A Stable Proteinaceous Structure in the Territory of Inactive X Chromosomes*

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Transcriptional inactivation of one copy of the X chromosome in female cells equalizes expression of X-linked genes between males and females. This “dosage compensation” is a multistep process that involves epigenetic modifications of chromatin and is induced by the expression of a large non-coding RNA termed Xist. In contrast to protein-coding mRNA molecules, which are free to diffuse and roam the entire nuclear interior, Xist is locally constrained to the territory of inactive X chromosomes by as yet unclear mechanisms. Recent results have suggested a contribution of scaffold attachment factor A (SAF-A)1 in the silencing of X-linked genes, maybe by inducing a local change in nuclear architecture. Here, in vivo mobility experiments demonstrate that SAF-A is a component of a highly stable proteinaceous structure in the territory of inactive X chromosomes, which might act as a platform for immobilizing Xist RNA during the maintenance phase of X inactivation.

The equal expression of X-linked genes in male and female mammals is achieved by transcriptional silencing of the second copy of the X chromosome in females. Silencing is a complex multistep process that leads to a heterochromatization of most of the inactive X chromosome, which becomes microscopically evident as the Barr body (1). On the microscopical level, the Barr body has been widely used as a cytogenetic marker to identify cells with an aberrant number of X chromosomes and is usually located close to nucleoli or the nuclear periphery together with other heterochromatic regions. On the molecular level, the processes that lead to silencing of the second X chromosome are induced by expression of a large, non-coding RNA that is essential for inactivation (reviewed in Ref. 2). The gene encoding this RNA, called Xist, is the only known gene that is expressed exclusively from the inactive X chromosome (3). Expression of Xist RNA is the first detectable event in X inactivation, later followed by large scale chromatin remodeling, a series of epigenetic modifications of chromatin such as the lysine methylation of histone H3, hypoacetylation of histone H4, the enrichment of the peculiar histone variant macroH2A, and finally methylation of the DNA itself (4–8). Importantly, even though Xist is present on inactive X chromosomes throughout the life of an organism, it is directly involved in transcriptional silencing only in early embryogenesis. Later, silencing becomes Xist-independent, and the inactive state can be perpetuated by redundant epigenetic mechanisms even in absence of Xist (6, 9, 10). Apparently, Xist imparts a chromosomal memory early in development (“initiation phase”), which facilitates histone methylation in Xi during the “maintenance phase” at later times (11). During maintenance, the chromosomal memory allows Xist to efficiently establish histone methylation, but Xist is no longer necessary for silencing itself (11). In both phases, Xist RNA is locally constrained to the territory of the inactive X chromosome (3, 12), in contrast to almost any other RNA species in the nucleus that freely diffuse and can roam the entire nucleus (13, 14). Xist remains in the vicinity of its expression site, exclusively spreading in cis to neighboring regions of the same chromosome (but not in trans to non-X chromatin regions that may even be closer), until it essentially “coats” the entire inactive X chromosome.

The molecular basis of the very low mobility of Xist, and the mechanism that imparts its local constraint, are currently unclear. Earlier experiments have suggested that diffusion of Xist is prevented by binding to components of a stable proteinaceous substructure of the nucleus (12). Indeed, we recently demonstrated that scaffold attachment factor A (SAF-A)1 is enriched in the territories of the inactive X chromosome (15). This protein has earlier been identified and characterized as a DNA and RNA-binding component of a putative nuclear scaffold (16–19) and appears to be involved in the regulation of gene expression and DNA replication (20–22). As the enrichment of SAF-A in inactive X territories depends on an intact RNA binding domain on the protein, and the protein can be extracted from Xi by mild RNase (but not DNase) treatment (15), SAF-A is a plausible candidate for a structural protein that might contribute to Xist immobilization. Indeed, the localization of Xist RNA results from cooperative binding of protein factors to redundant low affinity sequence elements (23), in exactly the same way in which SAF-A interacts with nucleic acids (24). Due to the strong self-assembly activity of SAF-A in presence of nucleic acids (25), Xist RNA and SAF-A might cooperate to form a stable structure in the territory of Xi and provide a self-organizing platform essential for the maintenance of the silenced state of the X chromosome. This paper provides first in vivo evidence in favor of this hypothesis, demonstrating the presence of a stable, locally constrained substructure in inactive X territories.

**EXPERIMENTAL PROCEDURES**

*Cell Culture and Transfection—*Human embryonic kidney (HEK293) cells were cultivated and transfected as described previously (15), and cells stably expressing SAF-A-enhanced green fluorescent protein were selected by selection with G418 for 4 weeks. Cells selected for further analysis expressed SAF-A:GFP to less than 5% of the level of endogenous SAF-A; to exclude potential problems due to overexpression.

**Live Cell Microscopy—**For live cell microscopy, cells were split onto 35 mm culture dishes with a glass bottom (Mattek), and analyzed 1–2 days after splitting. For the analysis, cells were placed on a heated stage of a Zeiss Meta 510 confocal laser scanning microscope. Fluorescence recovery after photobleaching (FRAP) was measured using a 531 nm laser and a confocal pinhole of 1.5 Airy units.

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cence recovery after photobleaching (FRAP) experiments were performed by bleaching a narrow strip across the nucleus by 20 iterations at 488 nm and 80% laser output. The prebleaching status, bleaching, and fluorescence recovery were recorded by the “time series” module of the Zeiss software (version 3.2), using 1% laser output to minimize further bleaching. 128 × 128-pixel resolution at Zoom factor 3–5, and a 3.5 Airy pinhole. For quantitative analysis, a minimum of 25 individual cells were measured, and mean pixel intensities in specified regions of interest were determined with the freeware image analysis software ImageJ by Wayne Rasband (rsb.info.nih.gov/ij/) on an Apple MacOs X computer. For comparison of fluorescence recovery in inactive X territories and non-X chromat regions, the bleaching strip was set to hit an entire Xi in addition to “normal” nuclear regions; for the analysis, two identical sized regions of interest were placed on Xi and a non-X reference region in the bleached area and a non-bleached region for data normalization.

For time lapse microscopy, stacks of images recorded by the Zeiss time series module were converted to Quicktime videos (see supplemental material), spanning variable time windows after bleaching. Single territory FRAP was performed by bleaching the entire Xi, and monitoring recovery over time, or in a reverse way by setting two bleaching regions to cover the entire cell except for a single Xi territory.

RESULTS AND DISCUSSION

Inactivation of the second copy of the X chromosome is an essential dosage compensation process to equalize the expression of X-linked genes between males and females. The inactivation process depends on the expression and exclusive localization of a 17-kb non-coding RNA, termed Xist, in the territory of the inactive X chromosome (reviewed in Ref. 2). Xist RNA is known to be exempt from free diffusion in the nuclear interior, presumably by interaction with protein components that are enriched in the Xi territory (12). The immobility of Xist RNA is in striking contrast to all other investigated RNA species, which are free to roam the entire nucleus by passive diffusion (13). It is likely that the immobility of Xist is essential to restrict the inactivation process to the right target and prevent illegitimate silencing of non-X chromatin that localizes in the vicinity of Xi. Elucidating the mechanism that constrains the mobility of Xist is therefore important to gain a complete molecular understanding of the X inactivation process and its maintenance.

It has been shown that Xist remains detectable even after near-complete removal of chromatin by DNase digestion and salt extraction, suggesting that it is part of a non-chromatin nuclear structure often referred to as “nuclear matrix” or “scaffold” (12). However, the existence and functional relevance of such a structure in living cells is a matter of debate, mainly because of the rather harsh procedures usually employed in its isolation. Thus, non-invasive in vivo methods need to be employed to investigate this point beyond experimental flaws. In vivo mobility experiments such as fluorescence recovery after photobleaching (FRAP), fluorescence loss induced by photobleaching, and fluorescence correlation spectroscopy can give insights into the mobility of cellular components. To study the mechanism of Xist immobilization, we performed FRAP experiments on SAF-A, a component of the putative nuclear scaffold that is enriched in the territories of inactive X chromosomes (15). In other regions of the nucleus, SAF-A is present in several populations that differ in their mobility and has a high immobile fraction of ~60% of total cellular SAF-A (compare Fig. 1B). When narrow strips containing an entire Xi territory are bleached and monitored for fluorescence recovery over time, Xi and non-X chromatin regions do not differ in the rate of fluorescence recovery (Fig. 1, A and B). This demonstrates that the mobile fraction of SAF-A can enter the Xi territory without any impediment, suggesting that the local viscosity or molecular crowding does not differ between Xi and non-X chromatin. However, the ~2-fold enrichment of SAF-A in Xi observed in untreated cells is not restored after bleaching.

FIG. 1. Fluorescence recovery (FRAP) experiments on SAF-A in living HEK293 cells. A strip across the nucleus containing an inactive X chromosome territory was photobleached, and fluorescence recovery was monitored over time. A, example of a typical cell; upper panel, confocal analysis of the cell before and after bleaching; lower panel, quantification of fluorescence in this cell, showing the mean pixel intensity profile in the box shown in the first picture of the upper panel, from left to right. Note that SAF-A is enriched ~2-fold in the untreated cell (prebleach), but the recovery is uniform with no preference for the Xi. B, quantitative FRAP experiment. The mobility of SAF-A was determined by photobleaching a strip across the nucleus and determining the fluorescence recovery on the inactive X chromosome and a non-X nuclear region nearby. The mean and standard deviation of measurements on 25 individual cells are shown, demonstrating identical mobility of SAF-A in the inactive X territory and non-X nuclear regions. C, the ratio of mean intensities of SAF-A fluorescence in Xi and a non-X reference region was calculated and monitored over time. Before photobleaching, SAF-A is enriched in Xi ~2-fold, but this enrichment is not re-established after bleaching.
Quantified as an “enrichment recovery,” following the ratio of mean fluorescence of Xi to non-X, there is a marked drop from the normal 2-fold enrichment to 1 (identical intensity after bleaching), which does not “recover” but remains at 1:1 ratio over time (Fig. 1C). Thus, the fluorescence recovers to typical non-Xi levels, but not further, and remains at this level over long periods of time (Fig. 2 and see supplemental movie M1).

These results suggest that the enrichment of SAF-A in Xi is due to the presence of a large protein fraction that does not dissociate from Xi, in addition to the background level of mobile SAF-A that is identical in Xi and the rest of the nucleus. The lack of exchange between the stably bound and the soluble fraction is more directly demonstrated in “reverse” territory FRAP, where two large regions of interest where used to bleach the entire nucleus except for the Xi territory (Fig. 3 and supplemental movie M2). In this experiment, the amount of fluorescent SAF-A in Xi drops to approximately half during the bleaching process, indicating the redistribution of the soluble fraction in the entire nucleus, but then stays unchanged for long periods of time. In fact, Xi territories were readily detectable in confocal analysis 2 h after reverse territory FRAP. Thus, binding of SAF-A in the Xi territory is extraordinarily stable, preventing its turnover with the soluble fraction of SAF-A. This is, to the best of our knowledge, the first in vivo demonstration of a stable protein structure in the inactive X chromosome territory.

Combined with earlier findings that SAF-A requires its RNA binding domain for enrichment in Xi (15), and the strong nucleic-acid induced self-assembly of SAF-A to large complexes (18, 25), the data presented here are compatible with a role of SAF-A for the self-organization of a stable structure that immobilizes Xist RNA. While SAF-A is certainly not needed for coating Xi chromatin with Xist, which can bind to chromatin by itself (23), such a structure could prevent spreading of Xist RNA in the nuclear interior to confine it to the Xi territory. In addition, the proteinaceous structure formed by SAF-A might act as a platform on which epigenetic modifications occur, especially during the maintenance phase of X inactivation. On first sight, a direct involvement of SAF-A in the initiation process would also appear plausible because SAF-A has recently been shown to physically interact with proteins involved in epigenetic chromatin modifications, such as the histone acetylase p300 (26) and protein arginine methyltransferase 1 (27). However, these proteins have been implied in gene (co)activation rather than silencing, a function clearly incompatible with X inactivation. Importantly, though, these proteins are not co-enriched with SAF-A in Xi, and the methylation/acetylation status of histones in Xi indicates that p300 and protein arginine methyltransferase 1 are not active there (28). Unless an interaction of SAF-A with a component of the silencing cascade that leads to transcriptional inactivation of on X chromosome is convincingly demonstrated, we tend to believe that the structure described in this paper may be important for the maintenance of the silenced state, rather than for its induction.

In this scenario, the stable proteinaceous structure containing SAF-A might be part of the “chromosomal memory” mechanism that ensures efficient histone methylation specifically in the territory of Xi (11). Future experiments will focus on the possible role of SAF-A in this “memory,” and should allow a more detailed understanding of the dosage compensation in mammalian cells.

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