Mutation detection using a novel plant endonuclease

Catherine A. Oleykowski, Colleen R. Bronson Mullins, Andrew K. Godwin and Anthony T. Yeung*

Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111, USA

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ABSTRACT

We have discovered a useful new reagent for mutation detection, a novel nuclease CEL I from celery. It is specific for DNA distortions and mismatches from pH 6 to 9. Incision is on the 3′-side of the mismatch site in one of the two DNA strands in a heteroduplex. CEL I-like nucleases are found in many plants. We report here that a simple method of enzyme mutation detection using CEL I can efficiently identify mutations and polymorphisms. To illustrate the efficacy of this approach, the exons of the BRCA1 gene were amplified by PCR using primers 5′-labeled with fluorescent dyes of two colors. The PCR products were annealed to form heteroduplexes and subjected to CEL I incision. In GeneScan analyses with a PE Applied Biosystems automated DNA sequencer, two independent incision events, one in each strand, produce truncated fragments of two colors that complement each other to confirm the position of the mismatch. CEL I can detect 100% of the sequence variants present, including deletions, insertions and missense alterations. Our results indicate that CEL I mutation detection is a highly sensitive method for detecting both polymorphisms and disease-causing mutations in DNA fragments as long as 1120 bp in length.

INTRODUCTION

Single-stranded nucleases such as S1 and mung bean nuclease nick DNA at single-stranded regions (1–3). However, the acid pH optima of these nucleases lead to DNA unwinding at A+T-rich regions and result in non-specific DNA degradation. For example, S1 nuclease was found not to cleave DNA at single base mismatches (4). The efficiency of mung bean nuclease at nicking supercoiled DNA is five orders of magnitude higher at pH 5 than at pH 8 (5). At neutral pH, a high concentration of mung bean nuclease is necessary to act on double-stranded DNA, mainly at A+T-rich regions (3). In this report, we show that celery and many plants possess novel endonucleases, characterized by neutral pH optima, that detect destabilized regions of DNA helices, such as at the site of a mismatch. The celery enzyme was named CEL I. The mismatch specificity of CEL I at neutral pH has enabled development of a highly effective and user-friendly mutation detection assay. We illustrate this CEL I method by detection of mutations and polymorphisms of the BRCA1 gene of a number of women affected with either breast and/or ovarian cancer and reporting a family history of these diseases.

MATERIALS AND METHODS

Preparation of plant extracts

Various plant tissues were homogenized in a Waring blender at 4°C and adjusted with a 10× solution to give the composition of buffer A [0.1 M Tris–HCl, pH 7.7, 10 µM phenylmethanesulfonyl fluoride (PMSF)]. The extracts were stored at −70°C. Equivalent data were obtained when the tissues were frozen in liquid nitrogen, ground to a powder with a mortar and pestle and then extracted with buffer A on ice.

Purification of CEL I

Celery stalks (7 kg) were extracted at 4°C with a juicer and adjusted with a 10× solution to give the composition of buffer A. The extract was concentrated with a 20–70% saturated ammonium sulfate precipitation step. The final pellet was dissolved in 250 ml buffer A and dialyzed against 0.5 M KCl in buffer A. The solution was incubated with 10 ml concanavalin A-Sepharose resin (Sigma) overnight at 4°C. The slurry was packed into a 2.5 cm diameter column and washed with 0.5 M KCl in buffer A. Bound CEL I was eluted with 90 ml 0.3 M α-D−-mannose, 0.5 M KCl in buffer A at 65°C. CEL I was dialyzed against buffer B (25 mM potassium phosphate, 10 µM PMSF, pH 7.0) and applied to a 100 ml phosphocellulose P-11 column that had been equilibrated in buffer B. The bound enzyme was eluted with a linear gradient of KCl in buffer B. The peak of CEL I activity was next concentrated by dialysis against saturated ammonium sulfate. The enzyme precipitate was dialyzed against buffer C (50 mM Tris–HCl, pH 7.8, 0.2 M KCl, 10 µM PMSF, 1 mM ZnCl2) and fractionated by size exclusion chromatography on a Superose 12 FPLC column in the same buffer. The center of the CEL I activity peak from this step was used as the purified CEL I in this study. Protein concentrations of the samples were determined by the Bicinchoninic acid protein assay (Pierce).

Preparation of mismatch-containing heteroduplexes

The oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer in the Fannie E.Rippel Biotechnology Facility of our Institution and purified using a denaturing PAGE gel in the

*To whom correspondence should be addressed. Tel: +1 215 728 2488; Fax: +1 215 728 3647; Email: at_yeung@fccc.edu
presence of 7 M urea at 50°C. DNA heteroduplex substrates of ~64 bp long containing mismatched base pairs or DNA loops (Fig. 1) were constructed by annealing partially complementary oligonucleotides. The single-stranded oligonucleotides were labeled at the 5′-termini with T4 polynucleotide kinase and [γ-32P]ATP prior to annealing with an unlabeled oligonucleotide. After annealing, all the substrates were made blunt-ended by the fill-in reaction of DNA polymerase I Klenow fragment using dCTP and GAP, and purified by non-denaturing PAGE as described (6) without exposure to UV light. DNA was eluted from the gel slices using an AMICON model 57005 electroeluter.

Mismatch endonuclease assay

5′-32P-labeled substrates (50–100 fmol) were incubated with CEL I preparation in buffer D (20 mM Tris–HCl, pH 7.4, 25 mM KCl, 10 mM MgCl2) for 30 min at 37 or 45°C in 20 μl reactions. Taq DNA polymerase (0.5–2.5 U) (Perkin Elmer) was added to each reaction where indicated. The presence of dNTP is not necessary for DNA polymerase to stimulate CEL I turnover. Ten micromolar dNTP was included only in the reactions of each reverse primer. PCR was performed in a reaction volume of 20 μl containing 100 ng genomic DNA as template, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 1 μM both forward and reverse primer, 60 μM each deoxyribonucleotide triphosphate, 5% dimethyl sulfoxide (DMSO) and 0.5 U Taq DNA polymerase. After an initial denaturation step at 94°C, the DNA was amplified through 20 cycles consisting of 5 s denaturation at 94°C, 1 min annealing at 65°C, decreasing by 0.5°C/cycle, and 1 min extension at 72°C. The samples were then subjected to an additional 30 cycles consisting of 5 s denaturation at 94°C, 1 min annealing at 65°C, decreasing by 0.5°C/cycle, and 1 min extension at 72°C, with a final extension for 5 min at 72°C. The PCR reactions were purified using Wizard PCR Preps (Promega). The sizes of the DNA fragments generated by PCR ranged from 211 to 1120 bp.

DNA templates for BRCA1 mutation analysis

Twenty-five pairs of PCR primers specific for 22 coding exons in BRCA1 were synthesized with 6-FAM dye (blue) at the 5′-end of each forward primer and with TET dye (green) at the 5′-end of each reverse primer. PCR was performed in a reaction volume of 20 μl containing 100 ng genomic DNA as template, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin, 1 μM both forward and reverse primer, 60 μM each deoxyribonucleotide triphosphate, 5% dimethyl sulfoxide (DMSO) and 0.5 U Taq DNA polymerase. After an initial denaturation step at 94°C, the DNA was amplified through 20 cycles consisting of 5 s denaturation at 94°C, 1 min annealing at 65°C, decreasing by 0.5°C/cycle, and 1 min extension at 72°C, with a final extension for 5 min at 72°C. The PCR reactions were purified using Wizard PCR Preps (Promega). The sizes of the DNA fragments generated by PCR ranged from 211 to 1120 bp.

Sample ascertainment

As part of a Fox Chase Cancer Center (FCCC) Institutional Review Board approved protocol, peripheral blood samples were obtained from consenting affected high risk family members through the Margaret Dyson/Family Risk Assessment Program (FRAP). Individuals participating in FRAP have agreed to allow their samples to be used for a wide range of research purposes, including screening for mutations in candidate cancer predisposing genes, such as BRCA1 (7). The participating individuals had previously been screened for BRCA1 mutations by the Clinical Genetic Testing Laboratory at FCCC and were screened for sequence alterations by CEL I mutation detection in this study in a blind fashion.
RESULTS

Detection of CEL I-like activities in plant extracts

By incubating plant extracts with a mismatch-containing heteroduplex, we detected a novel mismatch endonuclease activity. This activity performs a single-strand cut on the 3'-side of a mismatch site (Fig. 2). The activity appears to be present in many common vegetables and in a variety of plant tissues: root, stem, leaf, flower and fruit. From each tissue, we have found a similar amount of mismatch endonuclease activity per gram of tissue (Fig. 2A, lanes 2–13). We named the prominent activity present in celery CEL I. The substrate initially used was a 5'-labeled duplex with an extrahelical G nucleotide mismatch that can alternate between two consecutive G residues, thereby giving two CEL I cut bands. These gel mobilities are consistent with the production of a 3'-OH group on the deoxyribose moiety (6). All the CEL I-like mismatch endonucleases cut the DNA at the same two alternate positions on the 3'-side of the mismatch. The mismatch endonucleases of alfalfa sprout, asparagus, celery and tomato were each found to bind to a concanavalin A–agarose column and were eluted by α-D+-mannose (Fig. 2B). Thus, CEL I-like activities appear to be mannosyl glycoproteins.

Purification of CEL I

Celery stalks were chosen to be a source of model enzyme because of the year-round availability of celery, a low amount of chloroplast proteins and pigments in the extracts and the high mismatch specificity of CEL I. The CEL I purification procedure started with celery juice, containing ~350 g protein, from 7 kg celery stalk. The Superose 12 fraction contained 3 ml CEL I at 0.1 μg/μl and is estimated to be ~10 000-fold purified with a recovery of 9%. SDS–PAGE followed by staining with Coomassie Blue R250 indicated that the purest CEL I contains more than one protein band of 34–39 kDa (data not shown). It is not clear yet whether these bands represent glycoforms of CEL I or whether proteins with unrelated properties are present.

Incision by CEL I at mismatches of single nucleotide DNA loops and nucleotide substitutions

The mismatch incision by purified CEL I in substrates containing a single extrahelical nucleotide is shown in Figure 3A (lanes 2–5). This analysis shows that CEL I has a preference for G > A > C > T in the extrahelical position. The activity of CEL I is stimulated by the presence of Taq DNA polymerase (Fig. 3A, lanes 6–10). This stimulation of CEL I does not require dNTP (data not shown). Taq DNA polymerase stimulation of incision at the weak extrahelical T substrate is ~30-fold (Fig. 3A, comparing lanes 5 and 10), as measured by densitometry of the autoradiogram bands (data not shown). The DNA polymerase stimulation is less for extrahelical G and A substrates (Fig. 3A, lanes 7 and 8, respectively) because these substrates are already efficiently cut by CEL I. Because of base pairing slippage in the extrahelical nucleotide G and C substrates (Fig. 3A, lanes 2 and 4), two incision bands were seen. At the extrahelical nucleotide that is closer to the 5'-terminus, in the presence of Taq DNA polymerase and dNTP in lanes 7 and 9 mismatch slippage allows nick translation to occur after CEL I incision. As a result, the lower
Figure 3. Mismatch incision of the purified CEL I nuclease at different mismatches. (A) Taq DNA polymerase stimulation of purified CEL I incision at DNA mismatches of a single extrahelical nucleotide. Autoradiograms of denaturing 15% polyacrylamide gels are shown. F, full-length substrate, 65 nt long, labeled at the 5'-terminus (*) of the top strand. Lanes 1–5 and 6–10, 50 fmol substrates, in the presence of 10 µM dNTP, treated with 20 ng purified CEL I, without and with 0.5 U Taq DNA polymerase, respectively, for 30 min at 37°C; lanes 1 and 6, substrates containing no mismatch; lanes 11–14, substrates incubated with only Taq DNA polymerase in the presence of 10 µM dNTP, with the autoradiogram exposure time extended 3×. The two cuts (I and II) in lanes 2 and 4 are due to mismatch slippage in alternative base-pairing possibilities. One mismatched base at each cut site was repaired by DNA polymerase + dNTP in lanes 7 and 9. (B) pH profile of CEL I mismatch incision at a substrate with a single extrahelical G residue. S, substrate incubated without CEL I. Taq DNA polymerase and dNTP were not present in this study. If Taq DNA polymerase, but not dNTP, were included, the pH profile is similar, but the incision efficiency would be near completion in all lanes (data not shown). (C) CEL I incision at base substitutions. The top strands were 5’-labeled. Incubation with CEL I was for 30 min at 45°C in the presence of Taq DNA polymerase but no dNTP.

Figure 4. CEL I enzymatic mutation detection in the BRCA1 gene. (A) Schematic presentation of the exons of the BRCA1 gene and the polymorphisms and mutations detected in this report. The BRCA1 gene is divided into 24 exons (22 coding exons). For CEL I mutation detection, each PCR usually covers one exon. Exon 11 is divided into four regions of ~100 bp that overlap by at least 100 bp indicated by the diagonally shaded areas. All of exon 1, part of exon 2 and part of exon 24 are untranslated regions, as denoted by dotted areas. Exon 4 is not part of the mRNA (7). p, polymorphisms; m, mutations. (B) Electropherogram of CEL I mutation detection GeneScan analyses. Two color fluorescent heteroduplexes of the PCR products of the BRCA1 gene were prepared as described in Materials and Methods. All lanes have CEL I treatment. Vertical axis, relative fluorescence units; horizontal axis, DNA length in nucleotides. (A–D) Deletion of A in exon 19. The CEL I mismatch-specific peaks seen at sizes 106 and 146 nt in (C) and (D) for the 6-FAM-labeled and the TET-labeled strand, respectively, were not present in the wild-type control for the FAM (A) and the TET (B) strands. Full-length PCR product was observed at 249 nt length and residual primers at 20–30 nt. The signal in the full-length position exceeded the linear range of the detector. (E–H) Detection of C→T base substitution in exon 24. The PCR product was 286 bp. This C→T base substitution was detected as blue at fragment sizes 76 and 77 nt in (G) and as green at fragment size 206 nt in (H) for the 6-FAM-labeled and the TET-labeled strand, respectively, but not in the wild-type control for the FAM (E) and the TET (F) strands. (I–L) Detection of a C insertion mutation in exon 20. The PCR product was 410 bp long. This insertion of a single C residue was detected at fragment sizes 151 and 259 nt for exon 20 in (K) and (L), respectively, for the 6-FAM-labeled and the TET-labeled strand, respectively. The mutation-specific CEL I cuts were not observed in the wild-type control for the FAM (I) and the TET (J) strands. (M–P) Detection of mutations next to a polymorphism in exon 11. The PCR product was 1006 bp long. (M) Wild-type control treated with CEL I. (N) Polymorphism (2201 T→C) identified by CEL I. (O) Two polymorphisms (2210 T→C and 2196 G→A) detected by CEL I. (P) Polymorphism (2210 T→C) and mutation (K630ter; 2154A→T) detected by CEL I. Only the data from the TET-labeled strands are presented in (M–P).
bands of CEL I incision seen in lanes 2 and 4 was restored to full-length in lanes 7 and 9.

pH optimum of CEL I endonuclease

The pH optimum of CEL I appears to be in the neutral range although the enzyme is active from pH 5 to 9.5. The pH activity profile of CEL I cutting of the extrahelical G mismatch substrate without Taq DNA polymerase stimulation is shown in Figure 3B.

Incisions of CEL I at base substitutions

Base substitution mismatched substrates are also recognized by CEL I and cut on one of the two DNA strands for each mismatch duplex (Fig. 3C). Some of these substrates are less efficiently incised compared with those containing DNA loops. For the purpose of mutation detection \textit{in vivo}, all base substitution mismatches can be detected by CEL I at 45°C in the presence of 0.5 U Taq DNA polymerase (Fig. 3C). Substrates with the 5′-terminus of the top strands labeled were used in this study. CEL I substrate preference shown here is C/C ≥ C/A ∼ C/T ≥ G/G > A/C ∼ A/A ∼ T/C > T/G > G/T > G/A ∼ A/G > T/T.

Detection of mutations and polymorphisms in the \textit{BRCA1} gene

A CEL I-based assay was used to detect mutations and polymorphisms in various exons of the \textit{BRCA1} gene (Fig. 4). Strong incision bands were observed for heteroduplex alleles but not for wild-type alleles (Fig. 4B). The CEL I assay is also capable of detecting multiple sequence variants within the same DNA strand (Fig. 4, panels M–P).

A summary of the mutations and polymorphisms in the \textit{BRCA1} gene detected by CEL I in this study is shown in Figure 5. Sequence analyses of the coding regions and intron/exon boundaries confirmed that all known sequence variants were detected by CEL I. The DNA sequences flanking each mutation or polymorphism illustrate that CEL I detects mismatches in a variety of sequence contexts. Furthermore, no false positive or false negative conclusions were encountered.

DISCUSSION

Plants and fungi contain single-stranded specific nucleases that attack both DNA and RNA (8). S1 nuclease from \textit{Aspergillus oryzae} (1), P1 nuclease from \textit{Penicillium citrinum} (9) and mung bean nuclease from the sprouts of \textit{Vigna radiata} (2–3) are Zn proteases active mainly near pH 5.0. CEL I is similar to these enzymes in that the most purified enzyme fraction shows some single-stranded DNase activity and endonuclease activity on supercoiled plasmids, relaxed double-stranded DNA, UV irradiated plasmids and Y-shaped DNA duplexes (data not shown). However, CEL I is most active on mismatch substrates. The neutral pH optimum, incision primarily at the phosphodiester bond immediately on the 3′-side of the mismatch and stimulation of activity by a DNA polymerase are properties that distinguish CEL I from the above nucleases. The mechanism for DNA polymerase stimulation of the CEL I activity is presently unknown. One possibility is that DNA polymerase has a high affinity for the 3′-OH group produced by CEL I incision at the mismatch and displaces CEL I simply by competition for the site. Such protein displacement will allow CEL I to recycle catalytically. For the purpose of mutation detection, DNA polymerases with 3′→5′ exonuclease proofreading activity cannot be used. Such
DNA polymerases, of which the Klenow fragment of E.coli DNA polymerase I is an example, will excise the mismatch nucleotide after DNA polymerase displacement of CEL I at the site of mismatch incision. In the absence of dNTP, one will observe 3′→5′ exonuclease degradation of the DNA fragment produced by CEL I mismatch incision. In the presence of dNTP, a highly efficient in vitro mismatch correction system will have been reconstituted (data not shown). It is necessary to test whether or not other proteins, such as DNA helicases, DNA ligases and DNA terminus-binding proteins, can also assist CEL I at mismatch incision in vivo.

In the CEL I detection scheme used in this paper, two alleles will form two alternate heteroduplex mispairs such that at least one mismatch in each pair should be a good substrate for CEL I. G/G is paired with C/C, A/G is paired with C/T, A/C is paired with G/T and T/T is recognized least well by CEL I, but an A/A mismatch will be present in such a heteroduplex preparation and will be detected by CEL I. As shown in Figure 5, flanking sequence context apparently does not adversely affect the ability of CEL I to identify a mutation. Even mismatches flanked by GC-rich regions (Fig. 1) are recognized. The four PCR products of BRCA1 exon 11 are 889–1120 bp in length. Most of the time, mismatch incision will be observed as both colors in the electropherogram such that each independently confirms the position of the mutation/polymorphism. The sum of the two fragments is theoretically 1 nt more than the length of the PCR product. In the cases of mismatches that can wobble in alternative base pairings because of the sequence contexts and for large DNA loops the sum of the two fragments may deviate from the above rule. The principle of mismatch recognition by CEL I appears to be different from T4 endonuclease VII, which has also been used for enzyme mutation detection (11,12). The latter is a resolvase, which nicks one strand at the site of a mismatch and then in the other strand across from the DNA nick (12). Therefore, any nick can produce two corresponding fragments of the two colors. In the case of CEL I, the two fragments of the two colors represent two truly independent mutation detection events that complement each other to confirm the presence of the mutation. This distinction is because CEL I only nicks one strand of DNA in a mismatch heteroduplex at the site of the mismatch. There is no second cut in the opposite strand of the same DNA molecule after the first nick. Moreover, the CEL I mechanism allows the non-cut strand to be potentially useful as template for the removal of non-specific nicks, if any, by nick translation repair or ligation. Unlike resolvases, CEL I shows no tendency to nick duplex DNA at unique DNA sequences.

Other strengths of the CEL I mutation detection assay are its simplicity and its lack of preference for unique non-mismatch DNA sequences. Background non-specific DNA nicking is very low. The high signal-to-noise ratio of CEL I using fluorescent dye-labeled PCR products often allows mutations to be detected by visual inspection of the GeneScan gel image. CEL I is a very stable enzyme, during both its purification, storage and assay.

CEL I mutation detection provides a mutation detection method based on different principles than DNA sequencing and single-strand conformation polymorphism (SSCP) (13). In genes such as BRCA1, mutations can occur in numerous positions, making it very difficult for most mutation detection methods to screen for mutations in this gene. To date, >520 individual sequence alterations are known in the BRCA1 gene. The ability of CEL I to detect a mismatch at any one or more nucleotide positions without prior knowledge of the mutation provides promise of a very powerful method for screening mutations in cancer genes. Indeed, the ease of setting up and performing CEL I mutation detection should allow it to be established quickly in most laboratories.

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