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Molecular characterization of hybridoma subclones spontaneously switching at high frequencies *in vitro*

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Abstract

The hybridoma technology allows the production of large quantities of specific antibodies of a single isotype. Since different isotypes have special effector functions and are distributed distinctively throughout the body, it is often useful to have a library of switch variants from the original monoclonal antibody. We have shown previously that forced expression of activation induced cytidine deaminase (AID) in hybridomas increased their very low frequency of class switch recombination (CSR) *in vitro* only ~7–13 fold. Since we had previously identified rare hybridoma subclones that spontaneously switched at more than 100 times higher frequencies, we have now examined those higher switching variants to search for ways to further increase the frequency of isotype switching *in vitro*. AID was not responsible for the ~100 fold increase in CSR, so we used whole-genome gene expression profiling to provide a platform for studying candidate molecular pathways underlying spontaneous CSR in hybridomas.

Keywords

Class Switch Recombination; AID; Immunoglobulin; hybridoma; microarrays; real time RT-PCR

1. Introduction

Isotype class switching from IgM to IgG, IgA or IgE allows antibodies to retain the same antigen specificity but to carry out different effector functions and be distributed differently throughout the body (Stavnezer et al., 2008). Hybridomas produce monoclonal antibodies that express one isotype but these antibodies can be made more useful both for *in vitro* assays and for their effectiveness *in vivo* if they can be switched in culture to provide a library of monoclonal antibodies all with the same specificity but capable of carrying out different subsets of effector functions. Class switch recombination (CSR) juxtaposes the rearranged heavy chain V(D)J region that was expressed with the μ constant region to one or another of the downstream C γ 3-C γ 1-C γ 2b-C γ 2a-C ϵ -C α mouse constant regions by intrachromosomal recombination, with the deletion of the intervening DNA. *In vivo* and in some cell lines, this process is initiated

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by activation induced (cytidine) deaminase (AID) which deaminates cytidine residues in the single stranded DNA at donor and recipient switch (S) regions that are just upstream from the constant region genes, converting them to uridine residues. The G–U or G–abasic mismatches created are processed by base excision and mismatch repair in an error prone manner to produce staggered single stranded DNA breaks on each strand that can be converted into double stranded DNA breaks which are then processed by non homologous end joining pathways (NHEJ) (Stavnezer et al., 2008).

Most hybridomas switch from one isotype to another in culture at low frequencies of 10^{-5} – 10^{-6} (Radbruch et al., 1980), making it labor intensive and time consuming to obtain class switched variants *in vitro*. This was true for the 36–65 hybridoma, that makes an IgG₁ monoclonal antibody that binds to the hapten p-azophenylarsonate (Ars) (Marshak-Rothstein et al., 1980; Spira and Scharff, 1992). Hybridomas are generated by fusing primary B cells to malignant plasmacytoma cell lines (Kohler and Milstein, 1975) and represent a stage of B cell differentiation that does not usually express AID. We have previously shown that, like most hybridomas, 36–65 cells do not express AID (Iglesias-Ussel et al., 2006). We showed that the forced expression of AID in 36–65 cells, and some other hybridomas, increased the frequency of CSR only 7–13 -fold (Iglesias-Ussel et al., 2006). In contrast, using many iterative cycles of brute force sib selection we had in the past been able to isolate variants of the 36–65 hybridoma that spontaneously switched from IgG₁ to IgG_{2b} and IgG_{2a} at ~100 times higher frequencies of 10^{-3} – 10^{-4} (Spira et al., 1994). We have now compared the low-switching 36–65 hybridomas to their sister hybridomas that switched at a ~100-fold higher frequency and observed that AID expression could not be detected in the higher switching variants, indicating that different factors must be responsible for the ~100-fold increase in switching. We also found that the differences in the frequency of switching between the high and low spontaneously switching variants are retained even after forced expression of AID. We have used microarray gene expression profiling to compare these hybridomas that spontaneously switch at ~100 times different frequencies *in vitro* in an attempt to identify combinations of factors that might be responsible for spontaneous switching.

2. Materials and methods

2.1. Cell lines and cell culture conditions

Low and high spontaneously switching variants from 36–65, an A/J hybridoma that produces an IgG₁ anti-p-azophenylarsonate monoclonal antibody, were grown as previously reported (Spira et al., 1994). For convenience we have shortened the original names used in reference (Lin et al., 1996) throughout the text. Thus, 36–65.L derives from the low-switching variant 36–65.12.7.30.7.2, while 36–65.H is a high-switching variant newly isolated from 36–65.12.7.23.20.10.10.11.9. Naïve spleen B cells were obtained from two 6 week old C57BL/6 mice. Splenocytes were isolated, depleted of T cells, grown in RPMI 1640 medium containing 10% FCS and stimulated with 40 µg/ml LPS (Sigma-Aldrich, St. Louis, MO) and 25ng/ml IL-4 (R&D Systems, Minneapolis, MN) for 4 days. These animal experiments were approved by the Albert Einstein College of Medicine Animal Use Committee.

2.2 Soft agar cloning

4 ml of 0.4% SeaPlaque agarose (FMC Bioproduct) in 20% FCS medium was placed in a 60mm culture plate (Falcon-Becton Dickinson) and solidified at 4°C for 10 min. 10^3 cells in 1 ml of medium were laid over the top of the soft agar and placed at 4°C for 10 min. Cells were grown at 37°C for ~7 days and clones were collected and placed into a 96-well plate, as previously described (Iglesias-Ussel et al., 2006).

2.3. Transfection conditions

5×10^6 cells from two low (L25, L27) and two high (H23, H27) switching hybridomas were transfected with 10 μ g human AID expressing vector (pCEP4-hAID) or an empty vector control linearized with EcoRV and NruI using a GenePulser electroporator (BioRad) at 950 μ F, 450 V and 200 Ω . Cells were plated in 96-well plates at 10^4 cells/well, selected with hygromycin B (Calbiochem) and ~2 weeks later stable transfectants were picked and expanded in culture. Total RNA was isolated using Trizol (Invitrogen) and hAID mRNA expression was determined by RTPCR, as previously described (Iglesias-Ussel et al., 2006).

2.4. ELISA spot assay (ESA)

The assay was performed as previously reported (Iglesias-Ussel et al., 2006). Plates were pre-coated with a 1:500 dilution of the anti-mouse antibody against the corresponding isotype (Southern Biotechnology) and blocked with 2% BSA-TBS. Cells were plated and grown in culture for 18 hours. Spots were developed with biotinylated antibody against the corresponding isotype (Southern Biotechnology) and 5-BCIP substrate (Amresco) and counted using a dissecting microscope. Median frequencies of switching were calculated. When no spots were detected, one spot was assigned to allow determination of median frequencies.

2.5. Western blotting

5×10^6 Cells were lysed on ice for 30 minutes with 200 μ l lysis buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.5% NP-40, 0.5 mM EDTA pH 8 and complete protease inhibitor cocktail from Roche). The lysates were centrifuged at full speed and supernatants removed and boiled in 2 $\times$ Tris glycine SDS sample buffer (Invitrogen). Protein samples from 5×10^5 cells were separated on a 4–20% Tris-glycine gel (Invitrogen), transferred to a PVDF membrane (Invitrogen) and blotted with anti-AID mouse monoclonal IgG₁ antibody (Cell Signaling), followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Southern Biotech) or anti-beta actin mouse monoclonal IgG_{2a} antibody (Sigma, 5316) followed by HRP-conjugated goat anti-mouse IgG_{2a} antibody (Southern Biotech). Bands were visualized with a LAS-3000 imager (FujiFilm).

2.6. Gene expression profiling analysis by microarrays

Total RNA from the L25, L27, H23 and H27 hybridoma subclones (Fig. 1) was purified from 5×10^6 cells using RNeasy kit (Qiagen) and residual genomic DNA removed on column with DNase I (Qiagen). The RNA samples were labeled and hybridized to a mouse genome GeneChip 430 2.0 array (Affymetrix) at the Albert Einstein Genomics Facility, using standardized procedures. These arrays contain probe sets for 45,000 target sequences, corresponding to over 34,000 known mouse transcripts. Raw GeneChip data (.CEL files) were normalized at the probe level by Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003) and further filtered for variations in normalized intensity levels using GeneSpring 7.2 software (Agilent Technologies). The mRNAs that had a different abundance between the low and high spontaneously switching groups were identified by statistical filtering using an intersection of *t*-test results (with a *P* value cutoff of 0.05) and significance analysis of microarrays (SAM) with false discovery rate (FDR) set to 5% (Tusher et al., 2001). Pathway analyses and functional classifications of differentially expressed genes were conducted using a web based analysis tool, FatiGO of the Babelomics suite (Al-Shahrour et al., 2004) (<http://babelomics2.bioinfo.cipf.es/fatigoplus/cgi-bin/fatigoplus.cgi>). The RMA normalized data were deposited to the public Gene Expression Omnibus (GEO) repository under the accession number GSE15357.

2.7. Quantitative real-time RT-PCR (Q-PCR)

Total RNA was freshly isolated as described for the microarrays from a different batch of cells. Reverse transcription was performed by using iScript kit (Bio-Rad). Q-PCR was performed in triplicate using ABI PRISM 7700HT cyclor and SYBR green (Qiagen) double-stranded DNA product detection. The primers used are detailed in Supplementary Table 1. Relative fold-differences in mRNA/cDNA abundance were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. AID is not detectable in the hybridoma subclones switching at ~100 times higher frequencies

The isolation of 36–65 hybridoma variants that spontaneously switch at ~100-fold higher frequencies has been described previously (Spira et al., 1994). Since many of the factors responsible for CSR had not been discovered at that time, we have now characterized these variants further. We obtained, by agar subcloning, five fresh subclones of the original parental 36–65 hybridoma that switched from IgG₁ to IgG_{2a} and IgG_{2b} at low frequency (36–65.L) (Iglesias-Ussel et al., 2006) and five fresh subclones of the high-switching variant (36–65.H). The frequency of switching was determined using the ELISA spot assay (ESA) as described previously (Spira and Scharff, 1992). As we already reported, 36–65.L switched from IgG₁ to IgG_{2b} at a median frequency of 10^{-5} and to IgG_{2a} at 4×10^{-6} (Iglesias-Ussel et al., 2006), while 36–65.H variants have about 130-fold higher frequencies of switching to IgG_{2b} and 300-fold higher frequencies of switching to IgG_{2a}, confirming previous data (Spira et al., 1994; Lin et al., 1996).

Since AID had not been discovered when the high-switching variants were isolated and characterized (Spira et al., 1994; Lin et al., 1996), we examined whether AID was expressed differentially in the variants switching at spontaneously higher frequencies. In an earlier study, we had not been able to detect mouse AID in two subclones of the low-switching variants (L25, L27) by semi-quantitative RT-PCR or western analysis (Iglesias-Ussel et al., 2006). Similarly, in the present study two high-switching subclones (H23 and H27) do not reveal detectable expression of AID mRNA or protein (Fig. 1a and b).

3.2. Hybridomas switching spontaneously in vitro at low and high frequencies retain their different frequencies of switching after forced expression of AID

We had previously established that the stable expression of AID increased the frequency of switching in the low-switching variants by 7–13 fold (L25C and L27C vs L25A and L27A) (Iglesias-Ussel et al., 2006) and that is shown again here in the left hand part of Fig. 2a and b for comparison with the high-switching variants. At the same time that we had studied the low-switching variants, two subclones of the spontaneously higher switching variants (H23 and H27) were stably transfected with the same vector (pCEP4-AID) and expressed human AID protein (Fig. 1b). 10–20 independent transfectants with the empty vector (H23C and H27C) were compared with an equal number of clones transfected with pCEP4-AID, that were expressing hAID mRNA by RT-PCR, for their frequency of switching to γ 2b (H23A and H27A, Fig. 2a, right hand panel) and γ 2a (Fig. 2b, right hand panel) by ESA, as described in materials and methods. The median frequency of switching is shown below the scatter plot in Fig. 2.

Even though the ectopic expression of AID in the low-switching hybridomas increased their frequency of switching 7–13-fold (Iglesias-Ussel et al., 2006), AID did not bring them to the levels of spontaneous switching of the high-switching hybridomas (L25A and L27A vs H23C, H27C), Fig. 2a and b). Forced expression of AID in H23 and H27 resulted in an ~8 to 11-fold increase in the median frequency of switching to IgG_{2b} (Fig 2A) and IgG_{2a} (Fig. 2b). Thus,

after forced expression of AID, the frequency of switching increased in both the low and high-switching hybridomas, but they still retained the ~100-fold differences in switching frequencies.

We checked whether these variants were also switching to IgA at different frequencies. Frequencies of switching were determined by ESA in about 10 low and high switching subclones transfected with either AID or empty vector control. Switching to IgA occurred at very low frequencies of $\sim 10^{-6}$ in the non-AID expressing clones of both the low and high switching cells and increased ~5 fold in the AID expressing clones, but no differences were observed between the low and high switching variants (data not shown).

3.3. Gene expression profiling in hybridoma variants spontaneously switching at low and high frequencies in vitro

In order to comprehensively search for specific genes and pathways that might be involved in the increase of spontaneous switching, we used microarrays to examine gene expression programs in total RNA from two low (L25, L27) and two high (H23, H27) switching hybridoma subclones. Comparison of the two sister low-switching (grouped as Low) and the two high-switching variants (grouped as High), enable us to identify (see Materials and methods) a list of differentially and reproducibly modulated genes detected by 2091 unique probe sets (Supplementary Table 2). 1637 of them corresponded to known genes, 647 of which were up-regulated and 990 down-regulated in the variants spontaneously switching at higher frequencies.

The differentially expressed genes in the hybridoma variants switching at ~100 times higher frequencies were categorized according to Gene Ontology (GO) categories of biological process, molecular function, and cellular compartment (Fig. 3). This analysis suggests that the majority of genes differentially regulated between the low and high-switching variants are implicated in broad general processes that are also known to be involved in regular class switch recombination, such as transcription, response to DNA damage and DNA repair, DNA binding, chromatin modifications, RNA splicing, etc (Stavnezer et al., 2008), as well as other processes that could be involved but have not yet been explicitly studied.

3.4. Validation of microarray results by Q-PCR

We selected 18 of the 1637 (approximately 1%) genes identified as differentially expressed between the low and high-switching hybridomas in the microarray experiments (Supplementary Table 2), and examined their expression patterns by quantitative real-time PCR (Fig. 4). The validated genes included genes known to be involved in regular class switching (such as *Cd40*, *Lig4*, *H2afx*, *Ung*, *Xrcc4*, *Tcf2a*, *Id2*, *Apex1*, *Bcl6* and *Prdm1*) as well as genes involved in processes known to be necessary for regular CSR, such as regulation of transcription (*E2f5*, *Mta-1*, *Ell3*), response to DNA damage (*Gadd45a*, *Cdkn1a*), DNA replication (*Polm*), antigen receptor stimulation (*Card11*), and *Mcm3a*, since it has been associated with the appearance of double-stranded DNA breaks in the immunoglobulin variable gene (Kawatani et al., 2005).

The results obtained from the Q-PCR analysis agreed with those obtained from the microarray analysis (Fig. 4), even for the genes that showed less than 1.5-fold differences identified by microarrays, like *Lig4*, *Xrcc4*, *Tcf2a*, *Polm* and *Ganp*.

4. Discussion

It would be very useful to be able to routinely generate a battery of monoclonal antibodies expressing the same V regions of various isotypes, so they could access different parts of the

body and carry out different effector functions. Because hybridoma switch variants usually arise at very low frequencies of 10^{-5} – 10^{-6} (Radbruch et al., 1980; Spira and Scharff, 1992), we had in the past used sequential subcloning (Spira et al., 1984), also called sib selection, to isolate such variants using the 36–65 hybridoma (Marshak-Rothstein et al., 1980; Radbruch et al., 1980) as a model system. When we previously tried to address the molecular differences between the low and high-switching 36–65 cells, we found that the presence of easily detectable germ line transcripts of the $\gamma 2a$ switch regions was associated with the increase in switching to that isotype, but such germ line transcripts were not detected for the $\gamma 2b$ switch regions in either the high or low switchers, even though the increase in the frequency of switching to $\gamma 2b$ was comparable to that of $\gamma 2a$. We concluded that an increase in germ line transcription was not sufficient to explain the increase in CSR of the high switching variants (Lin et al., 1996).

Since AID, the primary molecule involved in initiating CSR, had not been discovered at the time, in this study we examined whether AID was in fact responsible for the ~100 higher frequencies of switching. Surprisingly, AID does not seem to account for the observed higher frequency of spontaneous switching, since neither AID protein nor mRNA were detected in the high-switching variants by western blot (Fig. 1b), RT-PCR (Fig. 1a) or microarray chip (Supplementary Table 2). However, it is still possible that AID is expressed transiently or only in a few rare cells in the population so its levels are below the limit of detection by those methods.

Spontaneous CSR can occur at low levels at all stages throughout B cell lymphopoiesis except plasma cells (Edry et al., 2007). The mechanism of CSR that is not induced by AID has yet to be elucidated, but it has been proposed to be carried out by either a different enzymatic machinery from the regular CSR or deregulated expression levels of its components, since it generates aberrant switch junctions characterized by increased mutations around the break points, reduction in microhomologies and increased switch region deletions (Edry et al., 2007). The fact that AID might be unnecessary for spontaneous class switching would be in accordance with the fact that chromosomal translocations in the immunoglobulin switch regions can be observed in the absence of AID (Unniraman et al., 2004).

In the studies reported here, the forced expression of AID increased the frequency of switching by ~7–13-fold in both the low and the high-switching variants, so the high-switching variants retained their ~100 times difference in its frequency of switching after AID overexpression (Fig. 2). Even the high-switching variants expressing AID still switch in culture at frequencies that are orders of magnitude lower than primary cells that were stimulated to carry out CSR *ex vivo* (Stavnezer et al., 2008). We therefore decided to look at the gene expression profiles in both the low and high-switching variants prior to the introduction of AID with the goal of not only understanding the mechanisms underlying spontaneous switching in hybridoma cells but with the hope of finding molecules that could then be used to increase the frequency at which hybridomas switch *in vitro* cultures. The microarray Chip results show that, although clonally related, the low and high-switching subclones have undergone numerous changes that are quite well shared between the two high and between the two low switching subclones (Supplementary Table 2 and Supplementary Fig. 1). This is perhaps not surprising, since repeated sib selections to obtain the higher switching variants were carried over the course of a year (Spira et al., 1994). It might be expected that in a hybridoma, that is a fusion of a plasmacytoma and a normal antibody producing B cell, the pattern of gene expression would be complex, representing a mixture of patterns from both parents that has undergone additional changes associated with many years in culture and chromosome loss and duplication. Perhaps it is not surprising that when the increased or decreased expression of genes known to be involved in regular switching or in B cell differentiation is superimposed on that already

complex pattern in the higher switching variants, the results are often unanticipated and sometimes confusing.

Of the genes previously reported to play a role in CSR (reviewed in Supplementary Table 3), only *H2afx*, *Lig4*, *Xrcc4*, *Ung*, *Apex1*, *Id2*, *Tcfe2a*, *Prdm1*, *Bcl6*, *Tnfrsf5*, *Nbn*, *Pten* and *Pcna* appeared differentially regulated in the low and high-switching variants in the array (Supplementary Table 2), even though some of them (*Lig4*, *Xrcc4*, *Tcfe2a* and *Pcna*) showed less than 1.5-fold differences in mRNA expression.

To gain confidence into our data set, we decided to confirm by Q-PCR (Fig. 4) not only genes known to be involved in regular CSR, but also genes that have not been reported to be involved in CSR but have a function in processes known to be necessary for regular CSR (like *Gadd45a*, *E2f5*, *Cdkn1a*, *Mta1*, *Polm*, *Card11*, *Ell3*, *Mcm3a*).

Cd40, which is a receptor that stimulates isotype switching and T-cell mediated B lymphocyte activation, was up-regulated in the variants switching at high frequencies (Fig. 4a), even though these hybridomas had not been stimulated with CD40L. mRNA for DNA ligase IV (*Lig4*), a protein involved in non homologous end joining resolution of dsDNA breaks that leads to recombination of S regions during class switch recombination (Pan-Hammarstrom et al., 2005), is also up-regulated in the high-switching variants (Fig. 4a). ID2 inhibits CSR, specifically to IgE, (Sugai et al., 2003) and its expression is down-regulated in the high-switching variants (Fig. 4b).

It has been reported that BLIMP1 inhibits regular CSR by blocking the expression of AID (Shaffer et al., 2002), and is expressed in plasma cells, like hybridomas, so it is not clear why *Prdm1*, that encodes BLIMP1, is up-regulated in the high-switching variants, both in our microarray (Supplementary Table 2) and Q-PCR data (Fig. 4a). It was however surprising that the high-switching variants have increased levels of *Bcl6*, since BLIMP1 is a plasma cell marker and BCL6 is a germinal center cell signature. Although BCL6 inhibits switching to IgE induced by CD40L+IL-4 stimulation, dependent upon STAT6 signaling (Harris et al., 1999), it is up-regulated in the high-switching variants in the microarray and Q-PCR (Fig. 4a). It was also surprising that some genes encoding for proteins known to be necessary for regular CSR, like H2AX (Petersen et al., 2001), UNG (Rada et al., 2002), XRCC4 (Soulas-Sprauel et al., 2007), E2A (Quong et al., 1999) and APEX1 (Guikema et al., 2007), were downregulated in the high-switching variants in the microarray and confirmed by Q-PCR (Fig. 4b).

We also validated by Q-PCR some genes that were observed by microarrays as differently regulated in the high-switching variants and had known roles in general processes required for switching, such as transcription or DNA damage repair. For instance, GADD45A, that has a known role in response to DNA damage, G2-M arrest in response to genotoxic stress, base excision repair through interaction with APEX1 and PCNA (Jung et al., 2007) and promoting DNA demethylation by AID (Rai et al., 2008), is up-regulated in the subclones spontaneously switching at higher frequencies (Fig. 4a). Another factor involved in response to DNA damage, *Cdkn1a* (encoding for P21), was also up-regulated by both microarray (Supplementary Table 2) and QPCR (Fig. 4a) in the high-switching variants. Of the proteins with a role in transcription, E2F5 and MTA-1 were more highly expressed in the variants switching to higher frequencies *in vitro*, but elongation factor RNA polymerase II-like 3 (*Ell3*) was down-regulated in these variants (Fig. 4). Since POL μ has been suggested to play a role in class switch recombination because it is involved in double stranded DNA breaks repair by non homologous end joining (Mahajan et al., 2002) and is expressed at high levels in the germinal centers of peripheral lymph nodes, we also confirmed the unexpected observation that *Polm* was down-regulated in the high-switching variants (Fig. 4b). Although in B cells antigen receptor-induced NF- κ B activation requires CARD11, a cytoplasmic scaffolding protein, this gene was shown

to be down-regulated, both by cDNA Chip (Supplementary Table 2) and Q-PCR in the higher switching variants (Fig. 4b).

It has been reported that *Mcm3ap* (encoding for GANP) is up-regulated in centrocytes of germinal centers (Kuwahara et al., 2000), contributes to affinity maturation (Kuwahara et al., 2004) and is associated with the appearance of double-stranded DNA breaks in the immunoglobulin variable region (Kawatani et al., 2005). Even though lack of GANP has been reported to not affect CSR (Kuwahara et al., 2004), we confirmed that it was down-regulated in variants spontaneously switching at high frequencies by Q-PCR (Fig. 4b). Perhaps this reflects the fact that GANP suppresses DNA recombination (Yoshida et al., 2007).

Our results suggest that it would require testing each of the identified pathways functionally to develop a way to increase the frequencies of spontaneous switching in existing hybridomas. An alternative might be to generate subclones of the high switching variants that no longer express their endogenous Ig, force the expression of AID and fuse the resulting clones to a pre-existing hybridoma. Such high switching cells could also be a useful fusion partner.

In summary, we have examined the impact of AID on a hybridoma that switches at low frequencies 10^{-5} – 10^{-6} *in vitro* and a somatic cell variant that was switching at ~100-fold higher frequencies, obtained by repeated rounds of selection for progressively higher switching during a period of a year. Expression of AID failed to explain the ~100 higher frequencies of switching, since i) AID was not detectable in the higher switching variants; ii) AID increased the frequency of switching of both the low and high-switching cells, but the presence of AID did not increase the level of CSR of the low-switching variants to that of the higher switching variants that were not expressing AID; and iii) the ~100-fold difference in the frequency of switching between the low and high-switching variants was retained after forced expression of AID. In an attempt to understand what was causing the high frequencies of spontaneous switching and to further increase class switch recombination (CSR) in hybridomas, gene expression profiling was performed to identify broad programs of gene regulation in the high-switching variants, compared to the variants switching at normal low frequencies. Our study provides a molecular profile of events associated with the 100-fold increase in spontaneous switching in hybridoma cells and should facilitate future studies aimed to the identification of genes, subsets of genes or pathways responsible for this phenomenon and perhaps for the normal process of CSR in primary cells as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Higher spontaneous frequency of switching is not due to AID expression. (a) Semiquantitative RT-PCR. Total RNA was extracted from the two high switching hybridoma subclones (H23 and H27) as well as naïve mouse spleen cells stimulated with LPS and IL-4 for 4 days (WT+), as described in materials and methods, and treated with DNaseI. RT-PCR one step (Invitrogen) was performed from 1 µg of total RNA and two five-fold dilutions using primers for mouse AID (mAID) (118 and 119 from (Muramatsu et al., 1999)) and GAPDH (Zhang et al., 2001). (b) Western blot. AID protein levels in 36–65 hybridomas switching at high frequencies before (H23, H27) and after AID transfection (H23.A1, H27.A18).

Fig. 2.

Forced expression of AID induces CSR in both the low and high-switching hybridomas, but the ~100 times difference in their frequencies of switching is retained. Two low (L25, L27) and two high (H23, H27) switching hybridoma subclones, that are shown in the first and 6th columns of panels a and b, were stably transfected with the hAID construct (A) or empty vector control (C). Frequency of class switch recombination from IgG₁ to IgG_{2b} (a) and IgG_{2a} (b) was determined by ELISA spot assay and fluctuation analysis. Every circle represents the frequency of switching of a particular subclone. When no spots were detected, one spot was assigned to allow determination of median frequencies (black circles). Median frequencies of switching are represented by horizontal bars and values are shown on the bottom of each column. Only hAID expressing clones were plotted. Data for low switch variants (left panels of Fig. 2a and b) has been published previously (Iglesias-Ussel et al., 2006) but is shown here for comparison purposes. Subclones H23 and H27 were used to measure mRNA AID levels by RT-PCR (Fig. 1a) and H23, H27, H23.A1, H27.A18 were used to measure protein AID levels shown in Fig. 1b.

Fig. 3. Gene Ontology (GO) analyses and KEGG metabolic pathways. Fatigo+ (<http://babelomics2.bioinfo.cipf.es/fatigoplus/cgi-bin/fatigoplus.cgi>) was used to determine the different categories of the 1637 genes that showed to be significantly up- and down-regulated in the variants switching at ~100 times higher frequencies by microarray analysis. Categories shown are among the pathways most significantly altered and for processes most probable to be involved in CSR (see Supplementary Table 2 for full gene list). The numbers show percentages of up-regulated (red) or down-regulated (blue) mRNAs, found represented in a specific GO category.

Fig. 4.

Real-time PCR validation of genes identified as differentially regulated in the variants spontaneously switching at ~100 times higher frequencies by microarray expression analysis. (a) Up-regulated genes in the high switching variants. (b) Genes down-regulated in the high switching variants. The graphs represent relative mRNA abundance for the depicted genes obtained by array (black bars) and Q-PCR (white bars) assays in hybridoma subclones spontaneously switching at low, L25, L27 (L) and ~100 times higher frequencies, H23, H27 (H). Error bars indicate coefficient of variation shown as percentage of a median measured value. Q-PCR results correspond to the $2^{-\Delta\Delta C_t}$ /median after normalization with the expression

of *Gapdh*. Baseline normalization relates to the average expression of the mentioned genes in the low-switching variants.