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Modulation of the Fas-apoptosis-signalling pathway by functional polymorphisms at *Fas*, *FasL* and *Fadd* and their implication in T-cell lymphoblastic lymphoma susceptibility

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In previous reports, we described germ line functional polymorphisms that differentiate *Fas* and *FasL* genes in two mouse strains (SEG/Pas and C57BL/6J) exhibiting extreme differences in susceptibility to γ radiation-induced T-cell lymphomas. Here, we provide new data reinforcing the importance of the extrinsic pathway of apoptosis mediated by Fas in T-cell lymphoma development and about the functional significance of polymorphisms located at intracellular and extracellular domains of *Fas* and *FasL*. Using DNA recombinant technology, we generate chimerical Fas and FasL proteins by combination of protein regions derived from the two strains and demonstrate that any Fas–FasL interaction involving chimerical proteins drive cell apoptosis to a significant lower extent than the wild-type SEG/Pas and C57BL/6J Fas–FasL systems. In addition, we report new polymorphisms in the coding sequence of *Fadd* and demonstrate that the interaction between Fas and Fadd is significantly stronger if Fas and Fadd are of SEG/Pas origin compared with the C57BL/6J system. Altogether, these results suggest a model in which functional polymorphisms at the three genes collaborate on the global ability of the Fas/FasL system to induce apoptosis. A complete analysis of these three genes in the pathway appears to be a *sine qua non* condition to accurately predict the effectiveness of the Fas system and to estimate susceptibility to T-cell lymphoma.

Introduction

Apoptosis in the immune system constitutes an essential process that regulates T-cell development within the thymus and peripheral homeostasis. Among the pathways involved is the Fas system, which has been reported to play a role in negative/positive selection of thymocytes during their development inside the thymus. The Fas system contributes as well to the elimination of peripheral lymphocytes after the clonal expansion phase and the autoreactive lymphocytes in the periphery, through the process called activation-induced cell death (1–9). However, the involvement of Fas-induced apoptosis in determining the genetic susceptibility to diseases affecting T cells is still under discussion (5,10,11).

We have previously reported that germ line functional polymorphisms at *Fas* and *FasL* genes may affect either their levels of expression and/or the biological activity of the Fas system in immature thymocytes, thus contributing to the susceptibility to develop γ radiation-induced T-cell lymphoblastic lymphomas (12,13). A large number of nucleotide changes were found to spread all along the coding sequence of both *Fas* and *FasL*, some of them involving a change of amino acid between mouse strains extremely different in their susceptibility to T-cell lymphoblastic lymphoma (C57BL/6J and SEG/Pas). Once demonstrated that the whole set of polymorphisms at *Fas* and *FasL* may modulate the genetic susceptibility to T-cell lymphoblastic lymphomas, the first goal of the present work was to separately assess the contribution of polymorphisms located at the extracellular or intracellular domains of both proteins. This objective was achieved using chimerical proteins generated by recombinant DNA technology.

In addition to Fas and FasL, Fadd is another essential element that should be taken into account to assess the global functionality of the Fas system. As a part of this system, Fadd is considered to be involved in the apoptosis of lymphocytes in peripheral lymphoid organs (14). However, the controversy about the implications of Fadd inside the thymus has been reopened in the recent years. Studies showing a critical role of Fadd inside the thymus (15) face those asserting that Fadd has a dispensable role in the development of thymocytes, although remaining essential for maintaining the homeostasis of peripheral T cells (16). This controversy prompted us to consider Fadd as the second goal in our study.

Abbreviations: cDNA, complementary DNA; DD, death domain; DED, death effector domain; PCR, polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Materials and methods

Mice and treatments

C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). SEG/Pas strain was provided by Drs J.L.Guenet and X.Montagutelli (Institute Pasteur, Paris, France). A cohort of mice was subjected to four sublethal doses of 1.75 Gy on a weekly basis, and the development of T-cell lymphoblastic lymphoma was followed during a 25 weeks latency period (13). Lymphomas were diagnosed as described previously (17). For animal experimentation, we followed the ethical considerations dictated by the European Commission (Directive 86/609/CEE) for the humane care and use of laboratory animals.

Chimerical Fas and FasL constructs

Fas-pcDNA3 and FasL-pcDNA3 constructs containing *Fas* and *FasL* complementary DNAs (cDNAs) from either C57BL/6J or SEG/Pas had been previously generated in our laboratory (13). Through digestion with XbaI (Fas-pcDNA3) or PinAI and BamHI (FasL-pcDNA3) (Roche Diagnostics GmbH, Mannheim, Germany), we released two fragments, one containing the sequence for the intracellular region and the other containing the sequence for the extracellular and transmembrane regions together with the plasmid. Cross-ligation of the fragments derived from C57BL/6J and SEG/Pas constructs allowed us to generate two new chimerical open reading frames combining the extracellular region of C57BL/6J with the intracellular of SEG/Pas and vice versa.

The functionality of the constructs was assayed through flow cytometry: we used phycoerythrin-conjugated hamster anti-mouse CD95 (clone Jo2) for Fas and biotin-conjugated mouse anti-mouse CD178.1 (clone KAY-10) for FasL together with phycoerythrin-conjugated streptavidin (all from BD Pharmingen, San Diego, CA). Primary antibodies were used in a 1:50 dilution, and secondary phycoerythrin–streptavidin was used in a 1:100 dilution. A FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) was used.

Cloning and sequencing of the mouse Fadd cDNA

Total RNA from testis of C57BL/6J and SEG/Pas mice was extracted using TriPure Reagent (Roche Applied Science, Indianapolis, IN). Reverse transcription was done using SuperScript First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR) (Invitrogen, Life Technologies, Carlsbad, CA) followed by PCR using the Expand High Fidelity PCR System (Roche Diagnostics GmbH). Conditions for PCR were as indicated by the manufacturer, with an annealing temperature of 55°C and an elongation

time of 45 s. The design of the primers was based on the previously reported sequence for *Fadd* cDNA from *Mus musculus* (Genbank accession no. NM_010175): Fadd-KpnI-Fw: 5′-GGTACCGATTTCCTATGTGGGATCGC-3′ and Fadd-XhoI-Rv: 5′-CTCGAGTCCGGGTGTTTCTGAGGAAG-3′ (restriction sites underlined).

Purified DNA fragments containing the *Fadd* cDNA were cloned into pGEM-T Easy Vector (Promega, Madison, WI). Fragments doubly digested with KpnI and XhoI (Roche Diagnostics GmbH) were subcloned into a pcDNA3 expression vector, which fused the FLAG epitope at the 3′ end (pcDNA3-FLAG, kindly gifted by M.Quintanilla's team).

C57BL/6J and SEG/Pas-derived *Fadd* cDNAs were sequenced using an ABI Prism 310 Automated Sequencer (Applied Biosystems, Life Technologies, Carlsbad, CA). All comparisons between sequences were made using the program L-Align from ExPASy Molecular Biology Server.

Cloning of the Fas cDNA sequences

Using the above-mentioned Fas-pcDNA3 constructs from C57BL/6J or SEG/Pas as a template, we amplified the cDNAs of *Fas* to clone them into a pcDNA3 expression vector that fused the Haemagglutinin epitope at the 3′ end (pcDNA3-HA, kindly gifted by M.Quintanilla's team). The primers included two distinct restriction sites for HindIII and NotI (underlined) and were as follows: Fas-HindIII-Fw: 5′-GCAAGCTTTTTTCCCTTGCTGCAGACATG-3′ and Fas-NotI-Rv: 5′-GCGGCCGCTTTCCCTCCAGACATTGTCCTT-3′.

PCR was performed using the Expand High Fidelity PCR System (Roche Diagnostics GmbH) and the aforementioned primers. Conditions for PCR were as indicated by the manufacturer, with an annealing temperature of 60°C and an elongation time of 60 s. Purified DNA fragments containing the *Fas* cDNAs were doubly digested with HindIII and NotI (Roche Diagnostics GmbH) and cloned into pcDNA3-HA plasmid.

Cell cultures and transient transfections

Human HEK-293 cells were cultured as described elsewhere (18). Transfections were done using LipofectAMINE Reagent (Invitrogen).

Terminal deoxynucleotidyl transferase dUTP nick end labeling assays

Apoptosis was determined using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (Roche Diagnostics GmbH) through microscopic observation. Glass-cultured HEK-293 cells were fixed in 3.7% paraformaldehyde (Panreac Química S.A.U., Castellar del Valles, Spain.), permeabilized in 0.1% Triton X-100 sodium citrate and then stained with TUNEL following the manufacturer's guidelines. Deoxyuridine triphosphate labelling of DNA strand breaks was visualized on a Zeiss Axiovert200 fluorescence microscope.

Caspases activation

For immunodetection of caspase-3 and caspase-8, 40 μg aliquots of total cell lysates obtained with radioimmunoprecipitation assay cell lysis buffer were separated by 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis under reducing conditions and electrotransferred to pure nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Rabbit anti-mouse polyclonal antibody H-277 against caspase-3 (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA) and mouse monoclonal antibody 1C12 against caspase-8 (1:500) (Cell Signaling Technology, Danvers, MA) were, respectively, used with appropriate secondary antibodies coupled to horseradish peroxidase. Subsequently, the peroxidase activity was obtained using Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK). Monoclonal anti-α-tubulin clone DM 1A antibody (1:10 000; Sigma–Aldrich, St Louis, MO) was used for loading control. Quantification of bands was made through densitometry using the Scion Image program.

Co-immunoprecipitation experiments

HEK-293 cells were transiently transfected with the pcDNA3-Fas-HA and pcDNA3-Fadd-FLAG constructs. Cells were lysed 24 h after transfection and protein expression was determined by western blotting or co-immunoprecipitation. For immunoprecipitation experiments, cells were lysed in IPH buffer [0.5% NP40 (IGEPAL), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid and 50 mM Tris–HCl; pH 8] and a cocktail of protease inhibitors: 2 mM Phenylmethylsulphonyl fluoride, 2.5 μl/ml Protease Inhibitor Cocktail and 10 μl/ml Phosphatase Inhibitor Cocktail 2 (Roche Diagnostics GmbH). Tagged Fas and Fadd proteins were immunoprecipitated using 1 μg of monoclonal antibody anti-HA (clone 3F10; Roche Diagnostics GmbH) or anti-FLAG (Sigma–Aldrich). Rabbit anti-rat or anti-mouse IgGs (Jackson Immunoresearch, West Grove, PA) coupled to protein A-Sepharose (Sigma–Aldrich) were used as secondary antibodies. Detection of co-precipitated proteins in western blots under non-reducing conditions was performed with anti-HA (1:500) and anti-FLAG (1:1500) monoclonal antibodies and appropriate secondary antibodies: Immunopure Goat Anti-Rat IgG, (H+L), Peroxidase Conjugated (1:5000) (Thermo Scientific, Pierce Biotechnology, Rockford, IL); ECL Mouse IgG, HRP-Linked Whole Ab (from sheep) NXA931 (1:1000) (GE Healthcare). A monoclonal sheep anti-mouse antibody for β-actin (GE Healthcare) was used in a 1:5000 dilution for loading control.

When specified, co-immunoprecipitation assays were carried out after *in vitro* FasL-induced activation of Fas system: 24 h after transfection, around 6×10^6 HEK-293 cells co-transfected with pcDNA3-Fas-HA and pcDNA3-Fadd-FLAG were co-cultured in P100 culture plates with a similar number of cells transfected with C57BL/6J or SEG/Pas-FasL-pcDNA3. Lysis using IPH buffer was carried out 8 h after plating.

Statistical methods

Statistical significances were determined using a one-way analysis of variance with a Tukey post-test, when multiple comparisons were performed or with a *T*-Student test in the case of two-group comparisons. All statistical tests were performed using the Statistical Package for the Social Sciences software (version 15.0; SPSS, IBM Corporation, Somers, NY).

Results

Functionality of the intracellular and extracellular polymorphic regions of Fas and FasL

In an attempt to elucidate the functional significance of polymorphisms located at the intracellular and extracellular regions of Fas and FasL, we separately assayed the effect of these polymorphic domains through the construction of chimerical Fas and FasL proteins combining the intracellular and extracellular regions from C57BL/6J and SEG/Pas mouse strains (Figure 1). Using co-cultures of human HEK-293 cells transfected with the chimerical receptors with cells transfected with the chimerical ligands, the *in vitro* levels of induced apoptosis were investigated in all the possible combinations. We chose HEK-293 as the recipient cells because they express a much reduced amount of FAS and FASLG compared with the exogenous expressions after transfections (data not shown).

The global effect on cell apoptosis was determined through TUNEL assay (Figure 2a). The non-chimerical systems (first four bars on the left) were used as reference. Interestingly, any Fas–FasL interaction involving chimerical proteins drive cell apoptosis to a significant lower extent than the non-chimerical SEG/Pas and C57BL/6J Fas–FasL systems. Using the SEG/Pas system (second bar) as the control reference for maximal apoptosis (see also ref. 13), our results showed that there exist significant reductions in the levels of induced apoptosis in any combination bearing at least one chimerical protein (Fas and/or FasL). However, not all the combinations exhibit the same level of reduction compared with the SEG/Pas system; we can distinguish two statistically different groups. On the one hand, a group exhibits apoptotic levels that fluctuate between 0.30 and 0.50 in comparison with the SEG/Pas system. This implies a reduction in the levels of Fas-induced apoptosis of between 50% and 70%. This group is constituted by the chimerical combinations BS-S, SB-B, SB-S, B-BS, S-BS, B-SB and S-SB. The second group exhibiting the maximal reduction (between 73 and 85%, apoptotic levels between 0.15 and 0.27) is formed by the four doubly chimerical combinations, together with the BS-B combination. These results clearly indicate that it is the accumulation of polymorphic amino acid along the Fas and FasL proteins what determines the different level of apoptosis observed between SEG/Pas and C57BL/6J. However, when only the intracellular region of Fas in the BS-B combination was substituted, a marked reduction in the level of apoptosis was also induced. Thus, it seems reasonable to think that the polymorphic amino acids located at this region may be of major importance in this process.

This generalized reduction in the levels of apoptosis detected by TUNEL assays was corroborated by measuring the levels of active caspase-8 and caspase-3. All chimerical combinations showed a reduction in the amount of active caspase-8 and caspase-3 when compared with the non-chimerical SEG/Pas system (Figure 2b).

To rule out the possibility that chimerical Fas and FasL proteins exhibit impaired expression or stability, which might account for the reduced apoptosis levels as compared with the wild-type systems,

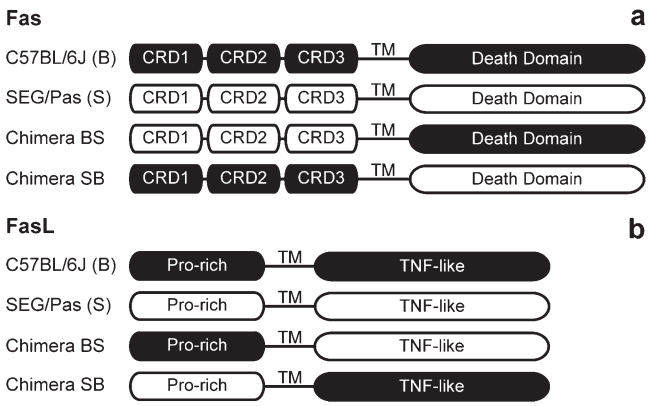

Fig. 1. Schematic representation of the chimerical constructs. (**a**) Fas protein structure, with the extracellular region comprising the three Cysteine-rich domains [(CRDs) 1, 2 and 3], the transmembrane region (TM) and the intracellular region consisting of the Death Domain. (**b**) FasL protein structure, with the intracellular region featured by the Proline-rich domain (Pro-rich), the TM and the extracellular region with the tumour necrosis factor-like domain (TNF-like). Black boxes represent regions of C57BL/6J origin, whereas white boxes represent regions of SEG/Pas origin. Nomenclature for the chimerical constructs defines always the intracellular domain first and then the extracellular; B, C57BL/6J; S, SEG/Pas.

we have determined the expression levels of every construct used for these experiments (supplementary Figure 1 is available at *Carcinogenesis* Online). Our results demonstrate that the chimerical constructs do not exhibit reduced expression compared with the wild-type constructs.

Fadd exhibits sequence variants between C57BL/6J and SEG/Pas

The receptor Fas interacts with Fas ligand (FasL) through its extracellular domain, whereas its intracellular region recruits the protein Fadd by domain homology. Given the importance of the polymorphic amino acids located on the intracellular region of Fas, through which it interacts with Fadd, the ensuing question was to find out whether the Fadd protein exhibits polymorphisms as well.

In order to tackle this problem, we analysed the *Fadd* coding sequence derived from the two strains. Interestingly, we identified five changes of nucleotide between SEG/Pas (Genbank accession no. EU917072) and C57BL/6J (Genbank accession no. NM_010175) (Figure 3a). We have compared the variations described in Ensemble database (http://www.ensembl.org/index.html) with these polymorphisms between C57BL/6J and SEG/Pas. Only one of them corresponds to the SEG/Pas versus C57BL/6J polymorphisms found in our study: T486C (Ensemble ID rs13472364). From those described in Ensemble, this is the only genetic variation affecting the coding sequence of *Fadd* since the rest of them are on either the 3′ untranslated regions or intronic regions. However, it is 'synonymous', so it causes no change of amino acid. In our case, three of the five nucleotide variations found between C57BL/6J and SEG/Pas result in a change of amino acid: A152G (E51G), C177T (H59R) and A565G (N189D) (Figure 3b).

Remarkably, two of the amino acid changes are situated at the death effector domain (DED), by which Fadd recruits Procaspase-8. The other amino acid change is located at the C-terminus near the death domain (DD) (Figure 3b).

The ability of Fas to recruit Fadd varies with the mouse strain

To assess the functionality of the two different *Fadd* variants, together with those polymorphisms located at the sequence coding the intracellular region of *Fas*, we have measured the ability of Fas and Fadd proteins to interact using co-immunoprecipitation assay. Combining Fas and Fadd proteins from either C57BL/6J or SEG/Pas in double transfected Fas/Fadd-HEK-293 cells, our results indicate that the interaction between them is stronger in the Fas system of SEG/Pas mice than in C57BL/6J ('Basal' assay in Figure 4a). Interestingly, when we combine Fas and Fadd in heterologous combinations, their interaction is stronger when Fas derives from SEG/Pas.

The same results were obtained after induction with the Fas ligand, through the co-culture of the double transfected Fas/Fadd-HEK-293 cells with FasL-transfected HEK-293 cells (FasL-induced assay in Figure 4b).

Results of co-immunoprecipitation have been complemented with studies of apoptosis by TUNEL assay in HEK 293 cells co-transfected with *Fas* and *Fadd* cDNAs in the same combinations as in Figure 4 (Figure 5). Our results show a significant correlation with the data obtained from the co-immunoprecipitation experiments. Those two combinations exhibiting the highest levels of interaction between Fas and Fadd (SEG/Pas-derived Fas + SEG/Pas-derived Fadd, second lane and SEG/Pas-derived Fas + C57BL/6J-derived Fadd, fourth lane) show as well the highest rates of apoptosis.

Discussion

Inactivating mutations at *Fas* and/or *FasL* have been involved in several forms of cancer (5,10,19–21). In particular, we have reported the importance of the Fas system in controlling T-cell lymphoblastic lymphoma induced by γ-irradiation (12). We demonstrated that γ-irradiation is able to initially induce the expression of *Fas* and *FasL* as a defensive strategy of the cells and that T-cell lymphoblastic lymphomas exhibit a clear reduction on the expression levels of both proteins. Thus, progression of these lymphomas appears to be favoured by the inactivation of the Fas-mediated extrinsic pathway. In addition, we reported germ line functional polymorphisms at *Fas* and *FasL* genes in two mouse strains exhibiting extreme differences in genetic susceptibility to γ radiation-induced T-cell lymphoblastic lymphoma. Nucleotide variations in the coding sequence of these genes result in amino acid changes in critical domains of their intracellular and extracellular regions. In the case of Fas, the amino acid changes were located at the tumour necrosis factor receptor-like extracellular cysteine-rich domain (CRD) 1, as well as in CRD2, CRD3, and its intracellular death domain. In the case of FasL, we detected two amino acid changes in its intracellular region and four amino acid changes in its extracellular region (12,13). Since the interaction between Fas and FasL clearly depends on the intracellular and extracellular regions of both genes (22–29), we wanted to assess the relative contribution of polymorphisms at each of these four regions to the functionality of the Fas system. To this end, chimerical Fas and FasL proteins combining intracellular and extracellular polymorphic regions from C57BL/6J and SEG/Pas were constructed and the levels of induced apoptosis were investigated in co-cultures of transfected HEK-293 cells using TUNEL assays and measuring caspase-8 and caspase-3 activation. Our data revealed a complex scene with multiple interactions showing striking differences in the biological activity of

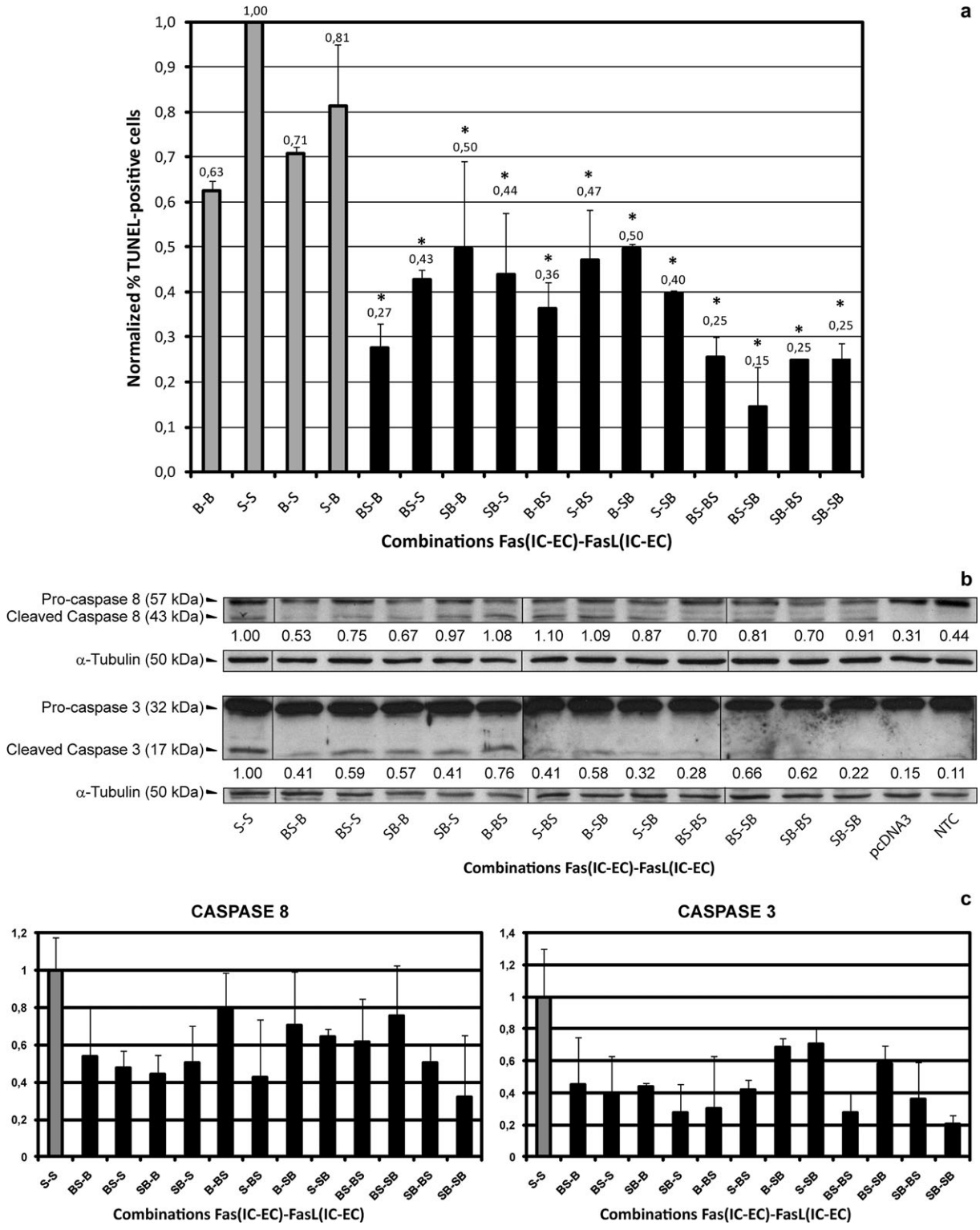

Fig. 2. Apoptosis induced by chimerical Fas–FasL systems. (**a**) Quantification of TUNEL-positive HEK-293 cells in co-cultures of cells transfected with chimerical and non-chimerical *Fas* and *FasL* cDNAs. Twenty-four hours after transfection, 1.5×10^6 HEK-293 cells transfected with Fas constructs were co-cultured in P100 culture plates with a similar number of cells transfected with FasL constructs, in the 16 possible combinations. Receptor–ligand combinations were allowed to interact for 8 h. The number of TUNEL-positive cells was counted from fields of at least 100 cells, estimated through optical microscopy, the percentage of positive cells was calculated as referred to the total number of cells, and the value for cells transfected with the empty vector was subtracted. The results are referred to the S–S combination. The combinations of transfected cells in co-cultures are indicated as Fas–FasL. For example, S-B denotes cells expressing Fas from SEG/Pas strain and cells expressing FasL from C57BL/6J. In the case of chimerical proteins, the first letter always corresponds to the strain origin of the intracellular region and then the extracellular. For example, SB-BS denotes a double chimeric combination where Fas is composed of the intracellular region from SEG/Pas and the extracellular region from C57BL/6J, whereas FasL is composed of the intracellular region from C57BL/6J and the extracellular one from SEG/Pas. *Denotes statistical significance compared with the S–S combination, with $P \leq 0.001$. (**b**) Representative western blots for caspase-8 and caspase-3 activation (cleaved). Protein extracts were obtained from co-cultures with the same combinations as those indicated in (a). The ratios (cleaved-caspase/procaspase) are referred to the S–S combination and indicated under the corresponding lanes. (**c**) Histograms show mean values of the ratios (cleaved-caspase/procaspase)—with their corresponding standard deviations—calculated from the quantification of various experiments of western blot performed for both caspase-8 and -3.

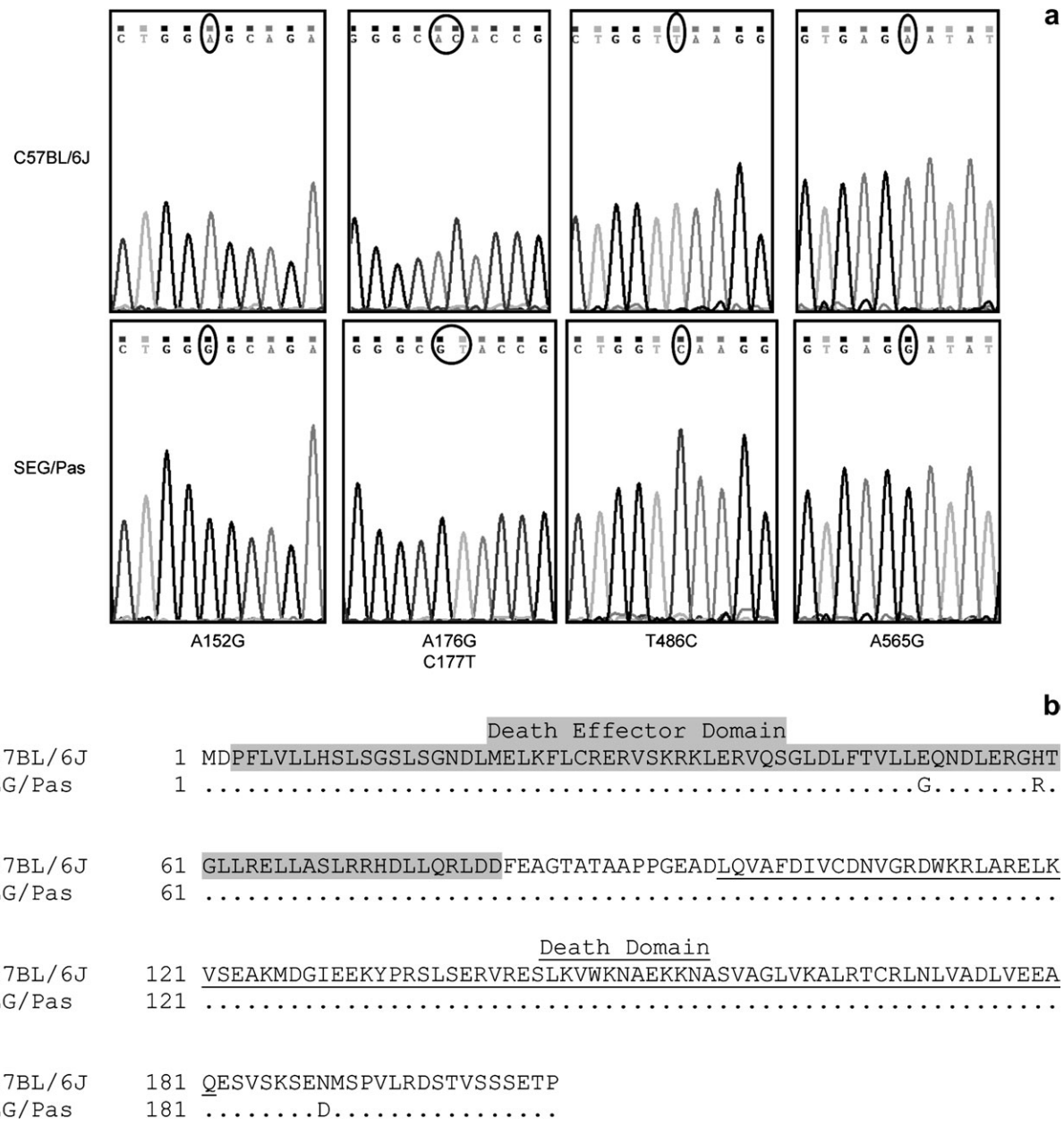

Fig. 3. *Fadd* cDNA variations and amino acid sequences comparison. (**a**) C57BL/6J- and SEG/Pas-derived *Fadd* cDNAs were sequenced and compared. Nucleotide changes (A152G, A176G, C177T, T486C and A565G) between C57BL/6J and SEG/Pas are circled, in a 10-nucleotide context. (**b**) Comparison between amino acid sequences of Fadd in C57BL/6J and SEG/Pas mice. Numbers on the left indicate the amino acid position from the initiation codon. Dots indicate sequence identity. Polymorphisms are indicated by the corresponding amino acid substitution. Fadd DED is shaded, and DD is underlined. Three nucleotide substitutions (A152G, A176G and C177T) involve two amino acidic changes at the DED (E51G and H59R). An additional nucleotide change (A565G) affects the amino acidic sequence near the C-terminus (N189D).

the Fas system. We confirm that the Fas system from SEG/Pas (strain resistant to γ radiation-induced T-cell lymphoma) is the most efficient one in terms of inducing the death signal (S–S combination) and demonstrate that any Fas–FasL interaction involving chimerical proteins drive cell apoptosis to a significant lower extent than the non-chimerical SEG/Pas and C57BL/6J Fas–FasL systems (Figure 2). It seems reasonable to think that the polymorphisms at both the intracellular and extracellular regions of Fas and FasL are involved in the differential ability of the system to drive cell apoptosis, as these variations might be affecting the folding of the proteins and thus their interaction and functionality. Alternatively, the chimerical constructs might as well produce proteins with reduced expression or stability, but this possibility has been ruled out by analysis of their expression levels (supplementary Figure 1 is available at *Carcinogenesis* Online), which confirms that the chimerical constructs do not show impaired expression compared with their wild-type counterparts, and suggests that the diminished apoptosis found when the system consists of at least one chimerical element is not an artefact derived from this problem, but a result of the different functionality of the elements of the system depending on their haplotype.

As mentioned previously, in addition to Fas and FasL, another element exists in the Fas signalling pathway, Fadd, which is essential for the functionality of the Fas/FasL system (23). This gene has been described as a tumour suppressor since mice lacking it are prone to develop T-cell lymphoblastic lymphoma (15). Furthermore, spontaneous thymic lymphoma has been induced using dominant-negative

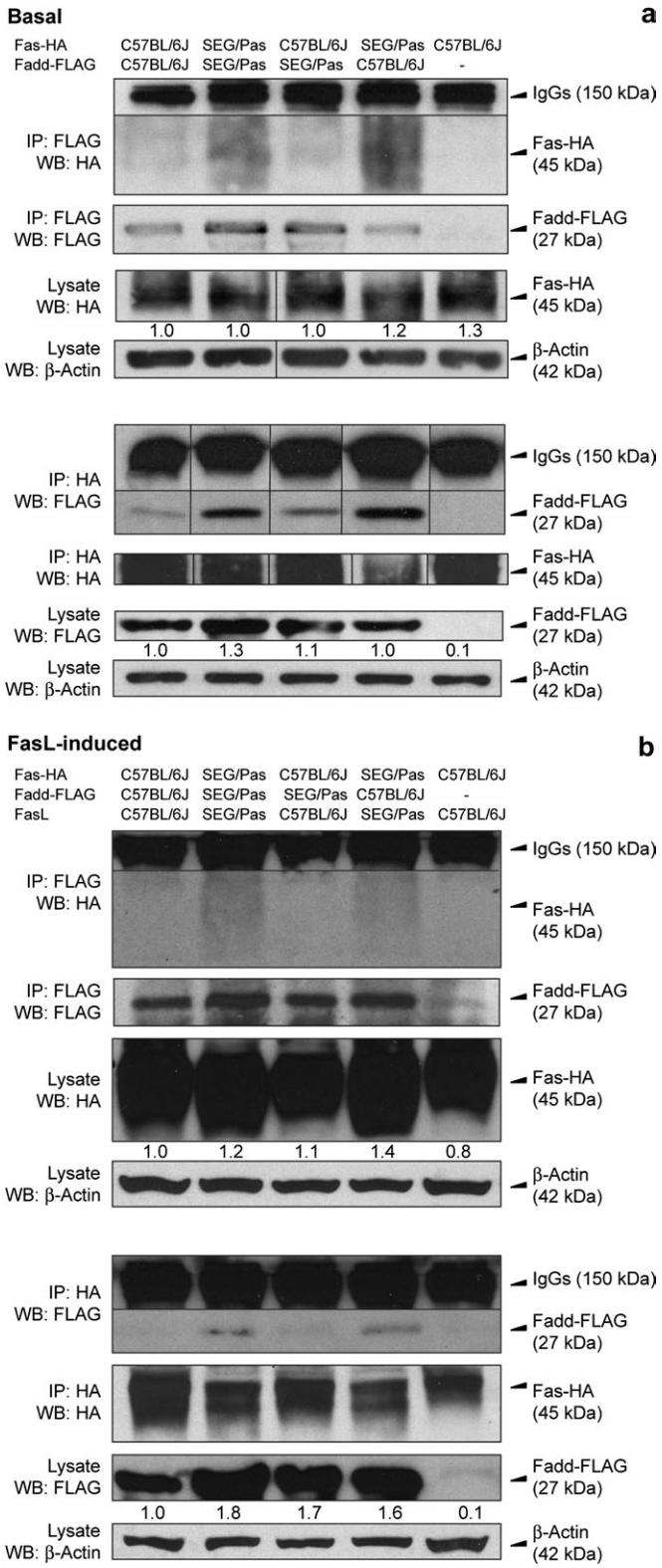

Fig. 4. Fas–Fadd co-immunoprecipitation. Interaction between Fas and Fadd depends on the strain of origin. (**a**) HEK-293 cells were co-transfected with C57BL/6J- or SEG/Pas-derived Fas-HA and Fadd-FLAG, as indicated. Total lysates were immunoprecipitated using specific anti-FLAG and anti-HA antibodies and then Fas-HA and Fadd-FLAG were detected by western blot using anti-HA and anti-FLAG antibodies, respectively. IgGs band (>150 kDa) appear due to lack of β-mercaptoethanol in loading buffer. As a control for transfection, the recombinant proteins Fas-HA and Fadd-FLAG were analysed in total lysates by western blot; β-actin was analysed as a loading control. The ratios (Fas-HA or Fadd-FLAG/β-actin) in the whole lysates are referred to the C57BL/6J-C57BL/6J combination and indicated under the corresponding lanes. (**b**) The same experiment was done after induction of the system through a co-culture of Fas + Fadd-co-transfected cells with FasL-transfected cells.

mutants for this protein (30). The Fadd protein consists of a DD by which it electrostatically interacts with Fas-DD and a DED that hydrophobically recruits Procaspase-8 (23). In humans, it has been additionally demonstrated that both domains need to be covalently attached for proper interaction with FAS (30). It thus seems that Fadd self-association by the DED, through the so-called hydrophobic patch (surface in the vicinity of F25) and/or the RXDLΦ motif (residues 72–76), stabilizes Fadd for its binding to active Fas and so is required for DISC formation (30–33). In our case, the sequence of Fadd in SEG/Pas mice differs from that of C57BL/6J mice in three changes of amino acid, none of them located at the DD. Notably, the E51G polymorphism we find in *Fadd* cDNA involves an amino acid, proposed to allow the structural superposition between DED and DD, that forms part of the Procaspase-8 binding surface (30). However, our results from the co-immunoprecipitation assays suggest that it is the origin of Fas and not Fadd, what mainly determines their level of interaction. As well, these results correlate with the ability of each variant to induce apoptosis, as demonstrated in Figure 5, where it is showed that the presence of SEG/Pas-derived Fas is able to induce the highest level of apoptosis. On the other hand, the polymorphism at N189 might affect the phosphorylation status of Fadd at S191, which has been proposed as a key factor for the functionality of Fadd (34–37). This should be subject of further studies in the future.

Altogether, these results are in agreement with our previous results (13) and reinforce the idea that SEG/Pas mouse strain carries a more effective Fas-dependent apoptosis pathway than the C57BL/6J strain, sustaining our hypothesis for a model in which the Fas system may play a role in modulating the genetic susceptibility of mouse strains to develop T-cell lymphoblastic lymphomas.

In conclusion, four important points emerge from these analyses. The first one highlights the idea that the inactivation of the Fas-dependent extrinsic pathway of apoptosis plays an essential role in the progression of T-cell lymphoblastic lymphoma. The second one indicates that there exist specific polymorphisms at *Fas*, *FasL* and *Fadd* genes that differentiate mouse strains exhibiting

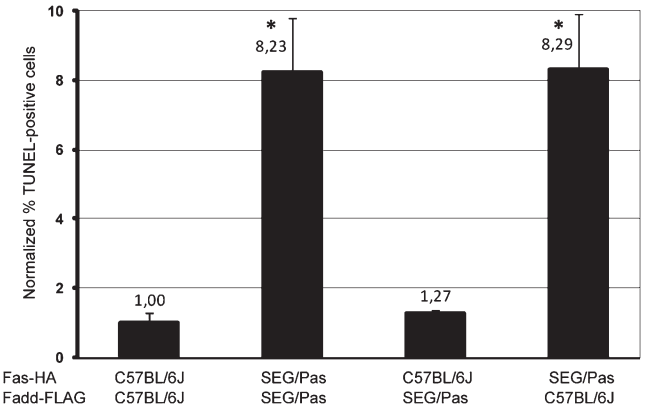

Fig. 5. Apoptosis is related to haplotype-determined Fas–Fadd interaction. Apoptosis analyses by TUNEL assay in HEK-293 cells co-transfected with C57BL/6J- or SEG/Pas-derived Fas-HA and Fadd-FLAG. The number of TUNEL-positive cells was counted from fields of at least 100 cells, estimated through optical microscopy, the percentage of positive cells was calculated as referred to the total number of cells and the value for cells transfected with the empty vector was subtracted. The results are referred to the C57BL/6J-C57BL/6J combination. *Denotes statistical significance compared with this combination, with $P < 0.001$.

extreme differences in susceptibility to γ radiation-induced T-cell lymphomas. These polymorphisms seem capable of modifying the biological activity of the Fas-apoptosis-signalling pathway. Thirdly, the study shows that the global functionality of the Fas-apoptosis-signalling pathway is dependent on the combined action of the functional polymorphisms at these three genes. Finally, a fourth point emerging from our research is that any conclusions based exclusively on the analysis of individual polymorphisms at only one of these genes is not enough to accurately predict the effectiveness of the Fas system or to estimate susceptibility to T-cell lymphomas. A complete analysis of at least the three genes appears to be a *sine qua non* condition for this.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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