic substrates are difficult to design and, hence, are available for only a limited number of enzymes. For example, fluorogenic substrates are not available for kinases, glycosyltransferases, and lipid transferases. In contrast to flow and image cytometries, chemical cytometry relies not only on optical resolution but also on physical separation of chemical species by means of electrophoresis or chromatography. This feature of chemical cytometry makes it an indispensable tool in studies of enzyme activities in single cells because it can operate with fluorescently labeled substrates, which can be designed for many enzymes including kinases, glycosyltransferases, and lipid transferases.

In this work we used chemical cytometry to study intracellular metabolism of S, a Ras-mimicking pentapeptide substrate. To deliver S into the cells, we added it to the cell culture media. Confocal fluorescence microscopy and chemical cytometry showed that S could permeate through the plasma membrane. While inside the cell, S was converted to three products, two of which were secreted into the cell culture media and one was retained inside the cells. None of the three intracellular products was identical to any of the three products, P_1, P_2, and P_3, of sequential farnesylation, proteolysis, and methylation of S by FTase, EPase, and MTase in solution. There are three major reasons why P_1, P_2, and P_3 were not observed in chemical cytometry. First, S may have intracellular localization different from that of Ras proteins, the native substrates of FTase, EPase, and MTase. Second, S can be cleaved by intracellular proteases before or after it undergoes the three reactions of interest. Third, S may undergo geranylgeranylation instead of farnesylation (12–14). Larger peptides with the Ca_1a_2X motif may be more adequate models of Ras proteins than S if the challenges of their synthesis and delivery into cells are overcome. Ultimately, exogenous or endogenous green fluorescent protein tagged Ras proteins can be used as substrates if the method of their CE separation from the products is refined.

To conclude, this work and our previous studies of the S enzymatic conversion in solution suggest a generic way for finding a suitable Ras-mimicking substrate to monitor the activities of FTase, EPase, and MTase in single cells. First, pure products of the farnesylation, proteolysis, and methylation of the candidate substrate are obtained in solution using FTase, EPase, and MTase. Second, CE separation of the substrate and the three products is optimized when using our conditions as the first step in optimization. Third, the delivery of the substrate into cells is optimized. Fourth, products of intracellular conversion of the substrate are compared with the products of substrate farnesylation, endoproteolysis, and methylation by using co-sampling of cells and pure products. When found, a suitable substrate will serve as a toll for finding inhibitors of FTase, EPase, and MTase; such inhibitors can be potentially anticancer therapeutic agents. Moreover, such a substrate can facilitate in monitoring the heterogeneity of a tumor's response to Ras-targeting anticancer therapies.

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