

LETTERS TO NATURE

infiltrate. This phenomenon is mediated by the downregulation of the production of TNF- α and by the action of IL-4. TNF- α is a mediator of cell recruitment in inflammatory infiltrates¹⁵ and is a critical cytokine in the pathogenesis of EAE, and probably of multiple sclerosis¹⁶; IL-4 is associated with recovery from disease, and is seen in multiple sclerotic lesions^{17,18}. By selectively targeting the initial trigger for inflammation, the secondary infiltrate can be controlled. This may be relevant for highly selective immune therapy of autoimmune disease in the face of determinant spreading¹³ and nonspecific amplification of the inflammatory response. □

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Augmented humoral and anaphylactic responses in Fc γ RII-deficient mice

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DESPITE its widespread distribution on both lymphoid and myeloid cells, the biological role of the low-affinity immunoglobulin-G receptor, Fc γ RII, is not fully understood. Defects in this receptor or its signalling pathway in B cells result in perturbations in immune-complex-mediated feedback inhibition of antibody production^{1–6}. We now report that Fc γ RII-deficient animals display elevated immunoglobulin levels in response to both thymus-dependent and thymus-independent antigens. Additionally, the effector arm of the allergic response is perturbed in these mice. Mast cells from Fc γ RII^{–/–} are highly sensitive to IgG-triggered degranulation, in contrast to their wild-type counterparts. Fc γ RII-deficient mice demonstrate an enhanced passive cutaneous anaphylaxis reaction, the result of a decreased threshold for mast-cell activation by Fc γ RIII crosslinking. These results demonstrate that Fc γ RII acts as a general negative regulator of immune-complex-triggered activation *in vivo* for both the afferent and efferent limbs of the immune response. Exploiting this property offers new therapeutic opportunities for the treatment of allergic and autoimmune disorders.

Immunoglobulin Fc receptors (FcRs) constitute a family of haematopoietic cell-surface molecules capable of eliciting intracellular signals and triggering numerous effector responses upon crosslinking by their ligand, the antibody–antigen complex⁷. Types I and III FcRs for IgG (Fc γ RI, Fc γ RIII) are expressed primarily on cells of the myeloid lineage and mediate effector functions, including phagocytosis, antibody-dependent cellular cytotoxicity and the release of inflammatory mediators, whereas type II receptors (Fc γ RII) are expressed on both myeloid and lymphoid lineages. *In vitro*, Fc γ RII inhibits the activation signal generated by the B-cell antigen receptor^{1–4}, mediated in part through the recruitment and activation of protein tyrosine phosphatases^{5,6}. Similarly, reconstitution studies have suggested that Fc γ RII can inhibit activation through other, immune receptor tyrosine activation motif (ITAM)-containing receptors⁸. We therefore investigated the *in vivo* consequences of disrupting this inhibitory pathway.

Mice homozygous for Fc γ RII^{–/–} were generated (Fig. 1a,b), which developed normally and were fertile. The absence of messenger RNA (not shown) and protein (Fig. 1c) for Fc γ RIB was demonstrated by northern and western blotting, respectively; the absence of membrane bound Fc γ RIB was demonstrated by flow cytometry of splenic B cells (Fig. 1d).

Expression of Fc γ RIB on effector cells has been shown to depend on their state of activation^{9,10}. Resident peritoneal macrophages (Fig. 1e) and bone-marrow-derived mast cells (Fig. 1f) reveal a >90% reduction in surface staining with the monoclonal antibody 2.4G2 in Fc γ RII^{–/–} mice (Fig. 1e,f), whereas thioglycollate-elicited macrophages retain 80% of their 2.4G2 staining (data not shown).

Development of myeloid and lymphoid cell lineages were not affected by the loss of Fc γ RII, as revealed by flow cytometric analysis of cells derived from the spleen, bone marrow, thymus and peritoneal cavity using a panel of lineage-specific markers (data not shown).

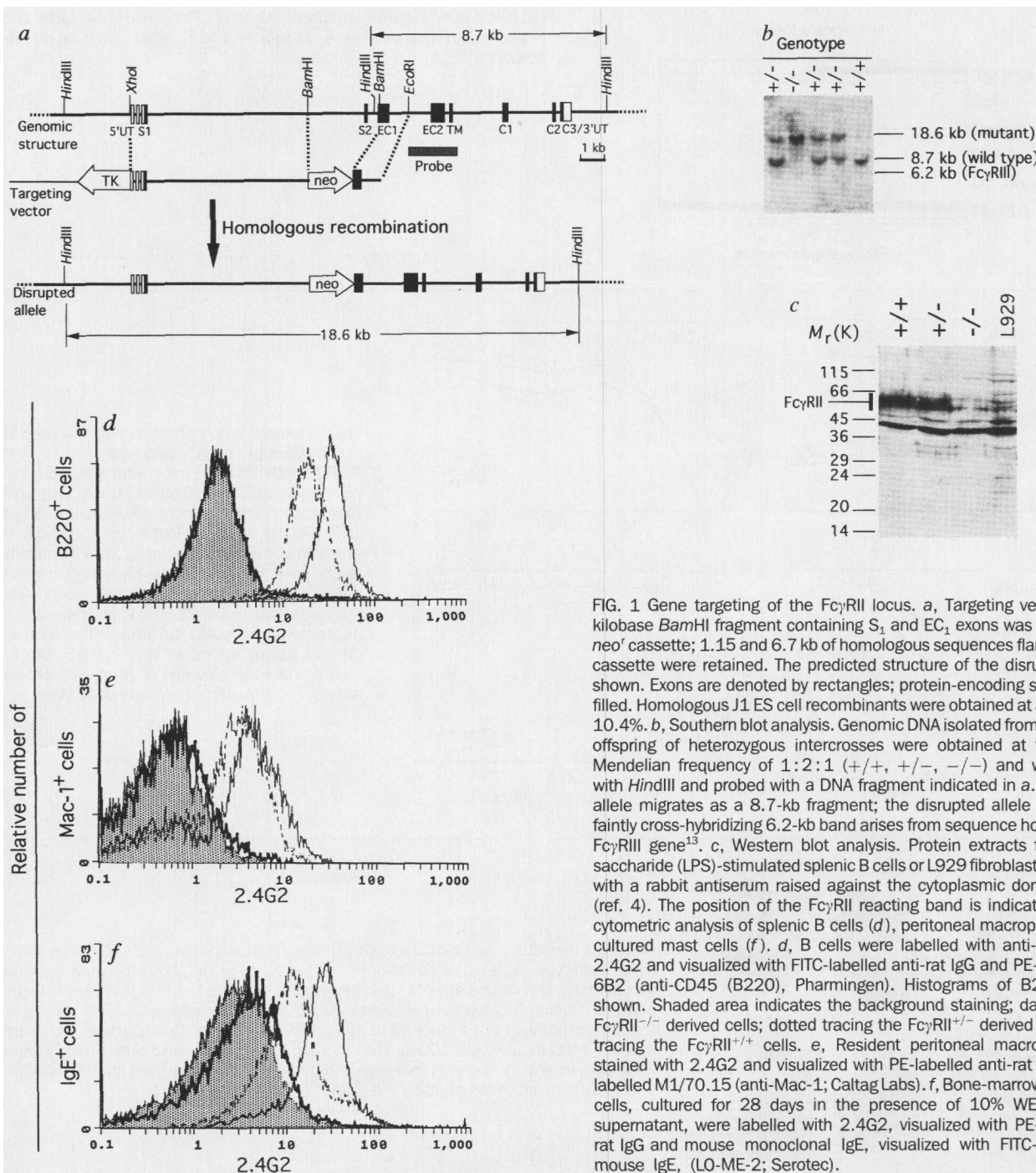
Crosslinking of surface IgM on resting B cells *in vitro* induces a proliferative response only when anti- μ F(ab')₂ is used as the stimulating antibody; intact antibody fails to stimulate B cell receptor-mediated cellular activation¹. In contrast, B cells derived from Fc γ RII^{–/–} proliferate in response to both intact anti- μ and F(ab')₂ (Fig. 2a). The significance of this pathway *in vivo* was determined by immunizing wild-type and Fc γ RII^{–/–} animals with sheep red blood cell (SRBC) or trinitrophenol keyhole limpet haemocyanin (TNP-KLH) as thymus-dependent antigens (Fig. 3a,b) or trinitrophenol lipopolysaccharide (THP-LPS) or TNP-Ficoll as thymus-independent antigens (Fig. 3c,d). In all cases, Fc γ RII^{–/–} showed significantly higher antibody titres than those of wild-type littermates (Fig. 3). This enhancement was evident after 14 days and persisted during the secondary response. Anti-SRBC IgM and IgG plaque-forming cell responses of splenocytes derived from deficient mice were significantly higher than those of wild-type mice at four days after the second immunization (data not shown). Isotype-specific enzyme-linked immunosorbent assays (ELISAs) indicated that the immunoglobulin isotypes that contribute to these differences were IgM, IgG1, 2a, 2b, 3 and IgA (data not shown).

Recent *in vitro* reconstitution studies in mast cell lines has suggested that Fc γ RII could inhibit FcR-triggered activation⁸. Bone-marrow mast cells derived from Fc γ RII-deficient or wild-type mice were stimulated through Fc ϵ RI or Fc γ RIII (Fig. 2b). Degranulation and serotonin release in response to IgE crosslinking were similar in wild-type and Fc γ RII-deficient mice. In contrast, wild-type mast cells show little degranulation or serotonin release above background upon crosslinking of Fc γ RII/III, whereas mast cells from Fc γ RII-deficient mice showed a substantial activation upon the same stimulation (Fig. 2b). Hence, Fc γ RII is sufficient substantially to repress mast-cell activation triggered by Fc γ RIII, but has no direct effect on Fc ϵ RI-mediated activation. Loss of Fc γ RII thus renders these cells sensitive to IgG-mediated triggering. IgG1-mediated passive cutaneous anaphylaxis (PCA)

in wild-type and $\text{Fc}\gamma\text{RII}$ deficient mice was evaluated (Fig. 4) to determine the potential role of $\text{Fc}\gamma\text{RII}$ in modulating this response *in vivo* (Fig. 4). PCA reactions were performed with a series of IgG concentrations, containing amounts ranging from 4 to 0.125 μg . Significant augmentation of this mast-cell-mediated response at a 5–10-fold lower antibody concentration was observed in $\text{Fc}\gamma\text{RII}$ -deficient mice as compared to wild-type littermate controls.

Our results indicate that $\text{Fc}\gamma\text{RII}$ functions as an inhibitory receptor for both B cells and mast cells *in vivo*. Inactivation of this receptor results in a defect in the ability to regulate antibody levels in response to antigenic stimulation for IgM, IgG and IgA, implying a network of interactions which are dependent on IgG immune complexes. Defects in $\text{Fc}\gamma\text{RII}$ function may therefore contribute to the development of autoimmunity by a failure of

feedback inhibition to regulate antibody production, as suggested by the analysis of the 'motheaten' (*me*) mouse, which is defective in protein tyrosine phosphatase 1C (refs. 5, 6, 11, 12). Loss of $\text{Fc}\gamma\text{RII}$ does not, however, result in uncontrolled antibody production after antigenic challenge *in vivo*, implying that additional pathways for maintaining antibody homeostasis are operating. The inhibitory role for $\text{Fc}\gamma\text{RII}$ on mast cells suggests that mast-cell activation by IgE may also be inhibited by recruitment of $\text{Fc}\gamma\text{RII}$, as might occur by IgG blocking antibodies in allergic desensitization. Indeed, co-ligation of $\text{Fc}\epsilon\text{RI}$ to $\text{Fc}\gamma\text{RII}$ renders mast cells anergic (M.O. and J.V.R., unpublished observations) and suggests new therapeutic methods by which mast cells may be repressed. $\text{Fc}\gamma\text{RII}$ -deficient mice are therefore an important tool for the dissection of both afferent and efferent arms of the immune response. □



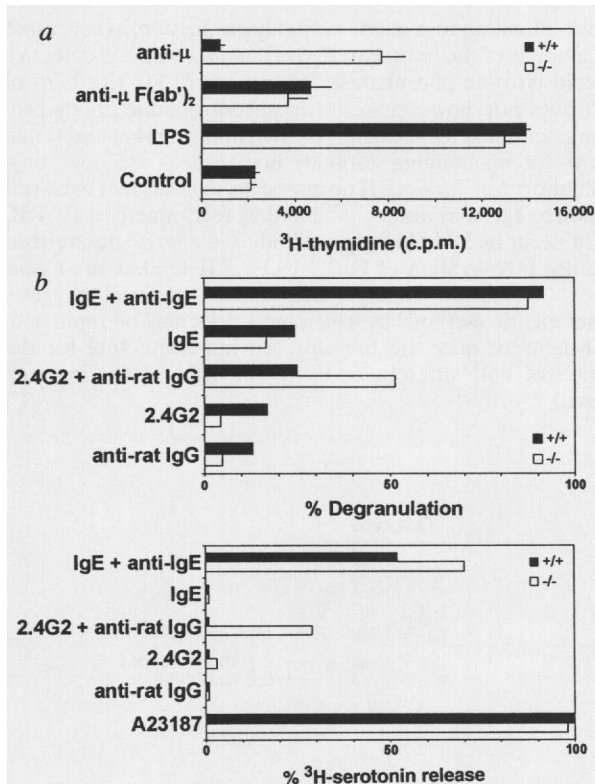


FIG. 2 B-cell and mast-cell stimulation *in vitro*. *a*, B-cell proliferative response to anti-μ stimulation. Splenic B cells were depleted of T cells with anti-Thy 1.2, anti-CD4, anti-CD8 and anti-CD-3 plus rabbit complement. The enriched B cells were stimulated with 5 μg ml⁻¹ LPS, 20 μg ml⁻¹ rabbit anti-mouse IgM (μ-specific; Serotec) or 20 μg ml⁻¹ goat anti-mouse IgM F(ab')₂. Mean [³H]thymidine uptake at 30–48 h and s.d. of triplicated samples for wild-type (+/+) and FcγRII-deficient (-/-) are shown. *b*, Mast-cell stimulation. Cultured mast cells were stimulated with 10 μg ml⁻¹ IgE plus 10 μg ml⁻¹ anti-mouse IgE, 5 μg ml⁻¹ 2.4G2 plus 20 μg ml⁻¹ anti-rat IgG (Cappel) or 5 μg ml⁻¹ ionophore (A23187). Per cent degranulation and [³H]serotonin release were determined 1 h after stimulation as described^{14,15}.

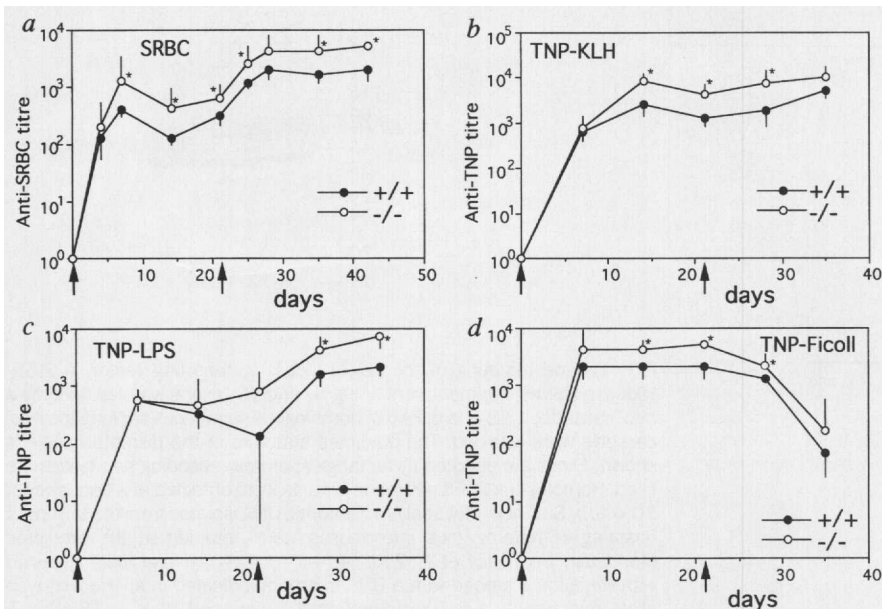


FIG. 3 Humoral response to antigen challenge in FcγRII-deficient mice. Wild-type (+/+) and FcγRII-deficient (-/-) mice were injected intraperitoneally with 2 × 10⁸ SRBC (*a*), 100 μg TNP KLH in complete Freund's adjuvant (*b*), 50 μg TNP-LPS (*c*) or 25 μg TNP-Ficoll (*d*) at day 0 and bled at the indicated times. Serum antibody levels were determined as haemagglutination titres using SRBC (*a*) or TNP-SRBC (*b-d*). Animals were boosted at 21 days to elicit a secondary response (denoted by arrow). The asterisk denotes values significant at *P* < 0.05. Experiments in *a* were repeated 5 times with similar results. Experiments in *b-d* were done twice.

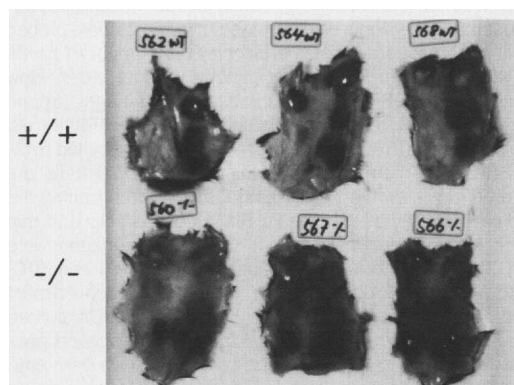


FIG. 4 IgG-mediated passive cutaneous anaphylaxis is greatly enhanced in FcγRII-deficient mice. Wild-type (+/+) and FcγRII-deficient (-/-) mice (in triplicate) were injected intradermally with mouse anti-TNP IgG1 ranging from 4 μg to 0.125 μg per site in steps of 2-fold dilution. The injections were arranged to contain decreasing amounts starting at the upper right (4 μg) to the lower left (0.125 μg) for each animal. One hour later mice were injected intravenously with 100 μg TNP-ovalbumin with 1% Evans blue. Animals were killed 30 min after intravenous challenge and the injected skin analysed for extravasation of blue dye, an indication of vascular leakage¹⁶.

Note added in proof: Since submission of this study, data have been presented¹⁷ which further substantiates the role of FcγRII as a genome inhibitor of ITAM-triggered activation.

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Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes

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DYSREGULATION of apoptosis can result in inappropriate suppression of cell death, as occurs in the development of some cancers¹, or in failure to control the extent of cell death, as is believed to occur in acquired immunodeficiency and certain neurodegenerative disorders, such as spinal muscular atrophy (SMA). Recently, we isolated a candidate gene, encoding neuronal apoptosis inhibitor protein (NAIP)², for SMA. This gene is homologous to two baculovirus inhibitor of apoptosis proteins^{3,4} (Cp-IAP and Op-IAP) and is partly deleted in individuals with type I SMA. A second SMA candidate gene encoding survival motor neuron (SMN), which is contiguous with the NAIP locus on 5q13.1, was also reported⁵. Here we demonstrate a NAIP-mediated inhibition of apoptosis induced by a variety of signals, and have identified three additional human complementary DNAs and a *Drosophila melanogaster* sequence that are also homologous to the baculovirus IAPs. The four open reading frames (ORFs) possess three baculoviral inhibition of apoptosis protein repeat (BIR) domains and a carboxy-terminal RING zinc-finger. The human *iap* genes have a distinct but overlapping pattern of expression in fetal and adult tissues. These proteins significantly increase the number of known apoptotic suppressors.

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To analyse the effect of NAIP on apoptosis, expression plasmids, either alone or encoding full-length NAIP or Bcl-2, were transfected into CHO, Rat-1 and HeLa cells followed by G418 selection. In a second approach, cells were infected with adenovirus, alone or expressing either NAIP, antisense NAIP or LacZ. Expression of NAIP was confirmed in both procedures by western blot analysis and immunofluorescence (data not shown). After infection with the recombinant adenoviruses, CHO cells were induced to undergo apoptosis by serum deprivation with survival rates of 48% (no insert), 51% (LacZ) and 45% (antisense NAIP) at 48 h (Fig. 1a). In contrast, CHO cells infected with adenovirus expressing NAIP demonstrate 78–83% survival. NAIP also induced survival in stably transfected CHO pools, albeit slightly less than is seen in adenovirus-infected cells: 44% of the vector transfectants and 65% of the NAIP transfectants survived at 48 h (Fig. 1b).

Overexpression of NAIP in CHO cells treated with 20 μM menadione (a potent inducer of free radicals⁶) resulted in 20–30% enhancement of survival compared with controls after 24 h (Fig. 1c, d). Overexpression of NAIP also protected menadione-treated Rat-1 fibroblasts from undergoing cell death (Fig. 1e–h). Only 15% of cells infected with LacZ expressing adenovirus were viable at 12 h, in contrast to 80% of NAIP-infected cells, an effect also detected with the pooled Rat-1 NAIP transfectants. Even greater survival was induced by NAIP overexpression at a lower menadione concentration (5 μM), with 98% of pooled NAIP transfectants and 33% of control transfectants viable at 24 h (Fig. 1g, h).

We next assessed the protective effect of NAIP on cells exposed to the cytokine tumour necrosis factor (TNF)-α. HeLa cells treated with TNF-α and cycloheximide were protected from apoptosis when infected with adenovirus expressing high levels of NAIP (139% survival at 48 h) at 48 h, an effect not observed with antisense NAIP (52% survival) (Fig. 1i, j). A similar effect was observed in pooled HeLa transformants. To confirm that cells surviving the apoptotic agents expressed NAIP, immunofluorescence with anti-NAIP antisera was performed on several of the cell death assays. A dramatic enrichment of NAIP-expressing cells was observed, with no alteration noted in the cytoplasmic distribution of NAIP (data not shown). Having demonstrated the apoptotic suppression activity of NAIP, we next sought to identify other human genes that might encode IAP-like proteins.

An initial search of the protein databases at NIH using the Blast program⁷ with IAP sequences from *Cydia pomonella* identified a homologous sequence-tagged site of 273 bp that encodes a potential RING zinc-finger domain located on the X chromosome at Xq24-25. A 240-bp product of polymerase chain reaction (PCR), generated using primers within this sequence, was used to probe a λZap-II human fetal brain cDNA library (Stratagene). Clones were isolated and sequenced, and the resulting cDNA, which encodes a protein with three BIR motifs and a RING zinc-finger, was termed *xiap* (for X-linked IAP). A search of the databases with *xiap* sequences led to the identification of an additional GenBank submission (accession number T96284), containing a 410-bp sequence with moderate homology to the *xiap* BIR domain sequence. A 159-bp fragment amplified by PCR from this sequence was used to screen a λUniZap human liver cDNA library (Stratagene), resulting in the isolation of two new human *iap* cDNAs, *hiap-1* and *hiap-2*. Both contain three BIR motifs and a RING zinc-finger (Fig. 2). In addition to the human IAP proteins, a *Drosophila* genomic DNA sequence (GenBank accession number M96581) was identified with the potential to encode a previously unrecognized IAP protein. By using oligonucleotide primers, we amplified by PCR a fragment of the cDNA sequence from a *Drosophila* adult cDNA library (Stratagene). This was used to probe a *Drosophila* embryonic cDNA library, from which full-length cDNAs were isolated and sequenced. The *Drosophila iap* (*diap*) ORF contains three BIR motifs, a RING zinc-finger, and a longer protein sequence element between the BIR domains, which is more characteristic of the human IAPs than either of the baculoviral viral IAPs (Fig. 2).

By using a human/mouse hybrid cell panel (BIOS Labora-