Contrasting roles of DAP10 and KARAP/DAP12 signaling adaptors in activation of the RBL-2H3 leukemic mast cell line

Nicolas Anfossi[1], Mathias Lucas[1], Andreas Diefenbach[2], Hans-Jörg Bühring[3], David Raulet[4], Elena Tomasello[1] and Eric Vivier[1]

[1] Centre d'Immunologie de Marseille-Luminy, CNRS-INSERM-Université de la Méditerranée, Marseille, France
[2] Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, USA
[3] University of Tübingen, Department of Internal Medicine, Tübingen, Germany
[4] Department of Molecular and Cell Biology and Cancer Research Laboratory, University of California, Berkeley, USA

A common feature of hematopoietic activating immunoreceptors resides in their association at the cell surface with transmembrane signaling adaptors. Several adaptors, such as the CD3 molecules, FcRγ and KARAP/DAP12, harbor intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAM) that activate Syk-family protein tyrosine kinases. In contrast, another transmembrane adaptor, DAP10, bears a YxxM motif that delivers signals by activation of lipid kinase pathways. We show here that the human signal-regulatory protein SIRPβ1 can associate with both DAP10 and KARAP/DAP12 in a model of RBL-2H3 cell transfectants. In association with KARAP/DAP12, SIRPβ1 complexes are capable of inducing serotonin release and tumor necrosis factor (TNF) secretion. By contrast, in the absence of KARAP/DAP12, engagement of SIRPβ1:DAP10 complexes does not lead to detectable serotonin release or TNF secretion by RBL-2H3 transfectants. However, triggering of SIRPβ1:DAP10 complexes co-stimulates RBL-2H3 effector function induced by sub-optimal stimulation of the endogenous FcεRI complex. Therefore, we report here a cellular model in which the association of a cell surface receptor with various signaling adaptors dictates the co-stimulatory or the direct stimulatory properties of the complex.

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1 Introduction

A dynamic balance of activating and inhibitory signals regulates cell activation [1, 2]. Cell surface receptors with intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) belong to a large family of molecules that transduce inhibitory signals upon engagement with their ligands [2, 3]. A striking feature of ITIM-bearing receptors resides in the co-existence of activating counterparts that are highly homologous for the extracytoplasmic domain [4]. Distinct genes clustered in a single locus encode ITIM-bearing molecules and their activating counterparts. Activating counterparts are devoid of ITIM and associate non-covalently with immunoreceptor tyrosine-based activation motifs (ITAM)-bearing adaptors, such as KARAP/DAP12 or FcRγ.

KARAP/DAP12 is a broadly distributed ITAM-bearing polypeptide, and is non-covalently associated with a variety of activating surface receptors [5, 6]. On natural killer (NK) and certain T cell subsets, KARAP/DAP12 associates with the activating forms of MHC class I-specific receptors, such as killer cell Ig-like receptors (KIR) in humans, Ly49 molecules in the mouse and the HLA-E/Qa-1-specific-receptors CD94/NKG2C in both species [7–9]. In human NK cells, KARAP/DAP12 also associates with a natural cytotoxicity receptor (NCR), NKp44 [10]. On monocytes and macrophages, KARAP/DAP12 associates with the signal-regulatory protein SIRPβ1 [9, 11], with the myeloid DAP12-associating lectin-1 (MDL-1) [12], as well as with members of the

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The first two authors contributed equally to this work.

Abbreviations: ITAM: Immunoreceptor tyrosine-based activation motif **ITIM:** Immunoreceptor tyrosine-based inhibition motif **KIR:** Killer cell Ig-like receptors **SIRP:** Signal-regulatory protein **RT:** Reverse transcriptase **TREM:** Triggering receptor expressed on myeloid cells

triggering receptor expressed on myeloid cells (TREM) group [13]. KARAP/DAP12-associated TREM include human/mouse TREM-1 on monocytes and neutrophils, human TREM-2 on monocyte-derived DC [14], and mouse TREM-2a, TREM-2b, TREM-3 on macrophages [15, 16].

DAP10 is a transmembrane signaling adaptor that shares with KARAP/DAP12, a short extracytoplasmic domain with no ligand-binding properties, a transmembrane domain with an Asp residue and an intracytoplasmic domain with a signaling motif [17, 18]. In contrast to KARAP/DAP12, DAP10 does not harbor a cytoplasmic ITAM, but displays an YxxM motif. YxxM motifs couple to phosphatidylinositol 3-kinase (PI 3-K)-dependent pathways, and are typically present in co-stimulatory molecules, such as CD28, ICOS and CD19. DAP10 has been shown to associate with NKG2D, an activating cell surface receptor that is expressed on NK cells, $CD8^+$ T cells and $\gamma\delta$ T cells in humans, as well as NK cells, activated $CD8^+$ T cells, $\gamma\delta$ T cells, $NK1.1^+$ T cells and activated macrophages in the mouse [19, 20]. Consistent with the presence of the YxxM motif in the cytoplasmic domain of DAP10, NKG2D acts as a co-stimulatory molecule in $CD8^+$ T cells and $\gamma\delta$ T cells [21–24].

We have shown that two alternative spliced products of the mouse *Nkg2d* gene, NKG2D-L and NKG2D-S, co-exist in activated NK cells, macrophages and T cells [25]. Whereas NKG2D-L selectively associates with DAP10, NKG2D-S can associate with both DAP10 and KARAP/DAP12 [25]. In association with DAP10, NKG2D serves as a co-stimulatory molecule in cells of the adaptive immune system (*i.e.* $CD8^+$ T cells). However, in association with KARAP/DAP12, NKG2D-S can serve as a primary recognition structure leading to NK cell and macrophage effector functions. Recently, the critical role of KARAP/DAP12 in NKG2D-induced IFN-γ production by NK cells was confirmed, but it was also shown that NK cells from KARAP/DAP12-deficient mice are still capable of *in vitro* lysis and *in vivo* rejection of certain target cells expressing NKG2D ligands [26]. It was also reported that human NKG2D, in association with DAP10, act as a primary triggering receptor for cytotoxicity in NK cells [27, 28]. Taken together these results were interpreted as a demonstration that DAP10 can mediate primary stimulation signals, and not only co-stimulatory signals, in NK cells [26, 28, 29]. To extend the analysis of the functional consequences of the promiscuous association of a single cell surface receptor with DAP10 and/or KARAP/DAP12, we report here the functional analysis of mast cell transfectants where the signal-regulatory proteins SIRPβ1 associate with these different adaptor molecules.

2 Results

2.1 DAP10 forms complexes with SIRPβ1 in RBL-2H3 cells

SIRP belong to a group of three Ig-like cell surface receptors that contain either a 110–113-amino acid (inhibitory SIRPα molecules) or a 5-amino acid intracytoplasmic domain (activating SIRPβ molecules). SIRPα and SIRPβ are encoded by separate genes clustered in the same locus (human chromosome 20p12.2–13 and mouse chromosome 2) [30, 31]. SIRPα1 (SHPS1, Myd-1, BIT, p84, MFR) are ITIM-bearing receptors which recruit the SH2-containing protein tyrosine phosphatases SHP-1 and SHP-2 *in vivo* [32–37]. SIRPβ are highly homologous to SIRPα in their extracellular region, but differ from them by the lack of ITIM, and the presence of a positively charged amino acid residue in the transmembrane domain (Lys) [30]. Several hematopoietic cell types express SIRP such as monocytes, granulocytes, dendritic cell subsets, a subset of $CD19^+$ B cell precursors, $CD33^+$ myeloid progenitor cells and mast cells [32, 38]. We and others have previously reported that SIRPβ1 associates with KARAP/DAP12 in the RBL-2H3 model of mast cell transfectants, in human monocytes, as well as in 293T and Jurkat cells upon transfection [9, 11]. Strikingly, SIRPβ1 can be stably expressed at the surface of RBL-2H3 cells (Fig. 1A), despite the failure to detect endogenous KARAP/DAP12 polypeptides in these cells by immunoblot analysis (Fig. 1B). Thus, it appears that SIRPβ1 can be stably expressed at the surface of RBL-2H3 cells in the absence of association with KARAP/DAP12. All cell surface receptors that associate with KARAP/DAP12 harbor a transmembrane Lys or Arg residue, the positive charge of which is involved in the interaction with the Asp residue in KARAP/DAP12 transmembrane domain [8]. Thus, we reasoned that SIRPβ1 might interact with an endogenous adaptor protein that also harbors an Asp residue in its transmembrane segment. Along this line, DAP10 and KARAP/DAP12 share an Asp residue at an identical transmembrane position (putative P9 position, starting from the junction with the extracellular domain) [6, 17, 18, 39]. Reverse transcriptase (RT)-PCR and immunoblot analysis show that RBL-2H3 cells express endogenous DAP10 polypeptide, as does the macrophage-like J774 cell line, and unlike CHO and 293T cells (Fig. 1B, C). SIRPβ1 proteins were immunoprecipitated from NP-40 lysates prepared from RBL-2H3 cell transfectants, and immunoblotted with anti-DAP10 and anti-KARAP/DAP12-specific antisera. As shown in Fig. 2, SIRPβ1 co-precipitates with DAP10 in both RBL-SIRPβ and RBL-SIRPβ/KARAP cells. As expected, KARAP/DAP12 and SIRPβ1 co-precipitate in RBL-SIRPβ/KARAP but not in RBL-SIRPβ cells (Fig. 2). The

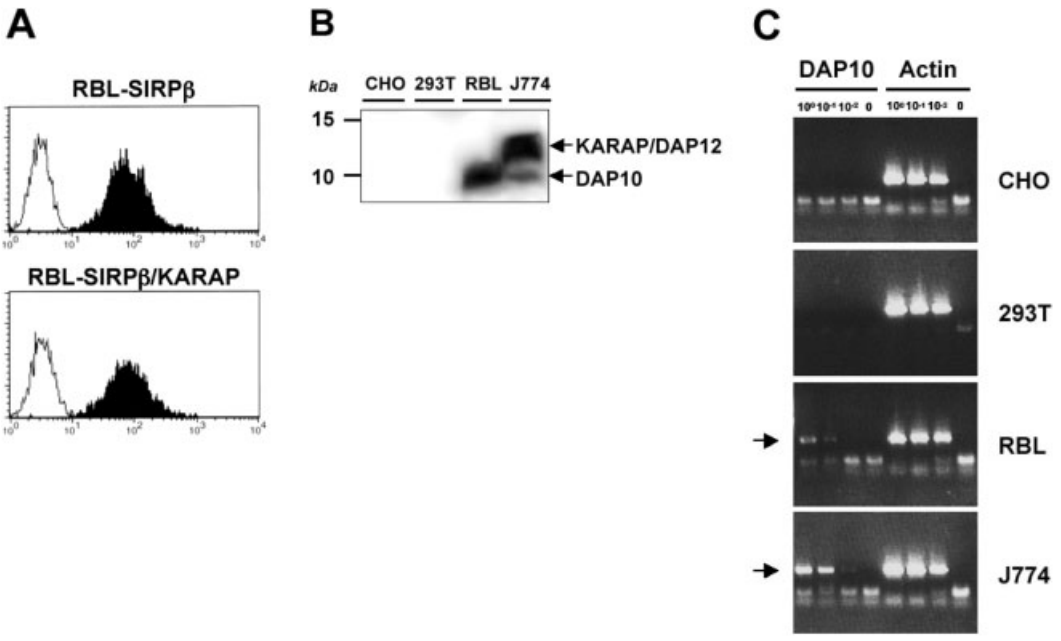


Fig. 1. Expression of SIRPβ1, KARAP/DAP12 and DAP10 in RBL-2H3 cells. (A) The cell surface expression of SIRPβ1 was assessed by flow cytometry on indicated cells using an anti-SIRPβ1 mAb (B4B6, filled histograms). Control stainings were performed using isotype-matched mAb (open histograms). (B) The expression of DAP10 and KARAP/DAP12 polypeptides was assessed by immunoblotting of NP40 lysates prepared from indicated cells, using a mixture of anti-DAP10 and anti-KARAP/DAP12-specific antisera. (C) The presence of DAP10 transcripts in indicated cell types was assessed using semi-quantitative RT-PCR. Amplification of DAP10 and actin transcripts were performed using 5 μl of undiluted (10^0), diluted cDNA (10^{-1}, 10^{-2}) or none (0).

cell surface expression of SIRPβ1 is reportedly up-regulated by the co-transfection of KARAP/DAP12 in Jurkat cell transfectants [11], but this was not observed in RBL-2H3 cells (Fig. 1A). The association of SIRPβ1 with DAP10 offers an explanation for this difference in the regulation of SIRPβ1 expression between RBL-2H3 and Jurkat cells. Indeed, RBL-2H3 and Jurkat cells do not express endogenous KARAP/DAP12 polypeptides but differ in the expression of DAP10. While DAP10 transcripts are present in Jurkat cells, only minute amounts of DAP10 protein have been documented [18], thus suggesting that DAP10 is a limiting factor for the cell surface expression of SIRPβ1 in Jurkat cells.

2.2 SIRPβ1 associates with DAP10 homodimers in RBL-2H3 cell transfectants

The juxta-membrane Cys residues in both KARAP/DAP12 and DAP10 are involved in the formation of disulfide-linked dimers, and it is thus possible that in RBL-SIRPβ/KARAP cells, SIRPβ1 associates with KARAP/DAP12:DAP10 heterodimers. However, no co-precipitation between KARAP/DAP12 and DAP10 could be detected in RBL-2H3 cell transfectants (Fig. 3A). In addition, anti-SIRPβ1 immunoprecipitates prepared from RBL-SIRPβ/KARAP cells only contain DAP10:DAP10 homodimers and KARAP/DAP12:KARAP/DAP12 homodimers, as revealed by two-dimensional diagonal SDS-PAGE (Fig. 3B). Finally, SIRPβ1 associates with DAP10 in absence of KARAP/DAP12 in RBL-SIRPβ cells (Fig. 1C, 2). Therefore, SIRPβ1 can independently associate with KARAP/DAP12 or DAP10 homodimers, depending upon the availability of the corresponding signaling polypeptides. Although not detected in RBL-2H3 cells, we cannot rule out the possibility that KARAP/DAP12 and DAP10 compete for SIRPβ1 association in other cells. Along this line, we failed to detect an association between SIRPβ1 and DAP10 in freshly isolated human monocytes or granulocytes, although the cell surface density of SIRPβ1 on these cells is comparable to that on RBL-2H3 transfectants (data not shown). It thus remains to be investigated whether the association of SIRPβ1 with DAP10 homodimers depends upon the cell type or the status of cell activation. Irrespective of this important issue, SIRPβ1 is the second example of a cell surface receptor with a propensity to associate with DAP10; so far only NKG2D has been reported to associate with this signaling polypeptide [17, 27]. More central to the focus of our study, SIRPβ1$^+$ RBL-2H3 transfectants allowed us

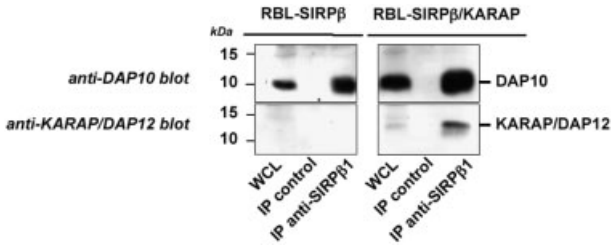


Fig. 2. SIRPβ1 associates with KARAP/DAP12 or DAP10 in RBL-2H3 cells. Immunoprecipitations (IP) of NP-40 lysates prepared from indicated cells were performed using an isotype-matched control mAb or anti-SIRPβ1 mAb (B4B6). Upper panels: Membranes were blotted using rabbit anti-DAP10 antiserum. Lower panels: Membranes were blotted using rabbit anti-KARAP/DAP12 antiserum. WCL: whole cell lysates.

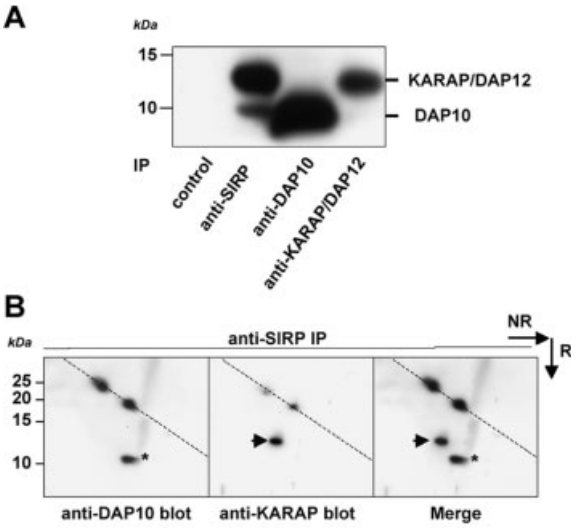


Fig. 3. SIRPβ1 associates with KARAP/DAP12 and DAP10 homodimers. (A) Immunoprecipitations of NP-40 lysates from RBL-SIRPβ/KARAP cells were performed using control mAb, anti-SIRPα/β mAb (P3C4), anti-KARAP/DAP12 or anti-DAP10 antisera as indicated. Samples were separated with a 15% SDS-PAGE and revealed using a mixture of rabbit anti-KARAP/DAP12 and anti-DAP10 antisera. (B) Anti-SIRPβ1 immunoprecipitates (using P3C4 mAb) prepared from RBL-SIRPβ/KARAP NP-40 cell lysates were separated using two-dimensional diagonal 20% SDS-PAGE. Immunoblots were first revealed using anti-DAP10 antisera (left panel), stripped using glycine buffer (50 mM, pH 1.9) for 30 min at RT, and then revealed using anti-KARAP/DAP12 antisera (middle panel). Images from anti-DAP10 and anti-KARAP/DAP12 immunoblots were electronically merged to generate the right panel image using Photoshop software. Dotted lines indicate the diagonal of the two-dimensional gels. NR: non-reducing conditions, R: reducing conditions.

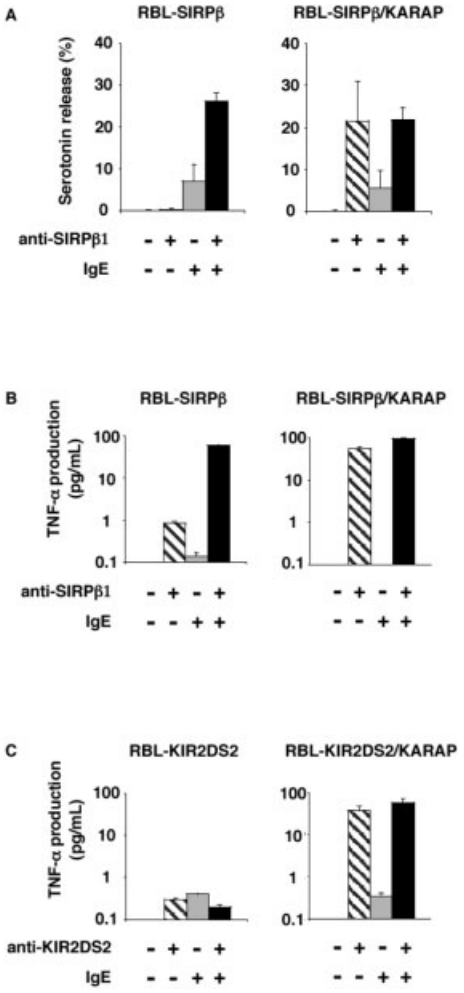
to further analyze the functional properties of SIRPβ1 complexes that contain DAP10 and/or KARAP/DAP12, as cellular models for analyzing the functional consequences of the promiscuous association of a cell surface receptor with these adaptors.

2.3 KARAP/DAP12 and DAP10 transduce distinct biological responses

Consistent with our previous data, anti-SIRP mAb cross-linking did not induce detectable serotonin release in RBL-SIRPβ cells [9] (Fig. 4A). However, engagement of SIRPβ1 in RBL-SIRPβ/KARAP cells led to substantial serotonin release [9] (Fig. 4A). Similar results were obtained when RBL-2H3 cell activation was monitored using TNF secretion (Fig. 4B). In light of the association of SIRPβ1 with endogenous DAP10, these results indicate that the engagement of SIRPβ1:DAP10 oligomers is not sufficient to induce RBL-2H3 cell activation, in contrast to SIRPβ1:KARAP/DAP12 oligomers. These results prompted us to investigate whether the SIRPβ1:DAP10 complex could rather act as a co-stimulatory receptor for ITAM-dependent signals. In this analysis, we took advantage of the endogenous ITAM-dependent FcεRI (α:β:γ_2) complex expressed on RBL-2H3 cells. Cells were stimulated using sub-optimal doses of IgE in the presence or absence of saturating amounts of anti-SIRPβ mAb, and activation was monitored using serotonin release (Fig. 4A) and TNF secretion (Fig. 4B). In RBL-SIRPβ cells, cross-linking of SIRPβ1 in the presence of sub-optimal doses of IgE led to an increase in both serotonin release and TNF secretion as compared to IgE alone. These results show that SIRPβ1:DAP10 oligomers are very efficient co-stimulatory molecules for FcεRI-dependent signals in RBL-2H3 cells, although they are inefficient in triggering RBL-2H3 cell serotonin release or cytokine production in the absence of ITAM-dependent primary stimulation signal. In RBL-SIRPβ/KARAP cells, cross-linking of SIRPβ1 in the presence of sub-optimal doses of IgE led to an increase in TNF secretion as compared to SIRPβ1 or IgE stimulation alone (Fig. 4B). This effect is likely the result of the co-stimulatory properties of the SIRPβ1:DAP10 complex combined with the direct stimulatory effect of SIRPβ1:KARAP/DAP12 engagement.

2.4 KIR2DS2 (CD158j) does not interact with DAP10 homodimers in RBL-2H3 cells

We finally asked whether the promiscuous association with KARAP/DAP12 and DAP10 was a common feature of activating receptors included in oligomeric complexes, and whether it could apply to other activating counterparts of ITIM-bearing molecules, in addition to



◀ *Fig. 4.* Function of DAP10 and KARAP/DAP12 in RBL-2H3 cell activation. (A) RBL-SIRPβ or RBL-SIRPβ/KARAP transfectants were incubated for 45 min at 37°C in the presence or absence (open bars) of anti-SIRPβ1 mAb (B4B6, 3 μg/ml, stripped bars), of mouse IgE antibodies (1:5,000, gray bars), or of both mouse IgE and anti-SIRPβ1 mAb (black bars). Cells were then stimulated with 50 μg/ml of GAM for 30 min before harvesting culture supernatants for serotonin release quantification. Data are expressed as mean ± SEM of duplicate samples and are representative of three independent experiments. In this experiment, 42.5±0.3% and 27.5±2.2% serotonin release were obtained with 1:1,000 IgE mAb stimulation of RBL-SIRPβ or RBL-SIRPβ/KARAP cells, respectively, and correspond to the maximum serotonin release detected in this representative experiment. (B) RBL-SIRPβ or RBL-SIRPβ/KARAP transfectants were incubated for 1 h at 4°C in the presence or absence (open bars) of anti-SIRPβ1 mAb (B4B6, 3 μg/ml, stripped bars), of mouse IgE antibodies (1:100, gray bars), or of both mouse IgE and anti-SIRPβ1 mAb (black bars). Cells were then submitted to an overnight stimulation using a $F(ab')_2$ goat anti mouse, and TNF production in the supernatant was tested with a WEHI-164 cell standard biological assay. In this representative experiment, the maximal TNF production upon IgE treatment (1:4 mAb dilution) was 17.5±1.2 and 21.5±5.0 pg/ml for RBL-SIRPβ and RBL-SIRPβ/KARAP cells, respectively. (C) RBL-KIR2DS2 transfectants were stimulated for 1 h at 4°C in the presence or absence (open bars) of anti-KIR2DS2 mAb (GL183, 10 μg/ml, stripped bars), of mouse IgE antibodies (1:100, gray bars), or of both mouse IgE and anti-KIR2DS2 mAb (black bars). Cells were then submitted to an overnight stimulation using a $F(ab')_2$ goat anti mouse, and TNF production in the supernatant was tested with a WEHI-164 cell standard biological assay.

SIRPβ1. We therefore tested whether activating KIR molecules (KIR-S) that have been shown to associate with KARAP/DAP12 could also bind to DAP10. RBL-2H3 cell transfectants expressing KIR2DS2 alone (RBL-KIR2DS2) or in combination with KARAP/DAP12 (RBL-KIR2DS2/KARAP) have been reported previously [39]. A large increase in KIR2DS2 expression was observed in the presence of KARAP/DAP12, although a substantial expression of KIR2DS2 was detected in RBL-KIR2DS2 transfectants [39]. Despite this limitation in the comparative analysis, both types of cells were stimulated with sub-optimal doses of IgE in the presence or absence of saturating amounts of anti-KIR2DS2 mAb, and assayed for TNF secretion. Consistent with our previous results on serotonin release [39], KIR2DS2 mAb trigger TNF secretion in RBL-KIR2DS2/KARAP, but not in RBL-KIR2DS2 cells (Fig. 4C). Yet, cross-linking of KIR2DS2 has no effect on IgE-induced TNF secretion on RBL-KIR2DS2 transfectants (Fig. 4C). Thus, in contrast to SIRPβ1, KIR2DS2 does not act as a co-stimulatory molecule on RBL-2H3 transfectants, despite the presence of large amount of DAP10 in these cells. In addition, immunoprecipitation experiments failed to detect any physical association of KIR2DS2 with DAP10 in RBL-2H3 cells [40], supporting previous data obtained in KIR2DS2 transfectants of the $DAP10^+$ human NK cell line NKL [27]. Thus, KIR2DS2 does not associate with DAP10 and is unable to transduce any co-stimulatory signal in RBL-2H3 cells.

3 Discussion

The main point of our results is to show that SIRPβ1 can be involved in two distinct types of immune cell activation depending on adaptor usage. In association with KARAP/DAP12, SIRPβ1 can act as a primary recognition structure that leads autonomously to cytokine secretion and serotonin release in RBL-2H3 cells [9]. Consistent with these data, SIRPβ1 cross-linking in Jurkat cell transfectants expressing SIRPβ1 and KARAP/DAP12 leads to tyrosine phosphorylation of extracellular-signal

regulated kinases (ERK-1 and ERK-2) and CD69 up-regulation [11]. In contrast, the association of SIRPβ1 with DAP10 does not confer stimulatory properties to this receptor in RBL-2H3 cells, as demonstrated by the inability of SIRPβ1:DAP10 cross-linking to induce significant serotonin release or TNF secretion. However, the SIRPβ1:DAP10 complex acts as a potent co-stimulatory molecule that enhances the FcεRI ITAM-dependent signals in RBL-2H3 cells, suggesting the existence of co-stimulatory signals in cells of myeloid origin.

These results are consistent with our previous data showing that KARAP/DAP12 contributes to NKG2D signaling in mouse NK cells, and that the association between NKG2D and DAP10 confers to NKG2D co-stimulatory rather primary stimulatory properties [25]. The role of KARAP/DAP12 in NKG2D function was established by the finding that: (i) DAP10$^{-/-}$ NK cells mediated significant residual cytotoxicity against target cells expressing NKG2D ligands, especially when the NK cells were recently activated [41], (ii) KARAP/DAP12$^{-/-}$ NK cells were partially defective in their cytotoxic activity against target cells expressing NKG2D ligands [25], (iii) DAP12$^{-/-}$ NK cells were highly defective in producing IFN-γ in response to NKG2D stimulation [25]. The qualitative distinction between KARAP/DAP12 and DAP10 signals was revealed by T cell studies. Indeed, cross-linking of NKG2D on activated CD8$^+$ T cells that ectopically expressed KARAP/DAP12 led to T cell receptor (TCR)-independent T cell proliferation and cytokine production [25]. In contrast, NKG2D acts as a co-stimulation molecule for the TCR in wild-type activated CD8$^+$ T cells [21–23, 42].

A recent study confirmed that NK cells from KARAP/DAP12-deficient mice are unable to produce IFN-γ upon NKG2D treatment [26]. Similar conclusions were drawn from studies with human NK cells and NK cell lines [27, 28], suggesting that KARAP/DAP12 is critical for cytokine production upon NKG2D triggering, consistent with our results. However, it has also been reported that in association with DAP10 (and in absence of KARAP/DAP12), NKG2D stimulation can lead to full activation of NK cell cytolytic program, as judged by redirected killing assays, killing of targets cells expressing NKG2D ligands upon transfection, as well as a granule release assay [26–28]. The involvement of multiple NK cell receptors-target cell ligands pairs in cytolysis complicates the interpretation of re-directed cytolysis and natural cytotoxicity assays.

However, the reported lack of association between NKG2D and KARAP/DAP12 in human NK cells [27, 28], as well as the induction of human NK cell granule release by plate-bound anti-NKG2D mAb supports a directly stimulatory function of NKG2D via DAP10 for the induction of NK cell cytolytic programs. Serotonin release by RBL-2H3 cells has been used as a model of granule release by NK cells [43, 44]. Our present data show that the triggering of DAP10-dependent pathways in RBL-2H3 cells does not lead to serotonin release. This difference suggests that transducing elements, other than KARAP/DAP12 participate in the signaling by NKG2D:DAP10 complexes in NK cells, but not in RBL-2H3 cells. This hypothesis is consistent with the reported coupling of DAP10 to critical component of NK cell cytolytic programs (*i.e.* PLC-γ2 and Vav1), despite the pharmacological blockade of PI 3-K-catalytic activity [28]. Clearly, the identification of such transducing effector/adaptor molecules remains to be performed and represent a next critical step in the dissection of NKG2D signaling.

Finally, the association of a receptor with various signaling adaptors documented here for SIRPβ1 is highly reminiscent of the variable composition of other oligomeric complexes, such as the TCR. Indeed, the stochiometry of the TCR complex varies with the nature and the state of activation of the T cell subsets. In some T cell lines, the αβ TCR can associate with various combinations of CD3ζ, and FcRγ dimers [45]. In intra-epithelial lymphocytes, FcRγ can substitute for CD3ζ, [46, 47]. In γδ T cells, the CD3δ subunit does not associate with the TCR [48]. Although not formally demonstrated, the combinatorial diversity generated by the association of these distinct signaling components with the TCR complex presumably creates a potential for a diversity of functional outcomes. Variations in the stochiometry of associated transducing components might also occur in NK cells, where several activating receptors (*e.g.* CD16, NKp46, NKp30) can associate with CD3ζ and FcRγ dimers [49–51]. Reinherz and colleagues have suggested earlier that the level of complexity created by the association between a given receptor and distinct signaling adaptors evokes the property of immunoglobulin isotypy [52]. As the immunological functions of the various immunoglobulin isotypes differ, isotypy allows the immune system to vary the response to the same antigen at different times or under different conditions [53]. By analogy, our results suggest that the distinct effector functions linked to cell surface receptors via their association with various combinations of signaling adaptors can be referred as to "isotypy of receptor complexes". It is striking to observe that isotypy of receptor complexes occurs via the reciprocal substitution or homo/heterodimer formation between closely related signaling adaptors. Indeed, DAP10 and KARAP/DAP12 share a similar protein architecture, and are encoded by adjacent genes separated by only 131 and 307 bp in the mouse and human genomes, respectively [54]. Similarly, CD3ζ and FcRγ are

closely related ITAM-bearing polypeptides whose genes are clustered on human and mouse chromosomes 1 [55]. Finally, the CD3γ, CD3δ and CD3ε components are also highly similar structures encoded by genes that are clustered on mouse chromosome 9 and human chromosome 11 [55]. It is therefore tempting to speculate that the ability of related signaling components to participate to the isotypy of receptor complexes has contributed to the emergence of families of signaling adaptor molecules via gene duplication.

4 Materials and methods

4.1 Cells

Stable transfectants of the rat basophilic leukemia cell line (RBL-2H3) have been described earlier. In brief, RBL-KIR2DS2 and RBL-SIRPβ have been transfected with human cDNA encoding KIR2DS2 and SIRPβ1, respectively [9, 44]. RBL-SIRPβ/KARAP and RBL-KIR2DS2/KARAP have been obtained by transfection of human KARAP/DAP12 cDNA in RBL-SIRPβ and RBL-KIR2DS2 clones, respectively [9, 44].

4.2 Semi-quantitative PCR analysis

Total RNA was extracted from indicated cell lines using RNeasy Mini Kit (Qiagen, Valencia, CA). Of the total RNA, 1 μg was used to produce cDNA in a final reaction volume of 50 μl using SuperScript II RT. Contamination by genomic DNA was avoided by treatment with RNase-free DNase (Invitrogen, Paisley, GB). Serial dilutions of cDNA (5, 0.5 and 0.05 μl) were then used to perform PCR in a final volume of 50 μl. Following primers were used: mouse DAP10 forward: ATGGACCCCCCAGGCTACCTC; mouse DAP10 reverse: TCAGCCTCTGCCAGGCATGTT; human DAP10 forward: ATGATCCATCTGGGTCACA; human DAP10 reverse: TCAGCCCCTGCCTGGCAT; actin forward: CATCCATCATGAAGTGTGACG; actin reverse: CATACTCCTGCTTGCTGATCC.

Reaction was performed with the following program: 94°C for 2 min, followed by 40 cycles of 94°C for 50 s, 56°C for 50 s, 72°C for 1 min, and finally 72°C for 7 min.

4.3 Immunoprecipitations

Cells (25×10^6 cells/ml) were harvested using trypsin-EDTA, washed once with PBS and resuspended in NP-40 lysis buffer (1% NP-40, 10 mM Tris, 150 mM NaCl, pH 8) supplemented with a complete protease inhibitors mixture (Roche Molecular Biochemicals, Mannheim, Germany). Samples were precleared three times with protein G-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Immunoprecipitations were performed using anti-SIRPβ1 mAb (B4B6, IgG1; B1D5, IgG2a), anti-SIRPα/β mAb (P3C4, IgG2a) [38], isotypic control mAb (mouse IgG1; Beckman-Coulter, Marseille, France), rabbit anti-DAP10 antiserum [25] or rabbit anti-human KARAP/DAP12 [56], in the presence of protein G-Sepharose beads overnight at 4°C. Samples (15×10^6 cells/lane) were electrophoresed under denaturing conditions onto one-dimensional or two-dimensional diagonal polyacrylamide gels and electroblotted to nitrocellulose membranes (Immobilon, Millipore, Bedford, MA) using a Trans-Blot Semi-Dry (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked overnight with 5% non-fat dry milk in PBS and then incubated with antisera diluted 1:500 in PBS 5% non-fat dry milk. Membranes were washed with PBS Tween 0.05% and incubated with horseradish peroxidase-coupled protein A/G (Pierce, Rockford, IL) diluted 1:10,000 in PBS Tween 0.05%. Proteins were detected using enhanced chemiluminescence reagents (ECL Plus; Amersham Pharmacia).

4.4 Serotonin release

RBL-SIRPβ and RBL-SIRPβ/KARAP transfectants were resuspended at the concentration of 1×10^6 cells/ml in DMEM 10% FCS and incubated for 1 h at 37°C with 2 μCi/ml of 5-hydroxy-[^{3}H]tryptamine creatinine sulfate (Amersham Pharmacia). Cells were then washed twice and incubated for 1 h at 37°C in DMEM 10% FCS medium, as described [44]. RBL transfectants were then resuspended at the concentration of 5×10^6/ml, distributed in 96 well/plates (2×10^5/well) and incubated for 45 min at 37°C with the indicated dilution of mouse IgE (2682-I), in the presence or absence of anti-SIRPβ1 mAb (B4B6, 3 μg/ml) in a final volume of 50 μl. After three washes, cell were then stimulated for 30 min at 37°C with 50 μl/well of goat F(ab')$_2$ anti-mouse (GAM) IgG (Beckman-Coulter, 50 μg/ml). Stimulation was blocked by adding cold DMEM 10% FCS medium, then plates were centrifuged for 5 min at 1,000 rpm and 50 μl of supernatant was mixed with 200 μl of emulsifier safe scintillation liquid (Packard Instruments, The Netherlands) and counted in a LS6000 Beckman counter. The percentage of serotonin release was calculated using as 100% the cpm contained in 50 μl harvested from wells containing the same number of cells and lysed in 100 μl of 0.5% SDS, 0.5% NP-40.

4.5 TNF-release assay

RBL-2H3 transfectants (2×10^5 cells/well in 200 μl) were incubated with mouse IgE (dilutions of 2682-I hybridoma supernatants) in the presence or absence of anti-SIRPβ1 (B4B6) or anti-KIR2DS2 (GL183) mAb for 1 h at 4°C. Cells were then submitted to an overnight stimulation using a F(ab')$_2$ goat anti mouse (GAM; 10 μg/ml; Beckman-Coulter). TNF production in the culture supernatant was then monitored using a WEHI-164 cell biological assay. Briefly, WEHI-

164 cells were incubated 20 h with supernatants from RBL transfectants, and their survival was assessed using MTT (2.5 mg/ml; Sigma-Aldrich, St Louis, MO) test. A standard survival curve was obtained using recombinant TNF-α (Preprotech, London, GB).

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Correspondence: Eric Vivier, Centre d'Immunologie de Marseille-Luminy, CNRS-INSERM-Université de la Méditerranée, Parc Scientifique de Luminy, Case 906, F-13288 Marseille Cedex 09, France
Fax: +33-491269430
e-mail: vivier@ciml.univ-mrs.fr

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