Gene Structure, Expression Pattern, and Biological Activity of Mouse Killer Cell Activating Receptor-associated Protein (KARAP)/DAP-12*

(Received for publication, July 23, 1998, and in revised form, October 19, 1998)

Elena Tomasello‡, Lucia Olesce‡, Frédéric Vély‡, Christophe Geourgeon§, Mathieu Bléry‡, Aziz Moqrich‡, Daniel Gautheret‡, Malek Djabali‡, Marie-Geneviève Mattei, and Eric Vivier‡***

From the ‡Centre d’Immunologie INSERM/CNRS de Marseille-Luminy, Case 906, 13288 Marseille cedex 09, France, the §IBBCP, CNRS UPR 412, 69 367 Lyon cedex 07, France, the ¶CNRS, E.P.91, 13402 Marseille cedex 20, France, the ¶INSERM U491, Faculté de Médecine de la Timone, Marseille, France, and the ¶¶Institut Universitaire de France, Paris, 75005 France

Natural killer cell and T cell subsets express at their cell surface a repertoire of receptors for MHC class I molecules, the natural killer cell receptors (NKRs). NKRs are characterized by the existence of inhibitory and activating isoforms, which are encoded by highly homologous but separate genes, present in the same locus. Inhibitory isoforms express an intracytoplasmic immunoreceptor tyrosine-based inhibition motif, whereas activating isoforms lack any immunoreceptor tyrosine-based inhibition motif but harbor a charged amino acid residue in their transmembrane domain. We previously characterized KARAP (killer cell activating receptor-associated protein), a novel disulfide-linked tyrosine-phosphorylated dimer that selectively associates with the activating NKR isoforms. We report here the identification of the mouse KARAP gene, its localization on chromosome 7 and its genomic organization in five exons. Point mutation and transfection studies revealed that KARAP is a novel signaling transmembrane subunit whose transduction function depends on the integrity of an intracytoplasmic immunoreceptor tyrosine-based activation motif. In contrast to previous members of the immunoreceptor tyrosine-based activation motif polypeptide family, KARAP is ubiquitously expressed on hematopoietic and nonhematopoietic cells, suggesting its association with a broad range of activating receptors in a variety of tissues.

Although NK cells have been initially defined as non-MHC restricted large granular lymphocytes (1), they have now been revealed as controlled in their effector function (i.e. cytotoxicity and cytokine production) by MHC class I molecules expressed at the surface of target cells (2). Multigenic and multiallelic families of NK cell surface receptors for classical and nonclassical MHC class I molecules have been identified in human and mouse. Human NK receptors belong to two structurally distinct groups: the immunoglobulin superfamily for the killer cell inhibitory receptors (KIRs), and the C-type lectins for the CD94/NKG2 heterodimers (3–5). NK receptors are members of the ITIM-bearing receptor family and recruit upon engagement with their cognate ligands, the intracytoplasmic SH2 tandem protein-tyrosine phosphatases, SHP-1 and SHP-2, which terminate NK cell activation programs (6). Isoforms of NK receptors have been identified that do not express intracytoplasmic ITIM and propagate activating rather than inhibitory signals (7–10). In this report, we identified and analyzed the structure and the function of a novel ITAM-bearing molecule, KARAP, which selectively associates with activating isoforms of KIRs, referred to as killer cell activating receptor (KARs). In contrast to KARs that are restricted to NK and T cell subsets, the KARAP transcription pattern revealed its expression on a wide variety of cell types including nonhematopoietic cells, such as neurons. Originally identified in lymphocytes, macrophages, and mast cells (11, 12), ITAM-bearing molecules are therefore more broadly distributed, because, in addition to the wide spectrum of KARAP expression, members of this family can also be encoded by viral genes or involved in platelet activation (13, 14).

EXPERIMENTAL PROCEDURES

Bio-informatics—The “select_hits” program has been developed to extract all potential KARAP sequences in a fully automatic manner, from sequence data bases. This program has been written in C (ANSI) and FORTRAN 77 and processes a data base in four consecutive steps: step 1, for DNA data base, translation of entries in all reading frames and selection of only potential peptides 50–200 amino acids in length; step 2, selection of entries with a predicted ITAM site (Y-X-[IL]-X(6,8)-Y-X-[IL]); step 3, on this subset, selection of entries with a predicted transmembrane region, as described (15); these regions must contain more than 12 amino acids; and step 4, selection of entries with charged amino acids in the transmembrane region (Asp, Glu, Arg, or Lys), a cysteine residue between the transmembrane region and the N terminus, and the ITAM between the transmembrane region and the C terminus.

Cell Transfection—RBL-2H3 cell transfectants expressing p50.2 CAR, i.e. KIR2DS2 (RBL-KAR: RT1b.g50 cells), have been previously described (16). Wild type mouse KARAP and human DAP-12 cDNAs were cloned in pNT-neo using PCR and cDNAs prepared from mouse spleen and human NK cell clone total RNA, respectively (17). Stable mouse KARAP and human DAP-12 transfectants of RBL-KAR cells were established by electroporation and culture in the presence of G418 (1 mg/ml); representative clones were selected for further investigation.

* This work was supported by institutional grants from INSERM, CNRS, and Ministére de l’Enseignement Supérieur et de la Recherche and specific grants from Association pour la Recherche contre le Cancer (to E. V.), the ‘‘Axe Immunologie des Tumeurs’’ de la Ligue Nationale contre le Cancer (to E. T.), ‘‘axe Immunologie des Tumeurs’’ de la Ligue Nationale contre le Cancer (to E. V.), ZEN- ECA (to F. V.), and from the Mobility and Training of Researcher Program (to L. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby identified as ‘‘advertisement’’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AF077829.

†† Member of the Institut Universitaire de France. To whom correspondence should be addressed: Centre d’Immunologie INSERM/CNRS de Marseille-Luminy, Case 906, 13288 Marseille cedex 09, France. Tel.: 33-4-91269444; Fax: 33-4-91269430; E-mail: vivier@ciml.univ-mrs.fr.

‡‡‡ Acknowledgment is due to the GenBank/EBI Data Bank with accession number(s) AF077829.

†††† The abbreviations used are: NK, natural killer; KIR, killer cell inhibitory receptor; ITIM, immunoreceptor tyrosine-based inhibition motif; ITAM, immunoreceptor tyrosine-based activation motif; KAR, killer cell activating receptor; PCR, polymerase chain reaction; mAb, monoclonal antibody; EST, expressed sequences tag; bp, base pair(s).
Point mutation constructs were generated by PCR-directed mutagenesis of the mouse KARAP and human DAP-12 constructs. Each point mutation involved a tyrosine replacement by phenylalanine. Fidelity of the constructs was verified by sequencing.

**Cell Activation and Immunoblotting**—Cells were resuspended at 10 × 10⁶ cells/ml in phosphate-buffered saline and pre-incubated for 10 min at 37 °C. Cells were then incubated for 5 min in the presence or absence of pervanadate (50 μl) prepared as described (18). Cells were immediately lysed in digitonin lysis buffer for 30 min on ice. After removing insoluble material by centrifugation at 12,000 rpm for 15 min, samples were subjected to immunoprecipitation for 2 h using indicated mAbs coupled to protein G-Sepharose beads (Amersham Pharmacia Biotech). Samples were then combined with reducing sample buffer (New England Biolabs) and boiled prior to fractionation on SDS-PAGE and immunoblotting (18). RBL cell serotonin release assays were performed as described previously (16).

**Genomic DNA Cloning**—A 129 mouse genomic DNA library cloned in EMBL3 phage was kindly provided by M. Malissen (Centre d’Immunologie INSERM/CNRS de Marseille-Luminy (CIML)); this genomic DNA was extracted from E14 clone of embryonic stem cells derived from 129/Ola substrain. The screening of the library was performed as described previously by Sambrook et al. (19). Mouse KARAP cDNA was labeled with [α-32P]dCTP using Klenow enzyme and then utilized as radiolabeled probe.

**Reverse Transcription-PCR Analysis**—Mouse DC2.7 T cell hybridoma and mouse BW5147 thymoma cell lines were gifts of B. Malissen (CIML); the mouse T cell line 2M (gift of J.-P. Kinet, Harvard, Boston, MA), IIA1.6 B cell line, and P815 mastocytoma have been previously described (20, 21). Mouse 3T3 fibroblasts, T end (endothelial cell line), 3.19 (perivascular cell line), and 1D (thymic epithelial cell line) were provided by P. Naquet (CIML). The mouse fibroblast cell line LTK was a gift of M. D. Cooper and H. Kubagawa (Birmingham, AL). Mouse cell lines N2A (neuroblastoma) and AZT20 (derived from a pituitary tumor) were provided by G. Rougon (IBDM, Marseille, France). Total RNA was prepared using TRIzol (Life Technologies, Inc.) according to the manufacturer’s protocol. Oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase were used for cDNA conversion in a total volume of 20 μl. PCR were performed in a 50-μl total volume with 5 μl of cDNA template. Used primer were: as follows: KARAP forward (5′-GGCTTCTGAGCCCTTCTTGGTC-3′) and KARAP reverse (5′-CTGGTGGTTGAGGCTACTGTA-3′). PCR was performed as follows: 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min for a total of 26 cycles. β-Actin was used as a control template with the following primers: β-actin forward (5′-TACCACTGGCATGATGGACT-3′) and β-actin reverse (5′-TCCTTTGCATCTGGGCAAT-3′). DNA was subsequently denatured in a NaOH 0.4% solution and transferred under alkaline conditions onto a Hybond N membrane. This membrane was hybridized with mouse KARAP cDNA, previously labeled with [α-32P]dCTP and then revealed by autoradiography. For β-actin, a mouse β-actin PCR product was labeled with [α-32P]dCTP and used as probe.

**Fluorescence in Situ Hybridization**—Metaphase spreads were prepared from a WMP male mouse, in which all autosomes except chromosome 19 were in the form of metacentric Robertsonian translocations. Concanaavalin A-stimulated lymphocytes were cultured at 37 °C for 72 h with 5-bromo-2-deoxyuridine (60 μg/ml) added to the final 6 h of culture. The KARAP λ phage was biotinylated by nick translation with the KARAP λ phage was biotinylated by nick translation with 16-UTP according to manufacturer’s protocol (Boehringer Mannheim). Hybridization to chromosome spreads was performed with standard protocols (22). The biotin-labeled DNA was mixed with hybridization solution at a final concentration of 10 μg/ml and used at 150 ng per slide. Before hybridization, the labeled probe was annealed for 45 min at 37 °C with a 150-fold excess amount of murine Cot-1 DNA (Life Technologies, Inc.) to compete with nonspecific repetitive sequences. The hybridized probe was detected by means of fluorescein isothiocyanato-conjugated avidin (Vector Laboratories). Chromosomes were counterstained and R-banded with propidium iodide diluted in anti-fade solution at pH 11.0.

**RESULTS**

**Bio-informatic Identification of the Mouse KARAP Gene as EST AA734769**—We previously identified KARAP as a 12-kDa polypeptide that selectively associates with KARs (23). Several biochemical observations revealed that KARAP shares striking similarities with members of the ITAM-bearing polypeptide family. First, KARAP is expressed as disulfide-linked dimer. Second, KARAP associates with KARs, which contain a charged amino acid residue in their transmembrane portion, similarly to ITAM-bearing polypeptides and their associated receptors (e.g. TCR, BCR, FceRI, and FcγRs) (24). Third, KARAP co-precipitates with a protein-tyrosine kinase activity and is tyrosine-phosphorylated, a critical signaling feature shared with ITAM-bearing polypeptides. We reasoned that these biochemical characteristics would enable us to elaborate a bio-informatic strategy in an attempt to identify the KARAP cDNA. The “select_hits” program was then generated as indicated under “Materials and Methods” and applied on ESTs. As reported in Table I, 467 potential candidates were detected. Among these hits, 131 were discarded because they present significant similarity with protein sequences of known function. On the subset of 336 hits with unknown function, we subsequently searched for the most similar entries with the CD3ζ and FceRI polypeptides by using the FASTA program set on default parameters (25). The most similar entry to CD3ζ and FceRI was the mouse EST AA734769. Table I indicates that four other ESTs were found to share high pairwise similarities (at least 96% identity) with AA734769. PCR primers were generated to obtain the full-length coding sequence corresponding to AA734769 from mouse spleen RNA. The nucleotide sequence corresponding to this PCR product revealed a 342-bp open reading frame (Fig. 1). The predicted amino acid analysis indicated the features of a type I transmembrane protein, including a 27-amino acid leader peptide (Met1 to Ala18), a 16-amino acid extracytoplasmic domain (Gln1 to Gly56), a 24-amino acid transmembrane domain (Val57 to Gly66), and a 47-amino acid long intracytoplasmic domain (Arg41 to Arg87). Consistent with our bio-informatic search strategy, the predicted mouse KARAP is a 9.6-kDa molecular mass mature polypeptide that contains N-terminal cysteine residues, a charged transmembrane amino acid (Asp85), and a typical ITAM based on Tyr65 and Tyr76: Y65QELQGQRPEVY76SDLN. Computer-based sequence alignment revealed a 73% amino acid identity of the AA734769 predicted protein with the recently described human DAP-12 ITAM polypeptide, which was found to associate with KARs (KIR2DS2) in transfected cells (26). A rabbit antisemur (KPI) was raised against an antigenic ITAM stretch present in human DAP-12 intracytoplasmic domain (I57AETESPYQELQGQRPEVYSDLN), which is highly homologous to that present in AA734769 (I57ETESPYQELQGQRPEVYSDLN) (28). Immunoblotting of KAR immunoprecipitates prepared from human interleukin-2 activated NK cells revealed a 12-kDa band reactive with KPI antibodies (data not shown). Taken together, these results show that DAP-12 corresponds to human KARAP and that AA734769 corresponds to mouse KARAP.

**Structure Function Analysis of Mouse and Human KARAPs**—We originally reported that the stable transfection of RBL cells with KAR cDNAs (i.e. KIR2DS2) leads to cell surface expression of a nonfunctional KAR molecule (16). RBL-
KAR transfectant cell lines were then transfected with a set of cDNAs corresponding to wild type or mutant mouse KARAP (AA734769) and human DAP-12. RBL transfectants coated with mouse anti-KIR2DS2 mAb or mouse IgE as a positive control were stimulated by the addition of goat anti-mouse antibodies in a standard serotonin release assay. As shown in Fig. 2A, both wild type mouse KARAP (mKARAP) and human DAP-12 (hDAP-12) reconstituted the activating property of KAR in RBL transfectants. Consistent with these results, anti-phosphotyrosine immunoblots performed on anti-KAR immunoprecipitates prepared from RBL-KAR + mKARAP transfectants revealed that mKARAP co-precipitates with KAR as a tyrosine-phosphorylated protein upon pervanadate stimulation (Fig. 2B), as well as upon KAR engagement (data not shown). In contrast, single Tyr(Y65F) (Fig. 2B), the mouse KARAP gene spans 3.56 kilobases (from the start methionine residue to the stop codon) and is divided into 9 exons of varying length. The leader peptide (dash), 27 amino acids; extracellular region (double line), 16 amino acids; transmembrane region (single line), 24 amino acids; intracytoplasmic region, 47 amino acids. The dot indicates a stop codon.

Genomic Organization and Chromosomal Localization of Mouse KARAP Gene—Southern blot analysis revealed that mouse KARAP is a single gene, as is its human DAP-12 ortholog (data not shown). An 18-kilobase mouse KARAP genomic clone was isolated by screening a 129 mouse λ phage library with mouse KARAP cDNA. This KARAP phage served as a probe for determining the chromosomal localization of the mouse KARAP gene using fluorescence in situ hybridization. A total of 50 metaphase cells were analyzed, and 90% of them showed specific fluorescent signal in the B band of murine chromosome 7. These results are consistent with the localization of human DAP-12 to chromosome 19q13, a region syntenic to mouse chromosome 7. The structure of the mouse KARAP gene was then determined by generating a set of primers spanning the corresponding cDNA sequence (Fig. 3A). As shown in Fig. 3B, the mouse KARAP gene spans 3.56 kilobases (from the start methionine residue to the stop codon) and is divided into 9 exons of varying length. The leader peptide (dash), 27 amino acids; extracellular region (double line), 16 amino acids; transmembrane region (single line), 24 amino acids; intracytoplasmic region, 47 amino acids. The dot indicates a stop codon.

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molecules have been documented only within the hematopoietic compartment. In contrast, reverse transcription-PCR analysis of KARAPtranscripts can be detected not only in NK cells (not shown) or in T and B lymphocytes but also in mast, endothelial, and epithelial cell lines as well as neuronal cells (Fig. 4).

DISCUSSION

A feature of the ITIM-bearing molecules such as KIRs is the existence of activating counterparts devoid of intracytoplasmic ITIMs and characterized by the presence of a charged amino acid residue in their transmembrane domain (28). We report here that the activating isoforms of KIRs associate with KARAPs, which function as transducing polypeptides coupling the engagement of KAR to the signaling machinery leading to RBL degranulation. The model of KAR reconstitution in RBL cells is directly relevant to the activation of lymphocyte-mediated cell cytotoxicity by KARs, because NK cells and cytotoxic T lymphocytes express intracytoplasmic granules that undergo regulated exocytosis upon interaction with target cells. Our molecular cloning strategy also revealed that mouse KARAP is the ortholog of human DAP-12 and is highly similar to the AA242315 mouse EST, which was identified during the course of our studies (26). We will thus refer to KARAP/DAP-12 for both human and mouse polypeptides hereafter.

KARAP/DAP-12 is a canonical ITAM-bearing polypeptide closer to CD3ζ and FcεRIγ than to any other ITAM-bearing molecule. CD3ζ, CD3δ, CD3ε, Igα, and Igβ polypeptides harbor an Ig-like extracytoplasmic domain, whereas FcεRIβ spans four times the membrane. In contrast KARAP/DAP-12 as well as CD3ζ and FcεRIγ are single transmembrane pass polypeptides that express a very short extracytoplasmic domain (5 amino acids for FcεRIγ, 9 amino acids for CD3ζ, and 16 amino acids for mouse KARAP/DAP-12), suggesting the absence of a specific extracytoplasmic ligand. CD3ζ and FcεRIγ are both present on mouse and human chromosome 1, whereas KARAP/DAP-12 are present on mouse chromosome 7 and human chromosome 19. It is thus tempting to speculate that genes encoding for ITAM-bearing molecules and their associated receptors might have originally evolved from the same locus, as FcγRI, FcεRIα, and FcεRIγ as well as KARAP/DAP-12 and KARs are respectively present in a close chromosomal vicinity.

CD3ζ, FcεRIγ, and other ITAM-bearing polypeptides have been shown to be involved both in the assembly and in the transducing properties of oligomeric complexes (11, 12). In contrast, the cell surface expression of KARs appears independent of KARAP/DAP-12 association in stable transfectants of RBL and Ba/F3 cell lines (16, 26). However, KARAP/DAP-12 also form noncovalent complexes with various activating counterparts of ITIM-bearing molecules, such as the lectin-like MHC class I receptors, NK2G2/C9D94 heterodimers in human as well as Ly-49H and Ly-49D homodimers in the mouse (29–31). The cell surface expression of these lectin-like receptors requires association with KARAP/DAP-12 for efficient cell surface expression (30, 31). It thus appears that KARAP/DAP-12 is required for the stable cell surface expression of lectin-like dimers, in contrast to Ig-like molecules. The expression of KARAP/DAP-12 in CD3ζ-deficient T cell lines, BWδ and 2M2, reveals that KARAP/DAP-12 cannot substitute for CD3ζ or FcεRIγ for the assembly of the TCR complex, because KARAP/DAP-12 association with the TCR components would have restored its cell surface expression (21). Reciprocally, the absence of KAR function in RBL-KAR transfectants as well as analysis of anti-KAR immunoprecipitates have shown that despite its transmembrane charged amino acid residue, KAR cannot associate with FcεRIγ in RBL cells (16). Therefore, the selective interaction between KARAP/DAP-12 and its associated receptors occurs through the interaction between charged transmembrane amino acid residues (26, 29–31) but is ensured by specific amino acid interaction motifs yet to be determined.
Similarly to other ITAM-bearing polypeptides, the integrity of KARAP/DAP-12 ITAM is mandatory to its transducing properties (32), as judged by receptor-induced RBL serotonin assays (Fig. 2A). The SH2-containing protein-tyrosine kinases ZAP-70 and Syk are the only effector molecules that associate in vivo with the phosphorylated form of ITAM-bearing molecules (33). The requirement of both tyrosines residues for complete KARAP/DAP-12 transducing function is consistent with the structure of ZAP-70 tandem SH2 domains, which dictates the simultaneous recruitment of both ITAM phosphotyrosines to a ZAP-70 SH2 tandem (34, 35). The recently reported in vitro association between KARAP/DAP-12 phosphorylated peptides and ZAP-70/Syk is consistent with our in vivo point mutation analysis (26). More surprisingly, no in vivo phosphorylation of KARAP/DAP-12 can be detected when a single KARAP/DAP-12 tyrosine is mutated (Fig. 2B). Similarly, analysis of Y-F Igβ single point mutants has revealed that Igβ tyrosine phosphorylation is dependent on the presence of both Igβ ITAM tyrosines (36). Therefore, it is possible that for some ITAM-bearing molecules, such as KARAP/DAP-12 and Igβ, the phosphorylation of ITAM tyrosines residues is the almost ubiquitous expression of KARAP, as judged by its transcription pattern (Fig. 4). Because physiological ligands of KARAP/DAP-12-associated receptors are still not fully characterized, the implications of KARAP/DAP-12 phosphorylation patterns remain to be unveiled.

Acknowledgments—We thank Bernard Malissen (CIML) for continuous encouragement and insightful advice, as well as Corinne Béziès La Fosse (CIML) for graphic expertise.

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33. We thank Bernard Malissen (CIML) for continuous encouragement and insightful advice, as well as Corinne Béziès La Fosse (CIML) for graphic expertise.

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