International Immunology, Vol. 17, No. 9, pp. 1257–1268
doi:10.1093/intimm/dxh302

Expression of the *Ian* family of putative GTPases during T cell development and description of an *Ian* with three sets of GTP/GDP-binding motifs

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Keywords: diabetes, GTPases, *lymphopenia*, rat, T lymphocytes

Abstract

Reports suggest that two members of the novel immune-associated nucleotide (*Ian*) GTPase family, *Ian1* and *Ian5*, play roles in T cell development. We performed real-time PCR analysis of the expression of *Ian* genes of the rat during T cell maturation, in macrophages and in cell lines. We found that all of the genes were expressed at relatively low levels at the early double-negative thymocyte stage but were expressed more strongly at later cell stages. Our study also revealed the fact that the previously reported *Ian9*, *Ian10* and *Ian11* genes are, instead, parts of a single gene for which we retain the name *Ian9*, potentially encoding a GTPase with a highly unusual triplicated structure. Antisera were developed against both Ian1 and Ian9. We established that Ian9 is produced as an ~75-kDa protein in both T cells and thymocytes. We observed that levels of both Ian1 and Ian9 proteins are profoundly reduced in T cells from *lymphopenic* rats as compared with wild-type rats. It was demonstrated that thymocytes and B cells from *lymphopenic* rats (Ian5 null) did not show enhanced sensitivity to γ-irradiation-induced apoptosis.

Introduction

Immune-associated nucleotide (*Ian*) genes encode a novel family of putative guanosine nucleotide-binding proteins (1). They are clustered in a 300-kb interval within human (h) chromosome 7q36.1 and a more compact 120-kb region in mouse (m) chromosome 6 and rat (r) chromosome 4. An analysis of genomic sequences has reported 10 *Ian* genes in human and 11 in the mouse (2). Ian proteins share a characteristic version of the GDP/GTP-binding domain observed in the different families of GTP-binding proteins and in some cases have been shown to bind GDP/GTP or to hydrolyze GTP (3–5). Sequence-based database mining has revealed distant relatives of the *Ian*'s in plants [AIG1 (6)] but not so far in yeast, nematodes or flies. Structural prediction programs have identified putative coiled-coil domains in most if not all of the predicted Ian polypeptides, suggesting participation in protein–protein interactions (1, 3, 5, 7). In addition, carboxy-terminal transmembrane domains are predicted for Ian2 [the numbering of Ian genes/proteins in this paper follows that used by MacMurray *et al.* (2)], Ian4, Ian5 and hIan12. Intracellular localization studies have yielded a variety of results, placing hIan2 in the endoplasmic reticulum (ER) (5), mIan4, rIan5 and hIan5 in the outer mitochondrial membrane (4, 8, 9) and hIan5 also in all three of the ER, the Golgi and the centrosomal regions (7).

Ian transcripts have been detected in many mammalian tissues but appear to be at their highest levels in cells of the immune system (1–4, 7, 10, 11). Consistent with this, it was in functional immunological studies that these genes first came to the attention of investigators. For example, Krücken and co-workers reported that the expression of the m*Ian2* (*imap38*) gene is up-regulated in the spleens of mice that develop immunity to the experimental malarial parasite *Plasmodium chabaudi chabaudi* (10, 12). These, and other, investigators have suggested that Ian proteins may be involved in the regulation of cell death versus survival. Recently, direct evidence supporting a role in the control of apoptosis was obtained when h*Ian5* was picked out from a functional screen as a cDNA capable of protecting against apoptosis induced by either okadaic acid or γ-radiation (7, 13).

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Received 1 October 2004, *accepted* 29 June 2005

Transmitting editor: A. Cooke

Advance Access publication 15 August 2005

Two separate lines of investigation have pointed to an important role(s) for Ian proteins in the development of T lymphocytes. First, a differential display study revealed that the expression of m*Ian1* is strongly induced at or after T cell-positive selection in the thymus; a wave of expression was also detected after the earlier β-selection stage (1). Second, positional cloning of *lymphopenia* (*lyp*), which is a major susceptibility locus for type I diabetes mellitus (*iddm1*) in the diabetes-prone BioBreeding (BB-DP) rat model of this disease (14, 15), has identified a frameshift (probably null) mutation in the r*Ian5* gene as the basis of this trait. In its homozygous state, the *lyp* mutation is associated with severe peripheral T cell lymphopenia (16, 17) and it has been demonstrated that both peripheral T lymphocytes and mature $CD4^+$ and $CD8^+$ single-positive (SP) thymocytes from *lyp/lyp* animals are much more susceptible to spontaneous apoptosis than their wild-type (w.t.) counterparts (18, 19).

Given these two independent indications of the importance of *Ian* genes in T cell development, one in mouse and the other in the rat, we undertook a study of gene expression of the entire *Ian* gene cluster in lymphomyeloid cells in order to assess expression levels of all *Ian* family members. We have commenced the study of Ian function by examining the susceptibility to γ-irradiation-induced apoptosis of thymocytes and B cells isolated from *lymphopenic* rats which are homozygous for an *Ian5* null mutation. We have developed polyclonal antisera against Ian1 and Ian9, and have used these reagents first to prove that the *Ian9* gene, which is predicted to have an unusual triplicated structure comprising domains encoded by *Ian11*, *Ian10* and *Ian9*, does indeed produce a corresponding protein product. Second, we determined levels of Ian1 and Ian9 proteins in various lymphoid populations.

Methods

Animals

Rats of the congenic strains PVG-*RT1*^{u}*RT7*^{b}, PVG-*RT1*^{u}*lyp/lyp* (18) and PVG (the latter used exclusively as a source of macrophages) and C57BL/6 mice were maintained in specific pathogen-free conditions in The Babraham Institute Small Animal Barrier Unit. Animals (males and females) were used between 8 and 12 weeks of age. In unpublished analyses we have confirmed that the PVG-*RT1*^{u}*lyp/lyp* strain, which derives its mutant *lyp* gene from the Edinburgh subline of BB-DP rats (20), carries the frameshift mutation in the *Ian5* gene described by Hornum *et al.* (11) and MacMurray *et al.* (2).

Sample preparation

Various rat primary cell sub-populations were isolated using combinations of mAbs with magnetic bead separations (Dynal, Oslo, Norway) and FACS as described in Supplementary Data Figure S1 and Supplementary Data, Table S1 (available at *International Immunology* Online). Primary cell populations were isolated and total RNA was extracted. All steps were conducted at 4°C. Thymus and lymph nodes (LNs) were recovered and disrupted in RPMI 1640 medium (Sigma, St Louis, MO, USA) containing 5% FCS (Biowest, Ltd, Ringmer, UK). After filtration the cells were centrifuged at 250 × *g* for 10 min and re-suspended in PBS containing 2% FCS. $CD11b/c^+CD172^+$ macrophages were obtained from peritoneal cells prepared by lavage of the peritoneal cavity with ice-cold PBS, containing heparin (50 units ml^{-1}), pH 7.4. Cells were centrifuged at 250 × *g* for 10 min and re-suspended in PBS containing 2% FCS.

Rat cell lines of lymphomyeloid origin were used and these are described in Table 1. The mAbs used in this study were anti-rat TCRαβ, R73 (31); anti-rat CD3, G4.18 (32); anti-rat CD4, W3/25 (33); anti-rat CD8α, MRC OX-8 (34); anti-rat CD8αβ, 341 (35); ant-rat CD43 (W3/13) (33); anti-rat CD11b/c, MRC OX-42 (36); anti-rat CD45RA/'B220', MRC OX-33 (37); anti-rat CD45RC, MRC OX-22 (37); anti-rat CD172 (SIRP), MRC OX-41 (Becton Dickinson, San Jose, CA, USA) (36); anti-rat Ig κ-chain, MARK-1 (38); anti-mouse CD4, YTS191.1 (39); anti-mouse CD8, YTS169 (39); anti-mouse-CD19, 1D3 (40) (BD PharMingen, San Diego CA, USA).

Total RNA was extracted from cells using TRIZOL reagent (Invitrogen, Paisley, UK) and used to generate first-strand

Table 1. *Ian* gene expression in peritoneal macrophages and lymphomyeloid cell lines of rat origin

		Cell type	Origin	Reference	*Ian1*	*Ian2*	*Ian3*	*Ian5*	*Ian6*	*Ian7*	*Ian9*
Primary cells	:'Control'	LN T cells			10 000	10 000	10 000	10 000	10 000	10 000	10 000
	:Macrophages	Rat no. 1			100	∇	507	∇	4438	104	∇
		Rat no. 2			49	∇	310	∇	2197	54	∇
Cells lines derived from	:T cells	Mg2(über)[b]	Thymoma		37	1817	122	978	881	130	871
		C58	Thymoma	(21)	56	1894	328	942	590	385	390
		Nb2	Lymphoma	(22)	421	1971	582	3285	3037	13	2687
		RL-1	Leukemia	(23, 24)	∇	∇	2137	*	∇	63	3277
	:B cells	Y3Ag1.2.3	Plasmacytoma	(25)	109	1692	387	134	778	11	9
		IR983F	Plasmacytoma	(26)	149	2751	783	202	2266	21	32
		YB2/0	Hybrid plasmacytoma	(27)	65	1585	234	74	3550	21	11
	:Myeloid cells	Mg2(mono)[b]	Thymic adherent		2	∇	17	∇	85	9	∇
		RBL-1	Basophilic leukemia	(28)	1	2506	7	24	33	1	∇
	:NK cells	CRNK16	Leukemia	(29)	∇	∇	14	∇	57	1	∇
		A181	Leukemia	(30)	10	42	14	65	25	3	67

The expression of *Ian* genes is given relative to LN T cells which are given the arbitrary value of 10 000. All the populations studied have a lower level of *Ian* gene expression than LN T cells. ∇, very weak expression of the *Ian* gene; *, *Ian* gene expression undetectable. [b]Gift from K. Wonigeit, Hannover Medical School, Germany.

cDNA with SuperScript III RNase H^- reverse transcriptase (Invitrogen).

Real-time PCR

Quantitative differences in expression levels of *Ian* transcripts in lymphomyeloid cells were determined using comparative real-time PCR. This was carried out using the ABI Prism 7700 system (Applied Biosystems, Cheshire, UK) and 'SYBR green master mix' according to the manufacturers' instructions (Applied Biosystems). PCR primer sequences specific for 'housekeeping genes' (HKGs) and *Ian* genes in mouse or rat are described in Supplementary Data, Table S2 (available at *International Immunology* Online). Two HKGs were used as internal controls: 6-phosphofructokinase C (6PFKc) and cirhin. These were selected because (i) they displayed optimal stability among the various samples tested as calculated by the M value (internal control gene-stability measure) (41) and (ii) they had at least two exons, enabling the design of primers, whose product will span an intron in genomic DNA, thus allowing the identification and exclusion of amplicons templated on contaminating genomic DNA on the basis of size, relative to those from the *bona fide* cDNA template. Cycling parameters were as follows: 50°C for 2 min, then 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. To detect non-specific PCR products (primer-dimers) a dissociation curve was performed at the end of the program: 95°C for 20 s, 60°C for 20 s and then 95°C for 30 s. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (C_T). This parameter was calculated for the genes of interest ($C_{T\ Ian}$) and the two different HKG ($C_{T\ HKG}$). The latter served to standardize the initial amount of material used, allowing an accurate comparison of the different cDNA samples (15 lymphocyte sub-populations from rat, 6 from mouse, 11 rat cell lines and rat macrophages). For each cDNA, $C_{T\ HKG}$ was subtracted from $C_{T\ Ian}$ giving a ΔC_T specific for each cell population. For each cDNA sample, the amount of target was normalized using the geometric average of the two HKG. This was calculated as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ is the difference between the ΔC_T of the two cDNA samples under investigation ($\Delta\Delta C_T = \Delta C_{T2} - \Delta C_{T1}$) (42). In all cases the standard deviation of triplicate C_T values was $<1.1\%$ of the mean.

γ-Irradiation

Thymus and LNs were recovered from PVG-*RT1^{u}RT7^b* (four animals) and PVG-*RT1^{u}lyp/lyp* (nine animals) rats and disrupted as describe above. LN T and B cells were isolated by negative selection using mAbs with magnetic bead separations (Dynal): to isolate B cells, anti-rat CD43 (W3/13) was coated on the beads; to isolate T cells, anti-rat Ig κ-chain (MARK-1) and CD45RA (MRC OX-33) were used. The three cell populations, at a concentration of 2.5×10^6 cells ml^{-1}, were each split into four different tubes in order to receive different doses of γ-irradiation (0, 0.2, 0.8 and 3.2 Gy) from a ^{137}Cs source (Schering IBL 437 Irradiator, Gif-Sur-Yvette, France) delivered at 0.87 Gy min^{-1}. Two hundred microliters (5×10^5 cells) of each cell solution was put in a 96-well plate and incubated at 37°C for 0, 3, 6, 9 and 24 h. At these different time points, the cells were centrifuged at $250 \times g$ for 2 min and re-suspended in 500 μl 1X binding buffer (10X: 0.1 M HEPES, 1.4 M NaCl, 25 mM $CaCl_2$). Fifty microliters of this suspension was added to 2.5 μl of Annexin V–Cy5 (BD PharMingen) and 2.5 μl of 7-amino-actinomycin D (7-AAD) (BD PharMingen). After 15 min incubation at room temperature in the dark, the samples were diluted in 200 μl of 1X binding buffer and analyzed by FACS. This experiment was performed in duplicate.

Cloning of rat and human Ian9

We designed primer pairs for human and rat *Ian9* (Supplementary Data, Table S2, available at *International Immunology* Online), which were then used to amplify cDNAs derived from human spleen and rat thymus. In both species, PCR products of ~2 kb were obtained after amplification with Advantage II DNA Polymerase (Clontech, Palo Alto, CA, USA). We then inserted human and rat PCR products into pCR4-TOPO (Invitrogen) and human and rat clones were sequenced by primer walking (Lark Technologies, Saffron Walden, UK).

Production of polyclonal antisera against Ian1 and Ian9

Mouse and rat Ian1 were PCR amplified from cDNA and cloned in appropriate frames into the glutathione-S-transferase (GST) fusion vector, pGEX-4T-1 (Amersham Biosciences, Bucks, UK). A polyclonal antiserum against mouse and rat Ian1 was raised by injecting rats with purified GST fusion proteins of mouse and rat Ian1. The antiserum raised detects Ian1 from both species. The C-terminal third of the Ian9 cDNA encoding amino acids 463–688 of the sequence shown in Fig. 5 was inserted, as above, into pGEX-4T-1. Custom production of a rabbit antiserum against the rIan9–GST fusion was undertaken by Harlan Seralab, UK.

Western blotting

Appropriate cells were lysed in SDS reducing buffer. Typically, proteins from 1 or 2×10^6 cells were separated by SDS-PAGE. Proteins were transferred electrophoretically onto Immobilon-P membranes (Millipore, Bedford, MA, USA). After overnight blocking, membranes were incubated for 1 h with the appropriate antiserum, washed extensively and then incubated for a further hour with goat anti-mouse IgG–HRP (Sigma), goat anti-rat $IgFc_\gamma$-specific HRP (Jackson ImmunoResearch Laboratories,) or goat anti-rabbit IgG–HRP (Dako, Ely, UK). Signals were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford IL, USA). Monoclonal mouse anti-actin (Sigma AS441) was used as a loading control.

Preparation of epitope-tagged transfectants

mIan1 and rIan9 were PCR amplified from cDNA and cloned into pCMV(EE) which provides an N-terminal EE tag (EEEEFMPMEF) (43). Stable transfectants were made by electroporation and selection in G418: mIan1 was transfected into the rat thymoma cell line C58 and rIan9 was transfected into HEK293 cells. Positive transfectants were identified by western blotting with both an anti-EE antibody and the relevant anti-Ian antiserum.

Results

Real-time PCR set up

The two HKGs (6PFKc and cirhin) were selected for their stable expression in the cell populations isolated and studied (Supplementary Data, Figure S1 and Table S1, available at *International Immunology* Online): the M value, which is a measure of this stability (41), was 0.56 for rat, 0.64 for mouse and 1.1 for cell lines and macrophages. The specificity of our real-time PCR products was confirmed by size and from the dissociation curves (as shown in Supplementary Data Figure S2, available at *International Immunology* Online), which showed a single peak.

Real-time PCR analysis of T cell lineage subsets

In mouse. The previously published results of Poirier and colleagues (1) had given clear evidence of differential expression of the *Ian1* gene within the thymus of the mouse. That study, however, did not measure *Ian1* expression in adult thymocyte subsets, nor did it employ real-time PCR as an analytical tool. Since our chosen strategy for the present study (see below) was to assess expression of all the *Ian* genes in adult rat thymocyte subsets using real-time PCR, we first performed a study of *Ian1* expression in adult mouse thymocyte subsets using this technique in order to make the results from mouse and rat directly comparable. Our data (Fig. 1) fit well with the earlier results of Poirier and coworkers, in showing a substantial rise (37-fold) in *Ian1* expression between the $CD4^+CD8^+$ double-positive (DP) and the $CD4^+CD8^-$ SP thymocyte stages, and the continuation of high *Ian1* expression into peripheral T cells.

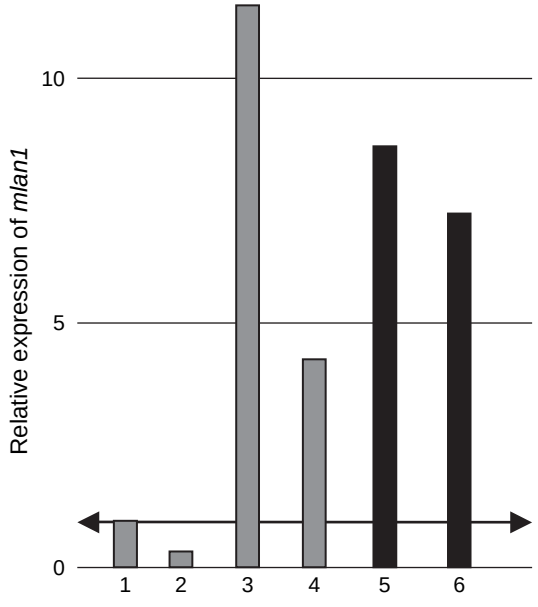

Fig. 1. Relative expression of *Ian1* measured by real-time PCR in the thymic and LN sub-populations of C57BL/6 mice (thymus, gray bar; LN, black bar); bar 1, DN thymocytes; bar 2, DP thymocytes; bar 3, CD4 SP thymocytes; bar 4, CD8 SP thymocytes; bar 5, LN CD4 T cells, and bar 6, LN CD8 T cells. The data are representative of two separate experiments conducted in triplicate.

In rat. Real-time PCR analysis of changes in *Ian* gene expression along the T cell development pathway in the rat is displayed in Fig. 2, as is a comparison between T lineage cells from normal rats and genetically *lymphopenic* (*lyp/lyp*) rats of a congenic strain. We make the following observations from these data. (i) Expression of all the *Ian* genes was relatively poor in the $CD4^-CD8^-$ double negative (DN) thymocyte subset but higher in later developmental stages. It was convenient to set the real-time PCR values from the w.t. DN cells at unity and to compare expression in other cell stages with this value. For all *Ian* genes, with the exception of *Ian1*, there was a substantial increase in gene expression between the DN and the DP stages. (ii) For *Ian2*, *Ian3*, *Ian6*, *Ian9*, *Ian10* and *Ian11*, the level of expression in the DP population was the highest observed. (iii) The highest expression of *Ian1*, *Ian5* and *Ian7* was observed in peripheral T cells. However, the progress towards this maximal peak was not the same for these three genes. *Ian1* showed a very steep rise from a value of 1.3-fold (relative to DN) in DP cells to values of 10.4- and 8.2-fold for $CD4^+CD8^-$ and $CD4^-CD8^+$ SP thymocytes up to values around 25-fold in LN T cells; the expression pattern for *Ian7* was similar to *Ian1*, although up-regulation of *Ian7* was already evident at the DP stage and the overall increase from DN to LN T cells was smaller; the expression of *Ian5* in peripheral T cells, although at its highest, was only slightly above that observed in DP and SP thymocytes. To some extent, the pattern observed for *Ian5* was intermediate between that seen for *Ian2*, *3*, *6*, *9*, *10*, *11* and that seen for *Ian1* and *7*. (iv) The expression values obtained for *Ian9*, *Ian10* and *Ian11* were remarkably similar, as discussed later. (v) *Ian* gene expression by B lymphocytes was generally modest, being higher than that of DN cells but lower than other T lineage populations. In these experiments, expression of *Ian6* in B cells was relatively high; that of *Ian7* was noticeably low. (vi) The data sets from the congenic normal and homozygous *lyp/lyp* rats were, overall, very similar. [Peripheral $CD8^+$ T cells were not prepared from the *lyp/lyp* strain because they are present at a very low frequency (18).] Clear differences in the profiles for *Ian5* probably relate to the fact that this gene is mutated in the *lyp/lyp* strain. In addition, the previously documented phenotypic changes in mature thymocytes and peripheral T cells associated with the rat *lymphopenia* trait might cause differences in expression of other *Ian* genes relative to the w.t. (18, 44–46). The consistent inversion of *Ian* gene expression in $CD4^+$ versus $CD8^+$ SP thymocytes between w.t. and *lyp/lyp* rats may be an example of this, although the reproducibility of these generally small differences has yet to be tested.

Ian gene expression in peritoneal macrophages and selected lymphomyeloid cell lines

In order to improve our general understanding of *Ian* gene expression, we extended our analysis to include normal rat $CD11b/c^+CD172^+$ macrophages (Supplementary Data Figure S1C, available at *International Immunology* Online) obtained from peritoneal lavages and a number of lymphoid or myeloid rat cell lines maintained *in vitro*. The most striking feature of these results (Table 1) is that none of the cell types expresses any of the *Ian* genes as strongly as LN T cells.

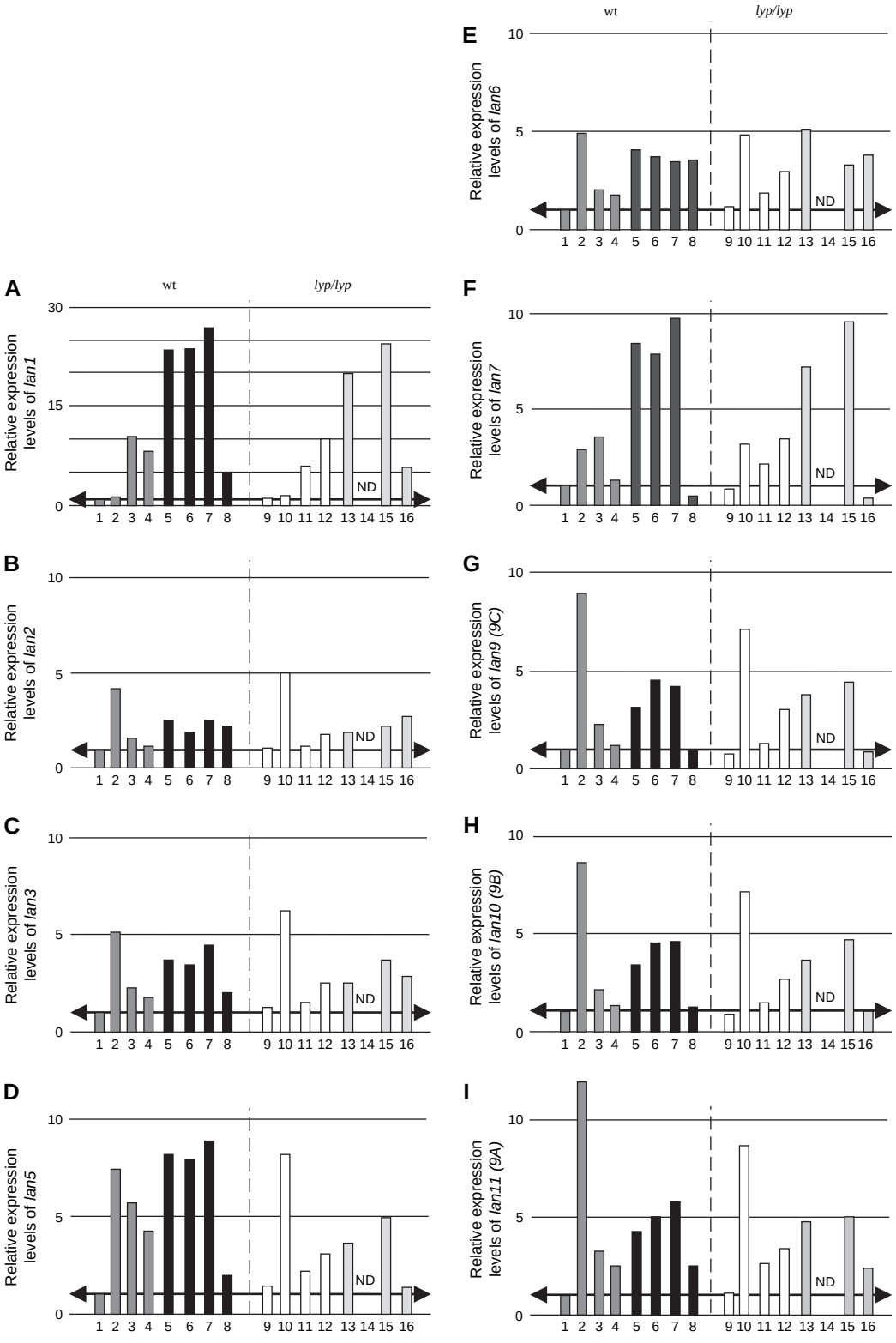

Fig. 2. Relative expression of *Ian* genes measured by real-time PCR in the thymic and LN sub-populations from PVG-*RT1*^{u}*RT7*^{b} (thymus, dark-gray bar; LN, black bar) and PVG-*RT1*^{u}*lyp/lyp* rat (thymus, open bar; LN: light-gray bar). Bars 1 and 9, DN thymocytes; bars 2 and 10, DP thymocytes; bars 3 and 11, CD4 SP thymocytes; bars 4 and 12, CD8 SP thymocytes; bars 5 and 13, LN CD4 T cells; bar 6, LN CD8 T cells; bars 7 and 15, LN T cells, and bars 8 and 16, LN B cells. (A–I) Expression level of, respectively, *Ian1*, *Ian2*, *Ian3*, *Ian5*, *Ian6*, *Ian7*, *Ian9*, *Ian10* and *Ian11* (note that we subsequently refer to Ian9 as Ian9C, Ian10 as Ian9B and Ian11 as Ian9A). Arrows indicate reference level (set at 1) corresponding to the PVG-*RT1*^{u}*RT7*^{b} DN population. The data are representative of two separate experiments conducted in triplicate. ND: not done (Bar 14). The paucity of CD8^{+} peripheral T cells in *lyp/lyp* rats precluded economic preparation of this cell population.

It should be noted that where any expression was seen in cell lines this was generally of the order of expression seen in DN thymocytes (from comparison of the data in Fig. 2 and Table 1 using LN T cells as the common standard). Expression of some of the *Ian*'s was detected in peritoneal macrophages, most notably *Ian6*. Some expression of all the *Ian*'s was detected in the T and B cell lines, the best overall expression being shown by Nb2, which, interestingly, is a T lymphoma (i.e. of peripheral origin). *Ian* expression in the myeloid and the NK cell lines was generally weak, with sporadic exceptions.

lyp/lyp lymphocytes show no enhanced sensitivity to apoptosis induced by γ-irradiation

The *in vitro* studies performed by Sandal and colleagues (7) indicated that hIan5 was capable of inhibiting γ-irradiation-induced apoptosis of Jurkat T cells. Given that rIan5 is implicated in the regulation of apoptosis in the rat T lineage (8, 47), we were interested to find out if primary cells from homozygous *lyp* mutant (*Ian5*$^{-/-}$) rats would show an enhanced sensitivity to γ-irradiation, consistent with these findings. Using flow cytometry to analyze cell populations (an example with w.t. cells after 3.2 Gy irradiation is shown in Fig. 3A), we quantified and compared apoptosis (by Annexin V staining) and necrosis (7-AAD staining) in thymocytes and LN B cells from w.t. and *lyp/lyp* rats. These two cell populations were chosen since both express Ian5 mRNA at detectable levels (ref. 11, and our own analysis, above) while neither displays gross abnormalities in *lyp/lyp* rats compared with w.t. The data in Fig. 3(B) demonstrate that, over the course of 24 h and at graded doses of γ-irradiation up to 3.2 Gy, neither cell population from *lyp/lyp* animals shows enhanced sensitivity

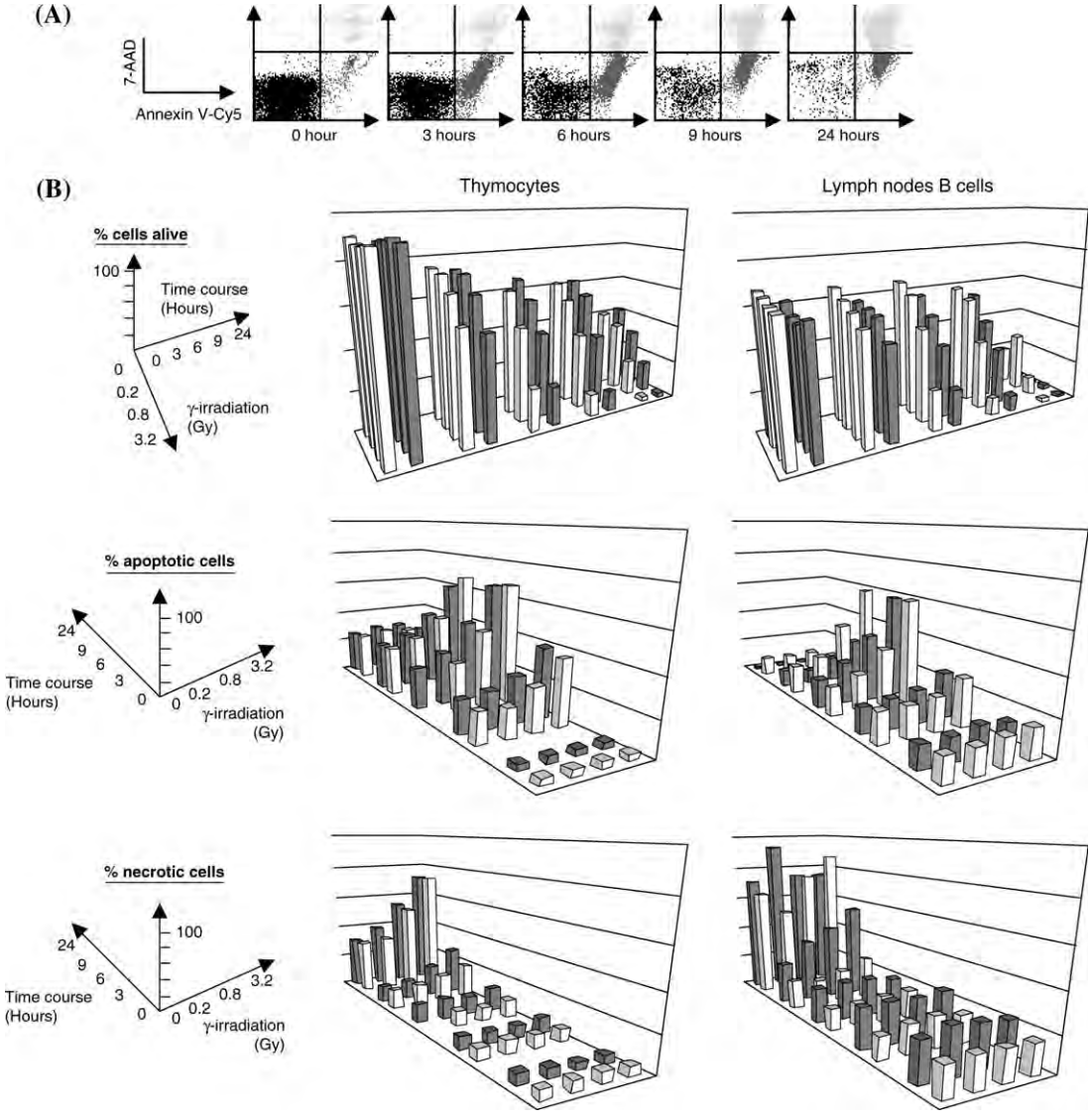

Fig. 3. (A) Flow cytometric analysis of apoptotic (Annexin V) and necrotic (7-AAD) thymocytes exemplified using data for w.t. rats after γ-irradiation (3.2 Gy) and after different hours of incubation (0, 3, 6, 9 and 24 h). (B) Comparison of survival, apoptosis and necrosis in thymocytes and LN B cells from w.t. (open bar) and *lyp/lyp* (filled bar) rats after γ-irradiation (0, 0.2, 0.8 and 3.2 Gy) and different hours of incubation (0, 3, 6, 9 and 24 h). These data were obtained using the markers set as shown in Fig. 3(A).

to γ-irradiation compared with cells from w.t. A slightly more rapid transition of *lyp/lyp* B cells to necrosis was the only difference observed. Similarly, preliminary experiments carried out on peripheral T cells gave no indication of enhanced sensitivity of *lyp/lyp* T cells compared with w.t. (data not shown). However, we considered the comparison of γ-irradiation-induced apoptosis in peripheral T cells to be inferior as an experiment to those using thymocytes and B cells since it has been demonstrated that *lyp/lyp* T cells are in a different differentiation/activation state compared with resting w.t. T cells (48).

rIan1 expression

The timing of the gross phenotypic defect in *lyp/lyp* (*Ian5*$^{-/-}$) rat T cell development parallels the normal expression of the *Ian1* gene more strongly than that of *Ian5* itself. We therefore wondered whether different members of the *Ian* gene family may have interacting functions in their contributions to T cell development. In order to begin to probe aspects of Ian function at the protein level, we have initiated the preparation of specific serological reagents to detect and quantify Ian proteins. We developed a polyclonal antiserum reactive with Ian1 proteins [as shown using stably transfected cell lines, Fig. 4(A)] and used it to probe lysates of cells from w.t. and *lyp/lyp* rats in western blots (Fig. 4B). The antiserum revealed a protein of the appropriate mobility (M_r ~38 kDa) which was at its highest level in w.t. LN T cells, but was also detectable in w.t. thymus, spleen and LN B cells. By contrast, the signals obtained from *lyp/lyp* cells were either very weak, or absent in spleen. *lyp/lyp* LN T cells gave only a very weak signal while thymocytes gave a signal that was *ca* half of that seen in the w.t.

Definition of an Ian polypeptide containing three sets of GTP/GDP-binding motifs

We have noted above that the real-time PCR results for *Ian9*, *10* and *11* in T lineage subsets were remarkably similar to one another. *Ian9*, *10* and *11* are physically close to one another in the published maps of the human and mouse *Ian* gene clusters (2). We aligned ENSEMBL genomic sequences in this area from human, mouse and rat and observed distinct blocks of sequence conservation, corresponding to exons. Scanning along the three genes, in each species, revealed the expected sequence motifs. However, only one termination codon was detected. This terminator was located at the 3′ end of *Ian9*, suggesting that *Ian11*, *10* and *9* (5′–3′) might form a continuous open reading frame (ORF). Our conclusion was supported by a human cDNA clone (AL834361), which contained *Ian11*, *10* and *9* as a single ORF. PCR primers, corresponding to the ends of the putative full-length human and rat coding sequences, were then used to amplify cDNAs derived from human spleen and rat thymus, respectively. In each case, a PCR product of the predicted size was obtained. These products were not found in control PCR reactions carried out on the original RNA used for cDNA production, indicating that they originated from cDNA and not from contaminating genomic DNA. After cloning and sequencing human and rat PCR products, we found several single-nucleotide changes relative to the original ENSEMBL entries: we presume these mutations were generated during PCR amplification. We were able, however, to identify human and rat clones (AJ633686, AJ633685) which contained single ORFs with minimal predicted amino acid changes with respect to the original ENSEMBL entries. Thus, we have confirmed that, in human and rat, *Ian11*, *10* and *9* are transcribed as one mRNA molecule, which contains a single stop codon.

We note that in mouse there is a database entry describing an essentially similar cDNA molecule, termed Ian9 (Accession AB178029). In order to resolve the potential nomenclatural confusion caused by this finding, we suggest that this gene and its protein product be designated as *Ian9* and that reference to *Ian10* and *Ian11* be dropped in future. For the purpose of the present description, we will refer to the three parts of Ian9 as follows: Ian9A, corresponding to the

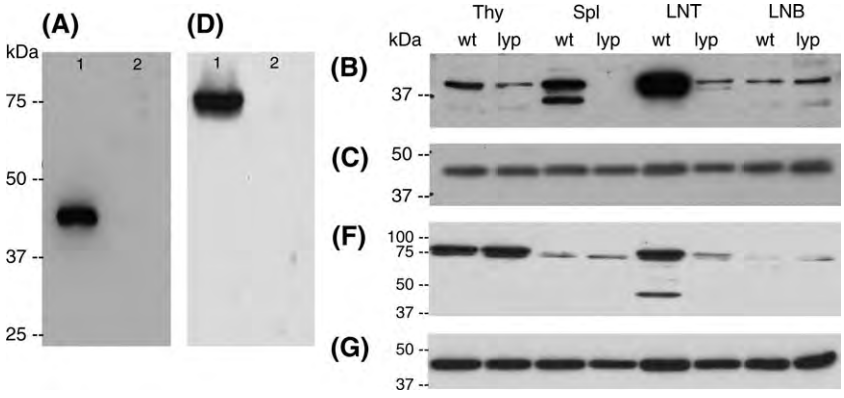

Fig. 4. Rat Ian1 and Ian9 expression in w.t. versus *lyp/lyp* rats. An antiserum reactive with murine (i.e. mouse and rat) Ian1 was developed (A), and used to investigate Ian1 expression in w.t. and *lyp/lyp* lymphoid cells (B and C). (A) Track 1: C58.pCMV(EE).mIan1 transfected cells. Track 2: C58 non-transfected cells. Blot developed with anti-mIan1 antiserum. (B and C) Lysates from 1 × 10^6 w.t. or *lyp/lyp* (lyp) cells were run in each lane. Cells were thymocytes (Thy), mononuclear splenocytes (Spl), LN T cells (LNT) or LN B cells (LNB). Western blots were developed with (B) the anti-murine Ian1 antiserum or (C) monoclonal anti-actin antibody as loading control. An antiserum, reactive with rIan9, was developed (D) and used to investigate Ian9 expression in w.t. and *lyp/lyp* lymphoid cells (F and G). (D) Track 1: pCMV(EE).rIan9 transfected HEK293 cells. Track 2: pEE vector transfected HEK293 cells. Western blots were developed with the anti-rIan9 antiserum. (F and G) Lysates from 2 × 10^6 w.t. or *lyp/lyp* (lyp) cells were run in each lane. Cells were thymocytes (Thy), mononuclear splenocytes (Spl), LN T cells (LNT) or LN B cells (LNB). The blots were developed using (F) anti-rIan9 antiserum or (G) monoclonal anti-actin antibody as loading control.

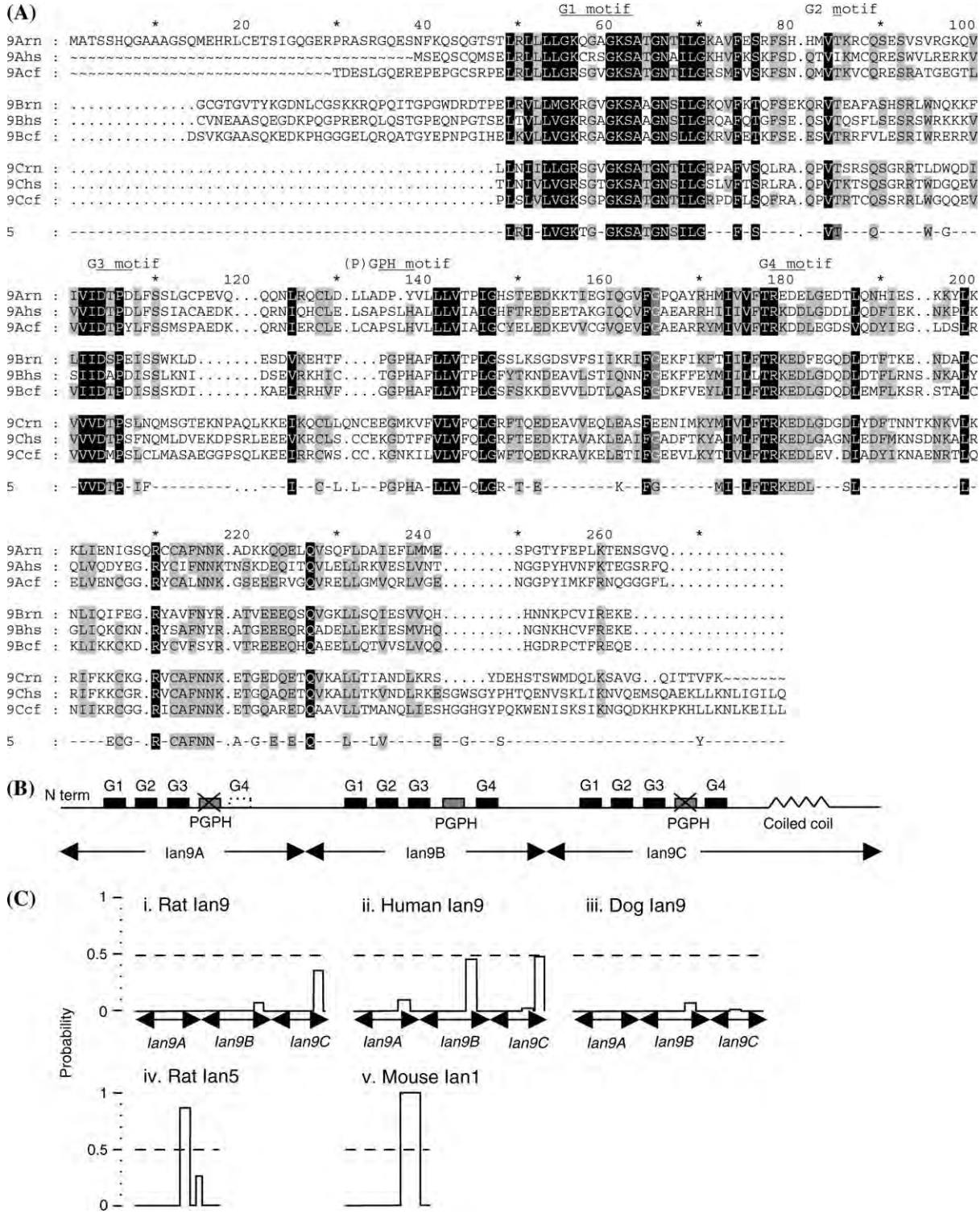

Fig. 5. (A) Alignment. The Ian9 sequences of rat (rn), dog (cf) and human (hs) were aligned using the program Clustal X (49) with its default settings. The starts of Ian9B and Ian9C and the ends of Ian9A and Ian9B were defined by the known intron–exon junctions. The end of Ian9C was determined by the first occurrence of an in-frame termination codon. The start of Ian9A was chosen as the first in-frame methionine. In the dog this exon was not identified. In order to obtain a working Ian consensus, mIan1, 2, 3, 4, 5, 6 and 7 were also aligned using Clustal X and a consensus with plurality of 5 was determined. This consensus was then added to the Ian9A, 9B and 9C alignment (line '5'). Shading was applied to the alignment by use of the GeneDoc program (50). Black highlight denotes an amino acid which is highly conserved and gray highlight represents a minimum of 60% conservation. The following symbols are used. Tilde appears upstream of a sequence start, period appears where there is a gap in the sequence. Amino acids on either side of a period are contiguous and dash represents a blank in the consensus. (B) A schematic representation of the predicted Ian9 polypeptide. This contains three sets of GTP/GDP-binding domains located in each of the three regions that

N-terminal third of the Ian9 triple (formerly Ian11); Ian9B, corresponding to the central third of the Ian9 triple (formerly Ian10), and Ian9C, corresponding to the C-terminal third of the Ian9 triple (formerly Ian9). Using trace data from the National Center for Biotechnology Information, we have also been able to assemble a dog Ian9 sequence that lacks probably only a few residues at its amino-terminus. Predicted Ian9 amino acid sequences for human, rat and dog are aligned in Fig. 5(A) where they are compared with an Ian consensus. This alignment compares the three internal 'repeats' within Ian9 (Ian9A, Ian9B and Ian9C) and highlights the conserved elements of the GTPase motif (3, 51). A schematic representation of Ian9 is shown in Fig. 5(B). Four aspects of these sequences merit comment. (i) A sequence motif shared by Ian polypeptides, namely, '(P)GPHxx(L)LV' beginning at position 135 on the alignment in Fig. 5(A), has been a valuable tool in our hands for the phylogenetic tracking of *Ian*-related genes. For instance, scanning of the human proteome databases with this predicted peptide brings back high scores only for the Ian family; scanning of other species yields only GTPases related to the Ian's. It is of interest that, within Ian9, only the Ian9B region preserves this motif in full. (ii) The length of the polypeptide region between the G3 and G4 motifs is generally longer in the Ian polypeptides than it is in, for example, the ras GTPases. However, Ian9B is substantially shorter than the Ian consensus in this region, while Ian9C is a little longer. (iii) There are a small number of non-conservative amino acid differences between the Ian consensus and the Ian9 sequence. The majority of these are concentrated in the Ian9B component of Ian9. These are T58R, T65A, Q89A/V/L, C128H, N216Y and E227Q. Two of these, *viz.* T58R and T65A, might have some direct impact within the GTP/GDP-binding pockets. (iv) Previous analyses of various Ian polypeptide sequences have predicted coiled-coil motifs in the carboxy-terminal portions. We have analyzed Ian9 sequences from mouse, rat, human and dog. In comparison with predictions for rIan5 and mIan1 (Fig. 5C, iv and v), we find, at best, weak coiled coils in Ian9 (Fig. 5C, i– iii). It is worth noting that the C-terminal portion of rat Ian9 (i.e. from Ian9C) gave a stronger coiled-coil score than either the Ian9A or Ian9B regions.

In order to confirm that this intriguing mRNA is actually produced as a protein product, we derived a polyclonal rabbit antiserum raised against rat Ian9C–GST. We used the antiserum to probe western blots of cell lysates from w.t. and *lyp/lyp* rats (Fig. 4). The antiserum detected a strong band in thymocytes from both w.t. and *lyp/lyp* cells, and a weaker band in both samples of spleen cells. In LN T cells, we observed a strong band in w.t. and a markedly less intense band in *lyp/lyp*. LN B cells gave weak signals. In all cases the band observed was of the size predicted from amino acid sequence, ~75 kDa. We observed an additional band of 40–45 kDa in w.t. LN T cells: this has yet to be further investigated.

Discussion

Real-time PCR set up

The main problems that can be encountered in gene expression analysis using real-time PCR are as follows. (i) The risk of genomic DNA amplification. This was solved using forward and reverse primers complementary to sequences in different exons. We note that Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), one of the most common HKGs employed, is unsuitable for use in murine rodents (unless working on a large scale where DNase treatment is practical) because it is expressed from a single exon (from ENSEMBL project: rat, ENSRNOG00000002494; mouse, ENSMUSG00000035059). (ii) The specificity of the product amplified. At low concentrations of specific cDNA, PCRs can generate spurious products in the form of 'primer-dimers'. This problem was apparent in some highly diluted samples (not unexpected, given the low numbers of cells contributing to our samples) but was never insuperable. The dissociation curves, used to detect non-specific product, all showed a single peak in the analyses underlying the data presented (Supplementary Data Figure S2, available at *International Immunology* Online). (iii) The choice of reference genes. The presumed stability of expression of the so-called HKGs was validated using the M value [using the geNorm program (41)]. After testing ubiquitin C, YWHAZ, 6PFKc and cirhin as candidate reference genes, the combination of 6PFKc and cirhin was found to give the lowest M value.

The quality of our real-time PCR analysis was emphasized by the remarkable similarity of the data obtained using primers originally designed to amplify separately *Ian11*, *10* and *9* (Fig. 2G–I). Subsequent bioinformatic and *de novo* cloning analysis demonstrated that these three 'genes' in fact constitute a single gene encoding a putative protein product that contains the principal Ian family features in triplicate. Hence, the close similarity of these three sets of PCR data was as it should be.

Ian expression in T cell development

Our results are fully consistent with a role for Ian proteins in the development and/or maintenance of mature T cells. This was already strongly indicated for Ian5 by the positional cloning of the rat *lymphopenia* gene (2, 11), which identified the basis of this mutant trait as a frameshift mutation in the *Ian5* gene such that functional Ian5 protein is absent. Phenotypically, the earliest cells in the T lineage to be definitively affected by the *lyp*/*Ian5* mutation are the mature (medullary) SP thymocytes, which fail to acquire the resistance to spontaneous apoptosis characteristic of the equivalent w.t. cells (18). It is, therefore, interesting to note that expression of *Ian*5 transcripts is elevated before the SP stage in the DP thymocytes, which, nevertheless, show no gross changes in population size or cell properties in *lymphopenic* animals (18, 46). Our observation of

we refer to as Ian9A, Ian9B and Ian9C. Each region possesses three or four G motifs with substitutions appropriate to Ian proteins, as described by Cambot *et al.* (3). G1 (GxxxxGKS) as well as G2 (T) is present in each case; G3 is present as DxxD/Y/E/S; G4 (RxxD) is present in both Ian9C and Ian9B in all three species and in Ian9A the motif is present in human and dog, but is altered to RxxE in rat. A (P)GPHxx(L)LV motif characteristic of the Ian family is present in the Ian9B region, but not in Ian9A or Ian9C. (C) Coiled-coil domains. The likelihood of coiled-coil domains (assessed using Coilscan [GCG] program) in Ian9 of (i) rat, (ii) human and (iii) dog is weak compared with rIan5 (iv) or mIan1 (v).

a similar expression of *Ian5* message in the DP and SP thymocyte compartments of normal rats is consistent with the *in situ* hybridization (ISH) results published by Hornum and colleagues (11), who observed an even distribution of r*Ian5* expression all over the thymus (cortex and medulla), and supports their suggestion that the expression of r*Ian5* may be important (albeit not essential) at all time points during thymocyte development, especially after the differentiation of DN to DP thymocytes. While the function proposed for the Ian5 protein as an anti-apoptotic regulator (7, 8) corresponds with the shortened life span of homozygous *lyp/lyp cf.* w.t. SP thymocytes and peripheral T cells (18, 47), it has no known significance in the DP population. MacMurray and colleagues (2), using northern blots, and Hornum and colleagues (11), using ISH and quantitative PCR, have described reductions in r*Ian5* mRNA levels in the thymi of *lyp/lyp* rats compared with w.t. These reduced levels may be due to instability of the mutated r*Ian5* transcripts (2, 52). In fact, our study shows a slight decrease only in SP thymocytes. A significant difference between our study and the previous ones, however, is the use of isolated thymocyte subsets rather than the analysis of whole thymus tissue, which obviously contains stromal epithelial and myeloid components in addition to lymphoid cells.

As mentioned above, hIan5 has previously been shown to be able to inhibit γ-irradiation-induced apoptosis of Jurkat T cells. However, we have shown here that *lyp/lyp* thymocytes and B cells which are deficient in rat Ian5 do not exhibit increased sensitivity to γ-irradiation when compared with cells from w.t. animals. Clearly, the experimental settings for the two sets of observations are substantially different. While the studies by Sandal and colleagues (7) used *in vitro* manipulation of a transformed T cell line, the present experiments employed freshly prepared *ex vivo* cells from w.t. or mutant animals. Although our test of the simplest prediction deriving from the human *in vitro* studies gave a negative result with respect to the γ-radiation sensitivity of *lyp/lyp* rat lymphoid cells, it will remain important in future to pay attention to T cell apoptosis-induction pathways related to those highlighted by Sandal and colleagues when trying to understand the origins of programmed cell death in the *lyp/lyp* animal model and, more generally, the *in vivo* function of Ian5.

Apart from Ian5, a strong case has also been made for the involvement of Ian1 in T cell development. Poirier and colleagues (1) observed a large increase in *Ian1* expression, at both message and protein levels, in the transition from DP to SP thymocytes in the mouse. Our real-time PCR data confirm this observation in the mouse and demonstrate a similar phenomenon in the rat, which shows a roughly 10- to 20-fold increase in transcript level between DP and SP thymocytes. These data also indicate for the first time a further marked increase (2- to 2.5-fold) in *Ian1* expression between SP thymocytes and peripheral T cells in the rat. It was interesting that similar changes were evident in both w.t. and *lyp/lyp* samples, despite the very different phenotypic status of peripheral T cells in the two cases, i.e. largely G_0 resting cells in the w.t. animals compared with a mixture of recent thymic emigrants and semi-activated cells in *lyp/lyp* animals (47, 53). However, when we used the anti-Ian1 antiserum, we observed that the level of Ian1 protein in LN T cells was ~10-fold greater in cells derived from w.t. as opposed to *lyp/lyp* animals. We also saw no Ian1 in the spleen of *lymphopenic* animals. This impact of the *Ian5* mutation on *Ian1* expression suggests either that Ian1 and Ian5 normally interact, directly or indirectly, in the same biochemical pathway or, alternatively, that Ian1 is a sensitive indicator, like $p27^{kip}$ (48, 54), of the altered activation state of *lyp/lyp* T cells. It is intriguing, too, that Ian9 protein expression shows a similar deficit in *lyp/lyp* T cells (see below).

A novel finding in our analysis is the behavior of rat *Ian7* transcription through T cell development. This *Ian* family member displayed a profile quite similar to that of *Ian1*, although the scale of changes was less marked, with clearly maximal expression in LN T cells. A single difference between these two genes was the higher relative level in DP thymocytes of *Ian7*, while *Ian1* expression in this population is barely different from the DN baseline. Both *Ian1* and *Ian7* carry predicted coiled-coil regions without the presence of a putative transmembrane domain (1, 3). It will be interesting to discover whether these two genes perform overlapping or distinct functions.

Aside from *Ian5*, *Ian1* and *Ian7*, the remaining genes under study, *viz. Ian2*, *Ian3*, *Ian6* and *Ian9*, showed similar and modest changes in expression, with generally their highest levels being detected in DP thymocytes. Indeed, the systematic increase in the expression of all the *Ian* genes (but for *Ian1*) between DN and DP stages is worthy of further investigation. Unlike MacMurray and associates (2), we saw no differences in the expression of r*Ian3* in thymus when comparing normal and *lyp/lyp* rats although, as mentioned above, this may relate to the use of isolated thymocyte populations as opposed to whole thymic tissue.

Our real-time PCR analysis of macrophages and rat *in vitro* cell lines yielded the important general finding that in none of these samples did expression of any of the *Ian* genes reach the (control) level observed in LN T cells. This may point to a particular importance of this gene family for the T cell lineage. The absence of an *Ian2* signal in macrophages was a surprise, given the data of Krücken, Stamm and co-workers (5, 12) who found high levels of *Ian2* in splenic macrophages in mice resistant to *P. c. chabaudi* malaria. This raises the possibility that expression of this gene may depend upon immune stimulation or is confined to highly specific types of cells. Alternatively, species differences may be responsible.

The triplicated Ian9 gene

We have reported our reasons for excluding the genes originally described as *Ian9*, *Ian10* and *Ian11* as separate members of the *Ian* gene family and subsuming them in the newly proposed *Ian9* gene. This reduces the known number of *Ian* genes by two to seven in rat, nine in mouse and eight in man. Our PCR cloning efforts from human and rat cDNAs confirm suggestions from database information that what we have named *Ian9* is a single gene containing triplicated *Ian* sequence elements and encompassing the previously designated *Ian11*, *10* and *9*. A protein with three GTP-binding/GTPase elements is an intriguing functional proposition, and we have now confirmed, by use of an antiserum, that such a protein does exist in rat spleen, thymus and T cells.

It has yet to be determined whether any of the three potential GTP-binding regions of Ian9 is active. Proteins containing multiple sets of guanosine nucleotide-binding domains are highly unusual but a precedent does exist in EngA (and its orthologues), present in all bacterial genomes and *Arabidopsis*. EngA contains two tandem GTP/GDP-binding domains. The crystal structure of the *Thermotaga maritima* EngA family member TM-Der has been solved (55). This study is particularly interesting in the context of the Ian9 'triple' because it presents evidence that the two guanosine nucleotide-binding sites of TM-Der are not equivalent. Thus, while both sites have GTPase activity, the more C-terminal site, GD2, has an exceptionally slow intrinsic rate of release of the product GDP. This suggests that the two GTPase domains make different contributions to the regulation of the protein, presumably through different interactions with extrinsic regulators. We are obviously far from knowing the function of Ian9 but the conserved sequence differences that we have pointed out between the three parts of the molecule suggest that similar subtle biochemical mechanisms may be at work as in TM-Der. We noted that in LN T cells, levels of Ian9 protein are reduced when the cells originate from an Ian5-deficient (*lyp/lyp*) animal. It is noteworthy that the decreases in protein levels of Ian1 and Ian9 observed in *lyp/lyp* as compared with w.t. LN T cells are discordant with the results obtained on mRNA levels by real-time PCR. This raises the possibility of post-transcriptional regulation of Ian1 and Ian9 expression: previously this has been suggested for hIan1 (3).

On a more general note, it will be important to find out whether all the Ian family of polypeptides are engaged in the same cellular activity (e.g. apoptosis) or have diverse roles. The field of known GTPases offers a broad selection of possible functions ranging from the intracellular signaling 'switch', for which ras is a paradigm (51), to involvement in cytokinesis and vesicle trafficking, of which the dynamins and the septins (both carrying coiled-coil domains) are good examples (56, 57).

Acknowledgements

We are grateful to Anne Cooke and Jenny Minson for providing antibodies. We thank Bethan Hughes, Michael Mimmack, Paul Evans, Steven Madison, Zoe Norgate and Jo Vandesompele for valuable advice on the real-time PCR. We thank Len Stephens for reading the manuscript. We thank Trevor Smith and Maureen Hamon for help with protein purification. This study was supported by the Biotechnology and Biological Sciences Research Council (BBSRC) initiative grant no. 202/GAN13085 to G.W.B. and J.R.M. and BBSRC Competitive Strategic Grant funding to the Laboratory of Functional Immunogenetics.

Note added in proof

Our attention has been drawn to the publication by Krűcken *et al.* 2004. *Gene* 341:291., who showed bioinformatic and northern blot evidence for the existance of the Ian9 'triple'.

Abbreviations

7-AAD	7-amino-actinomycin D
BB-DP	diabetes-prone BioBreeding
DN	double negative
DP	double positive
ER	endoplasmic reticulum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GST	glutathione-S-transferase
h	human
HKG	housekeeping gene
Ian	immune-associated nucleotide
ISH	*in situ* hybridization
LN	lymph node
lyp	*lymphopenia*
m	mouse
ORF	open reading frame
6PFKc	6-phosphofructokinase C
r	rat
SIRP	signal regulatory program
SP	single positive
w.t.	wild type

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