Premature condensation induces breaks at the interface of early and late replicating chromosome bands bearing common fragile sites

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Various studies suggest a tight relationship between chromosome rearrangements driving tumor progression and breaks at loci called common fragile sites. Most of these sites are induced after perturbation of the replication dynamics, notably by aphidicolin treatment. We have mapped the majority of these sites to the interface of R and G bands, which calls into question the previous assignment of aphidicolin-sensitive sites to R bands. This observation suggests that most of them correspond to loci that ensure the transition between early and late replicating domains. We show that calyculin A, which triggers chromosome condensation at any phase of the cell cycle but does not markedly impair replication, induces damage in the chromosomes of human lymphocytes treated in G₂ but not in G₁ phase. We demonstrate that these lesions colocalize with those induced by aphidicolin treatment. Hence, common fragile site stability is compromised, whether aphidicolin delays replication or calyculin A advances condensation. We also show that, in cells that go through an unperturbed S phase, completion of their replication and/or replication-associated chromatin reorganization occur all along the G₂ phase, which may explain their inability to condense properly after calyculin A treatment during this phase of the cell cycle.

genome instability | DNA damage | calyculin A | aphidicolin | cell cycle

One major property of the oncogenic process is the early emergence of cells displaying genetic instability. A statistical correlation has been established between genome remodeling and DNA breaks at chromosomal sequences termed common fragile sites (1–4). Cytogenetically, these sites appear as loci displaying recurrent breaks, gaps, or constrictions (BGCs) in cells grown under specific tissue culture conditions (5), notably exposure to various mutagens and carcinogens (6). Most of these sites specifically respond to stresses that partially inhibit DNA replication, such as cell treatment with aphidicolin (Aph), an inhibitor of DNA polymerases α , δ , and ϵ (7, 8). Hypoxia, shown to impair S phase progression (9, 10), was also identified as a potent fragile site inducer in mammalian cells, suggesting a route by which fragile site activation might drive genome remodeling in tumors (11).

Aph-sensitive sites (AphS) are present in the chromosomes of all members of a species, although they may be expressed at different frequencies in different individuals. They appear to be evolutionarily conserved in mammalian species examined to date (12, 13). Therefore, they are considered to be integral elements of the normal chromosome structure. The consequences of their instability are now well documented. In cultured cells, they constitute hotspots for sister chromatid exchanges (14, 15) and are preferred sites for integration of exogenous DNA (16, 17). Their activation also triggers and drives intrachromosomal gene amplification (18). In tumors, with a few exceptions suggesting a relationship with inherited diseases (13, 19, 20), AphS were essentially correlated with somatic events leading to deletions, translocations (1–4), and oncogene amplification (21, 22). The physiological impact on tumor development of deletions target-

ing AphS was best demonstrated by FRA3B, which encompasses the FHIT gene (23). Indeed, functional assays in deficient mice show that reexpression of FHIT prevents tumor development *in vivo* (24, 25).

Because Aph impairs DNA replication, it long has been proposed that the drug elicits damage in regions along which elongation is unusually susceptible to perturbation. In support of this hypothesis, several reports have suggested that Aph treatment, which slows down replication in general, specifically delays replication along AphS (22, 26–29). Nucleotide sequence analysis has revealed that all cloned AphS span megabase-long AT-rich regions enriched in peaks of high flexibility (30–33). Interestingly, Zlotorynski *et al.* (34) recently showed that flexible regions contain interrupted runs of AT dinucleotides, which have the potential to form secondary structures and, thereby, may perturb the progression of replication forks.

New insights into the mechanism underlying fragility at AphS also came from the identification of proteins that contribute to the stability of AphS. Casper *et al.* (35) showed in cultured cells that the checkpoint kinase ataxia telangiectasia-mutated and Rad3-related (ATR), which plays a major role in the stabilization of stalled replication forks, is critical for FRA3B stability. A role for this protein was confirmed by the finding that instability at AphS occurs *in vivo* in cells of patients bearing a heterozygous mutation for ATR (Seckel syndrome) (36). Instability also occurs in cells deficient for BRCA1, a mediator protein involved in the intraS and G₂/M checkpoints (37). In the absence of an intact Fanconi Anaemia pathway, which is supposed to monitor S-phase progression downstream of ATR, the stability at AphS is also strongly hampered (38). Finally, the cohesin SMC1, which contributes to the stability of stalled replication forks through an ATR-dependent mechanism, also participates in maintaining the stability of AphS (39). Altogether, these results suggest that AphS are regions intrinsically difficult to duplicate and particularly susceptible to treatments or mutations impairing replication fork stability.

These findings, and the observation that AphS are chromosomal regions that often fail to compact properly for mitosis (40), suggest that the relationships linking replication to chromatin condensation along these loci may be pivotal to their stability. Here, we study the impact of premature chromosome conden-

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Abbreviations: Aph, aphidicolin; AphS, Aph-sensitive sites; ATR, ataxia telangiectasia-mutated and Rad3-related; BAC, bacterial artificial chromosome; BGCs, breaks, gaps, and constrictions; Cal, calyculin A; PCC, premature chromosome condensation.

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Table 1. BGCs are induced by Cal and Aph in the chromosomes of human lymphocytes

Treatments	Donor 1			Donor 2			Donor 3			Donor 4		
	—	Cal	Aph	—	Cal	Aph	—	Cal	Aph	—	Cal	Aph
Total no. of cells	42	50	42	102	30	31	47	44	43	50	30	30
Cells without breaks	28	19	15	42	6	0	32	8	11	20	0	0
Highly damaged cells	0	0	0	0	4	0	0	4	0	0	2	0
Chromatid BGCs	9	107	34	60	122	217	11	107	101	30	319	170
Chromosome BGCs	9	15	15	17	8	15	6	3	6	14	15	17
Mean BGCs per cell	0.43	2.5	1.17	0.76	5	7.48	0.36	2.75	2.49	0.88	11.93	6.23

Quantification of the number of BGCs found in mitotic chromosomes of untreated (—) and Aph-treated lymphocyte populations from four donors and in mitotic-like chromosomes obtained upon Cal treatment of the same cell populations.

sation (PCC) on AphS stability by treating human lymphocytes with calyculin A (Cal), a specific inhibitor of serine-threonine phosphatases types 1 and 2A, that triggers PCC at any stage of the cell cycle (41, 42). We took advantage of condensation occurring in G₁, S, and G₂ phases to study the cytogenetic status of AphS at those stages of the cell cycle. We show that Cal treatment induces chromosome breaks targeted to specific sites, the vast majority of which colocalize with AphS at the cytogenetic level. Our results also show that most AphS were incorrectly localized and suggest that they lie actually at the interface of early and late replicating bands. Moreover, our findings support the hypothesis that AphS contain incompletely duplicated sequences in cells exiting an unperturbed S phase, a feature progressively corrected before mitotic entry at each cell cycle.

Materials and Methods

Culture and Treatment of Human Lymphocytes. Whole peripheral blood was obtained by venipuncture from volunteer donors and grown for 96 h as described in ref. 43. Common fragile site activation was performed by treating the cells with 60 ng/ml Cal (Sigma) for 50 min or with 0.1 μ g/ml Aph (Sigma) for 20 h before cell harvesting. High-resolution prometaphase banding was achieved according to ref. 43. Briefly, cells were blocked by overnight thymidine (350 μ g/ml) treatment. Cells were then rinsed twice with Hanks solution (GIBCO) and grown for 4.5 or 7 h (for optimum banding of chromosomes 3 and X, and 16, respectively) in complete medium containing 10 μ g/ml BrdUrd (Sigma). Twenty minutes before harvesting, cells were treated with 50 ng/ml of colchicine (Sigma). Slides were prepared according to standard cytogenetic procedures (43).

Preparation of R- or G-Banded Chromosomes. BGCs were observed on preparations stained by Giemsa (Prolabo) without pretreatments to obtain an homogeneous staining of the chromosomes. Preparations were then destained and treated as described in ref. 43 to reveal R- or G-banding. Preparations were restained by Giemsa, and the chromosome alterations previously detected were then located relative to the bands. Breaks were generally scored from 50 cells from each donor and each experiment.

Genomic Localization and Preparation of the Bacterial Artificial Chromosomes (BACs). BACs were selected from the human genome project RP11 library. FRA3B has been assigned to band 3p14.2 (44). This site was probed with BAC 641C17, which bears the STS markers D3S2757 and D3S1481 close to its telomeric and centromeric ends, respectively. FRA16D has been assigned to band 16q23.2 (45, 46). We selected BAC 571O6, covering markers D16S3138, D16S3125, and D16S3101 in the region of fragility. FRAXB has been assigned to band Xp22.3 (47). We chose BAC 483M24 that covers the GS1 gene and the STS marker DXS1130 at the near distal end of FRAXB. Bacterial strains containing the BACs were spread on LB agar supplemented with 12.5 mg/ml chloramphenicol and grown at 37°C

overnight. Clone identity was verified by PCR with probes corresponding to known STS markers. BACs were then extracted according to the manufacturer's instructions (Qiagen). Probes were biotinylated by Nick Translation (Invitrogen) and recovered by filtration through Quick Spin columns (Roche).

Fluorescent *in Situ* Hybridization (FISH) and BrdUrd Detection. FISH was performed essentially as described in ref. 48 without the proteinase K step. Biotin was revealed with alternating layers of fluoresceinated avidin (FITC) (Vector Laboratories) and biotin-conjugated goat anti-avidin (Vector Laboratories). Two layers of avidin/antiavidin were applied. For BrdUrd detection, chromosomes were denatured as for FISH, and the analog was revealed with three layers of mouse anti-BrdUrd (Becton Dickinson), rabbit anti-mouse Alexa 350, and goat anti-rabbit Alexa 350 (Molecular Probes). Slides were washed three times with PBS solution (Sigma) after each layer. Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) or with propidium iodide (PI, Sigma) and mounted in Vectashield (Vector Laboratories).

Results

Cal Induces Chromosome Damage. PCC was obtained by treating human lymphocyte cultures for 50 min with Cal. Chromosomes condensed in G₁ phase have a spiral appearance. In S phase, they present interspersed blocks of condensed and noncondensed chromatin. In late S and G₂ phases, they resemble mitotic chromosomes and display classical G- and R-banding patterns (Fig. 4, which is published as supporting information on the PNAS web site). Because they are not easily distinguishable from each other, chromosomes in late S, G₂, and mitosis will be collectively referred to below as mitotic-like chromosomes. Cytogenetic analysis of mitotic-like chromosomes from lymphocyte populations of four donors revealed the presence of BGCs. Their frequency was compared to those found in metaphase chromosomes of the same donor in the absence of treatment or after Aph treatment. The mean number of BGCs per genome ranged from 0.36 to 0.88 in untreated cells (mean 0.6), from 2.5 to 11.93 in Cal-treated cells (mean 5.55), and from 1.17 to 7.48 after Aph treatments (mean 4.34). The percentage of BGCs involving a single chromatid of the chromosomes was 70% in untreated cells and reached 94% and 91%, respectively, after Cal or Aph treatment (Table 1). Hence, the chromosomal consequences of both drugs appeared quantitatively very similar.

Cal Induces BGCs at Specific Loci. To determine whether Cal-dependant damage is targeted to specific chromosomal sites, we analyzed BGCs with a two-step procedure that helps minimize observation biases. First, BGCs were detected on homogeneously stained mitotic-like chromosomes of lymphocytes from eight donors. After destaining, chromosomes were R-banded and 839 of these BGCs were localized with respect to the bands. Thirty-five loci displayed a minimum of eight breaks (at least 1%

Table 3. Aph- and Cal-induced BGCs colocalize at the molecular level

	FRA3B (BAC 641C17)		FRA16D (BAC 571O6)		FRAXB (BAC 483M24)	
BGC	Aph	Cal	Aph	Cal	Aph	Cal
Proximal	8	6	19	13	6	6
Distal	16	15	9	5	14	14
Split	3	1	9	4	7	4
In	5	3	1	0	1	1
Total BGCs	32	25	38	22	28	25

The position of the FISH signals relative to the BGCs (proximal, distal, split, or in) is indicated.

confirm, at the molecular level, the colocalization of BGCs induced on Aph or Cal treatment.

FRA3B, FRA16D, and FRAXB Lie at the Interface of R and G Bands. The difficulties encountered in mapping BGCs on banded chromosomes and the availability of BAC probes prompted us to verify the localization of FRA3B, FRA16D, and FRAXB by FISH approaches. We focused on prometaphase chromosomes displaying high resolution R-banding revealed by detection of BrdUrd incorporated during early S phase (49, 50). Because FISH signals may travel along relatively large regions at this banding resolution, we performed a statistical analysis of the position of the spots relative to the bands. We studied 60 to 74 chromosomes hybridized with each BAC and obtained results suggesting strongly that FRA3B maps at the interface of 3p14.1 and 3p14.2 (Fig. 2A) and FRA16D at the interface of bands 16q23.1 and 16q23.2 (Fig. 2B). Even though bands were poorly resolved in the chromosomal region bearing FRAXB, our results suggest that the site maps close to the interface of bands Xp22.32 and Xp22.31 (Fig. 2C). Note that Yunis *et al.* (6) have assigned FRA3B and FRA16D to small R bands within G bands and FRAXB to the terminal R band of Xp arm. Hence, our high-resolution mapping supports the conclusion that most AphS lie at the junction of chromosome bands, i.e., at the boundary of domains replicating at different times through S phase.

Cal Does Not Markedly Impair S Phase Progression. Cal has been routinely used to trigger PCC, but because it perturbs the phosphorylation status of various proteins involved in cell cycle control, the drug may also impact on S phase progression (51). To clarify this point, we treated lymphocyte populations with both Cal and BrdUrd for 1 h, or with Cal alone for 30 min followed by 30 min of incubation with both drugs. Cytogenetic analysis showed that BrdUrd was incorporated in the DNA of all S phase cells we observed, whatever the labeling protocol (Fig. 3). Moreover, the mean length of the BrdUrd fluorescent tracks roughly paralleled the length of the labeling period (Fig. 3A). These results indicate that elongation occurs in the presence of Cal and does not notably slow down during Cal treatment. As indicated by the normal patterns and intensities of labeling observed in mitotic-like, in comparison with normal mitotic chromosomes (Fig. 3B and C), completion of S phase is not affected in the presence of Cal. This observation strongly suggests that Cal-induced damage at AphS results from PCC rather than from replication perturbation. It is generally admitted that the interspersed blocks of condensed and noncondensed chromatin observed in cells undergoing PCC reflects the inability of unreplicated sequences to condense properly in S phase. In good agreement with this view, we observed that BrdUrd labeling is associated with at least partially condensed chromosome domains (Fig. 3A and B). Why unreplicated DNA is

Fig. 2. FISH localization of BACs 641C17 (FRA3B), 571O6 (FRA16D), and 483M24 (FRAXB) on R-banded high resolution prometaphase chromosomes. (*Left*) Ideograms of chromosome or chromosome arms. The rightmost numbers indicate the number of observed chromosomes exhibiting the FISH signals at the indicated positions (note that only the most recurrent localizations were taken into account), and the red and blue forms refer to the situation illustrated in *Right*. (*Right*) Examples of FISH signals on R-banded chromosomes (reverse propidium iodide-FITC staining). *Left*, banding alone; *Right*, banding and FISH (green). (A) Short arm of chromosome 3 (FRA3B). (B) Chromosome 16 (FRA16D). (C) Short arm of chromosome X (FRAXB).

incompetent for condensation in S phase, and possibly in G₂, while it condenses efficiently in G₁, is not known.

Postreplicative Sensitivity of FRA3B to Cal. Because Cal treatment allowed us to observe condensed chromosomes in G₁ and G₂ phases, we compared the frequencies of damage induced by PCC before and after replication. The recognition of the different chromosomes by their banding pattern in G₁ phase being difficult, we used a probe for band 3p14 to study FRA3B in Cal-treated lymphocytes from one donor. We examined 140 cells displaying chromosome morphology typical of G₁ phase. Eight of them had BGCs in or close to the FISH signal on one homologue (5.7%), a percentage roughly similar to that found in metaphase chromosomes of untreated lymphocytes of this donor. In contrast, we found that among 193 cells displaying mitotic-like chromosomes, 76 ($\approx 40\%$) had damage within or close to the signal: 58 on one homologue and 18 on both homologues (Fig. 6, which is published as supporting information on the PNAS web site). Altogether, these results reveal that Cal sensitivity is cell cycle-dependent.

Fragile Sites Are Committed to Break in G₂ Cells Exiting an Unperturbed S Phase. To determine the lifespan of the DNA features responsible for postreplicative sensitivity to Cal, cells were grown for 1 h in the presence of BrdUrd, then Cal was added for 50 min

hypothesis. The specific role of ATR is better explained whether replication and/or chromatin reorganization after replication remain incomplete in G₂, rendering these loci incompetent for condensation, even in untreated normal cells. In contrast to the S phase exit, the G₂/M transition being stringently controlled, ATR would delay mitotic onset until their complete duplication via its downstream target BRCA1

(37). Hence, these sites might be integral components of the G₂/M checkpoint.

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