2F1 antigen, the mouse homolog of the rat "mast cell function-associated antigen", is a lectin-like type II transmembrane receptor expressed by natural killer cells

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Inhibitory lectin-like receptors expressed on the surface of hematopoietic cells are critically involved in regulation of their effector functions. Here we report that a novel mAb specific for mouse NK cells, 2F1, recognizes the mouse homolog of the mast cell function-associated antigen (MAFA), an inhibitory lectin-like transmembrane receptor expressed on rat mast cells. The 2F1 antigen (2F1-Ag) and rat MAFA are structurally highly conserved and contain a cytoplasmic motif similar to the immunoreceptor tyrosine-based inhibitory motif that is presumably utilized for inhibitory signaling. We also identified a human homolog that is closely related to the rodent MAFA/2F1-Ag proteins. Like rat MAFA, 2F1-Ag is probably encoded by a single gene, which exhibits relatively little polymorphism. Strikingly, while rat MAFA is considered a mast cell antigen, we have been unable to detect cell surface expression of 2F1-Ag by mouse mast cell lines, bone marrow-derived mast cells, or peritoneal mast cells. Furthermore, mouse bone marrow-derived mast cells were devoid of 2F1-Ag mRNA. Instead, we find that approximately 40 % of mouse NK cells express 2F1-Ag. Thus, MAFA/2F1-Ag may modulate immunological responses on at least two different cell types bridging the specific and innate immune system.

Key words: NK cell / 2F1 antigen / Inhibitory receptor / Mast cell / Lectin

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1 Introduction

Inhibitory receptors expressed on the surface of hematopoietic cells regulate immunocyte effector function through interaction with specific ligands. A paradigm is the recognition of MHC class I molecules by NK cells. MHC class I binding by inhibitory NK cell receptors results in the down-regulation or termination of NK cell activation, *i.e.* the cytolytic activity against tumor or lymphoblast target cells or the production of inflammatory cytokines [1–3].

Three families of inhibitory class I-specific NK cell receptors have been described. The CD94/NKG2 receptors are related to C-type lectins and have been identified in both the human and in rodents [4–6]. The Ly49 receptors are also lectin like, and have been identified in functional form only in rodents [7, 8]. The Ig-KIR receptors are members of the Ig superfamily (IgSF) and have been identified only in humans [9, 10]. All of these inhibitory receptors contain a cytoplasmic ITIM (immunoreceptor tyrosine-based inhibitory motif) with the conserved amino acid sequence I/VxYxxL/V. Upon MHC I engagement and tyrosine phosphorylation of the ITIM, intracellular tyrosine protein phosphatases such as SHP-1 are recruited to the ITIM [11]. The subsequent inhibitory signal cascade leads to the abrogation of the NK cell activation program induced by a variety of stimuli [12].

In addition to the class I-specific receptors expressed predominantly by NK cells, inhibitory receptors expressed by B cells, monocytes and mast cells have been described. LIR/ILT receptors are IgSF members expressed by monocytes, B cells and some NK cells in humans [13, 14], and PIR receptors are IgSF members expressed by B cells and monocytes in mice [15]. At least two inhibitory receptors have been identified on mast cells, the IgSF member gp49B1 on mouse mast

[I 18851]

Abbreviations: BMMC: Bone marrow-derived mast cells **ITIM:** Immunoreceptor tyrosine-based inhibitory motif **MAFA:** Mast cell function-associated antigen

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cells [16], and a lectin-related type II transmembrane receptor called "mast cell function-associated antigen" (MAFA) expressed by the rat mast cell line RBL-2H3 as well as rat lung mast cells [17, 18]. Aggregation of FcεRI, the high-affinity receptor for IgE on mast cells, leads to activation and secretion of inflammatory mediators, but activation is prevented if gp49B1 [19] or MAFA [20] is co-aggregated with FcεRI. Interestingly, gp49B1 is also expressed on activated NK cells [21, 22].

Here, we have employed a novel mAb to clone a cDNA encoding the presumptive mouse homolog of rat MAFA. The tissue distribution of the mouse receptor, called 2F1-Ag, suggests that it functions primarily if not exclusively in NK cells rather than in mast cells. Implications for the immunological function of MAFA/2F1-Ag are discussed.

2 Results

2.1 The 2F1-Ag is the mouse homolog of the rat MAFA protein

The derivation of the 2F1 hamster mAb reactive with mouse IL-2 activated NK cells will be described elsewhere (L. C. and D. H. R., in preparation). Representative data in Fig. 1A demonstrate that 40–50 % of NK1.1$^+$ splenocytes from B6 mice express the 2F1 antigen (2F1-Ag), whereas few if any NK1.1$^-$ splenocytes express the antigen detectably. A similar fraction of IL-2-activated NK cells [lymphokine-activated killer (LAK) or adherent lymphokine-activated killer (A-LAK) cells] stained with 2F1-mAb (data not shown). Since NK cells from C.B-17 SCID mice also reacted with mAb 2F1 (data not shown), we employed a modification of the COS cell transfection cloning method of Seed and Aruffo [23] to isolate a cDNA encoding the 2F1-Ag from a C.B-17 SCID LAK cell cDNA expression library. After three rounds of expression and enrichment, three 1.4-kb cDNA clones (clones # 3, # 20, # 34) were isolated that gave rise to 2F1 reactivity. Fig. 1B shows that COS-7 cells transfected with cDNA clone # 3, but not control-transfected COS-7 cells, readily stain with mAb 2F1. The transfected COS-7 cells did not stain with any other available NK cell-reactive mAb that we tested (data not shown).

Figure 1. Cell surface expression of 2F1-Ag by NK cells and transfected COS-7 cells. (A) Nylon wool-passaged, 2.4G2 preincubated spleen cells from B6 mice were stained with PK136-biotin/SA-TC and 2F1-FITC. (B) COS-7 cells were transfected with pME18S containing an irrelevant cDNA (dotted line) or cDNA clone # 3 (filled curve) and stained with 2F1-FITC.

Sequence analysis revealed that the three cDNA clones are identical and contain an open reading frame (ORF) of 564 bp predicting a protein of 188 amino acids (Fig. 2). The coding region sequence is very similar (86 % nucleotide identity) to the ORF sequence of rat MAFA [17]. The predicted amino acid sequences of mouse 2F1-Ag and rat MAFA also display a high degree of sequence identity (80 %), suggesting that the sequences represent homologs. A related human cDNA ($p = 2.4 \times 10^{-48}$ versus rat MAFA) was also identified by a search of human expressed sequence tag (EST) in the XREFdb website. The corresponding clone was sequenced and found to contain an ORF of 567 bp, predicting a polypeptide of 189 amino acids. This apparent human MAFA homolog is 49 % identical to both rat MAFA and mouse 2F1-Ag at the amino acid level.

Like rat MAFA, 2F1-Ag and the related human protein are predicted to be type II transmembrane proteins with a predicted unglycosylated molecular mass of 21–22 kDa (Fig. 2 and data not shown). They have a very similar

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2F1-Ag      MADSSIYSTLELPEAPQVQDESRWKLKAVLHRPHLSRFAMVALGLLTVILMSLLMYQRIL
rat MAFA    MADNSIYSTLELPAAPRVQDDSRWKVKAVLHRPCVSYLVMVALGLLTVILMSLLLYQRTL
human MAFA  MTDSVIYSMLELPTATQAQNDYGPQQKSSSSRPSCSCLVAIALGLLTAVLLSVLLYQWIL
            *.*  *** **** * . * .   . *.   **  *    .****** .*.*.*.**  *
               ITIM-like                           transmembrane

2F1-Ag      CCGSKDSTCSHCPSCPILWTRNGSHCYYFSMEKKDWNSSLKFCADKGSHLLTFPDNQGVK
rat MAFA    CCGSKGFMCSQCSRCPNLWMRNGSHCYYFSMEKRDWNSSLKFCADKGSHLLTFPDNQGVN
human MAFA  CQGSNYSTCASCPSCPDRWMKYGNHCYYFSVEEKDWNSSLEFCLARDSHLLVITDNQEMS
            * **    *. *  **  * . * ******.* .****** **  . ****   *** .

2F1-Ag      LFGEYLGQDFYWIGLRNIDGWRWEGG-PALSLRILTNSLIQRCGAIHRNGLQASSCEVAL
rat MAFA    LFQEYVGEDFYWIGLRDIDGWRWEDG-PALSLSILSNSVVQKCGTIHRCGLHASSCEVAL
human MAFA  LLQVFLSEAFCWIGLRNNSGWRWEDGSPLNFSRISSNSFVQTCGAINKNGLQASSCEVPL
            *   .. . * *****   ***** * *     * .** .* **.*.. **.****** *

2F1-Ag      QWICKKVLY
rat MAFA    QWICEKVLP
human MAFA  HWVCKKVRL
            .*.* **
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Figure 2. Comparison of 2F1-Ag, a human homolog, and rat MAFA amino acid sequences. The amino acid sequences are deduced from the cDNA for 2F1-Ag (Genbank accession number AF097357), a human homolog (Genbank accession number AF097358, EST clone obtained from I.M.A.G.E. Consortium, Genbank accession number AA188327), and rat MAFA [17], Genbank accession number X79812). Identical residues are marked with asterisks and similar residues with dots. The shared cytoplasmic ITIM-like motif and the predicted transmembrane regions are underlined. Potential N-linked glycosylation sites are also underlined.

N-terminal cytoplasmic domain of 37 amino acids harboring the consensus sequence IYSxLELP. This motif is similar to that of the functional ITIM (V/IxYxxL/V) in the cytoplasmic tail of inhibitory receptors expressed by NK cells and other lymphocytes. The cytoplasmic domains of the MAFA-related proteins are particularly similar to the corresponding regions of the human lectin-like inhibitory NKG2A receptor [24], with conserved residues extending the ITIM-like motif (IYSxLxLP).

The MAFA-related proteins all contain a 133 (134 in human) amino acid extracellular region including a "NK cell domain" (NKD) [25] which is related to carbohydrate recognition domains (CRD). Motifs (CYYF, WIGL) conserved in the NKD of most other lectin-like receptors on NK cells like CD94, CD69, NKG2A/B, NKR-P1 and Ly49 family members are shared by the MAFA/2F1-Ag proteins. Residues critical for calcium-dependent carbohydrate binding (both sites 1 and 2 as defined by Weis et al. [25]) are missing in MAFA/2F1-Ag. The mouse 2F1-Ag sequence, like rat MAFA, contains two potential N-glycosylation sites, while the human sequence contains four N-glycosylation motifs.

2.2 The 2F1-Ag is encoded by a single, non-polymorphic gene

Southern blots of genomic DNA from different inbred mouse strains were hybridized with a cDNA probe corresponding to the 3' half of the coding region and the 3' UTR of the mouse 2F1 cDNA clone (Fig. 3). All six strains tested, representing four independent genetic backgrounds, exhibited the same simple banding pattern. Similar results were obtained with additional restriction enzymes (data not shown), suggesting that the gene encoding 2F1-Ag is relatively non-polymorphic. The presence of a single band hybridizing to the EcoRI-digested genomic DNA, and a single major band in the BamHI digest, suggests that the gene encoding the 2F1-Ag represents a "single gene family". Similarly, the rat MAFA gene exhibits a simple Southern pattern with little polymorphism (Fig. 3, [18]). Although we observed no hybridization of the 2F1-Ag cDNA probe to human DNA in Fig. 3, we did observe cross-hybridization using less stringent hybridization conditions. Similar to the mouse and rat MAFA genes, the band pattern was simple and was conserved among four different DNA donors suggesting limited polymorphism of the human MAFA-like gene (data not shown).

2.3 The 2F1-Ag is expressed specifically on NK cells

Northern blot analysis of RNA from various mouse tissues demonstrated strong expression of 2F1-Ag mRNA in A-LAK cells, a cultured preparation of activated NK cells. The transcript of approximately 1.4 kb corresponds closely to the size of cDNA clones we isolated (Fig. 4 and data not shown). There was no evidence for alternatively spliced forms of the message in mouse cells. In contrast, evidence has been presented for at least two rat MAFA transcripts [18]. No expression of

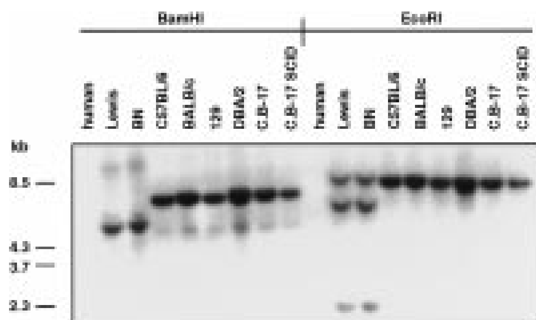

Figure 3. Detection of 2F1-Ag/MAFA related gene sequences by Southern hybridization. The Southern blots were hybridized with a 1.1-kb EcoRI/XbaI fragment from the mouse 2F1-Ag cDNA (clone # 3) consisting of coding and 3' UTR sequences, and washed three times with 2 × SSC/1 % SDS at room temperature and once with 0.5 × SSC/1 % SDS at 65 °C.

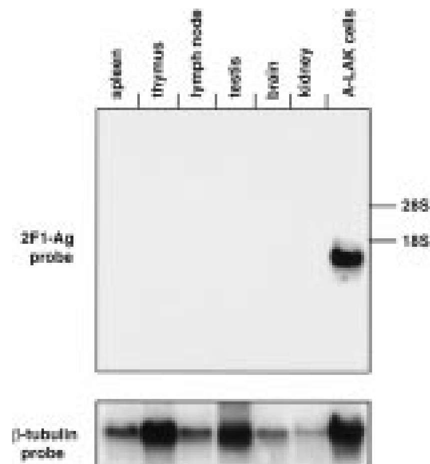

Figure 4. 2F1-Ag mRNA is expressed in IL-2 activated NK cells but not in most other tissues. The A-LAK cell preparation contained > 95 % NK1.1$^+$CD3$^-$ cells. RNA of the indicated tissues was separated on a 1 % agarose gel and transferred to a nylon membrane. The blots were probed and washed as in Fig. 3, stripped and hybridized to a β-tubulin-specific probe as a control.

2F1-Ag was detected in unfractionated cells from spleen, thymus, lymph node, testis, brain or kidney (Fig. 4). The failure to detect expression in spleen cells probably arises because NK cells represent only approximately 3 % of splenocytes. The low levels of mRNA detected in various organs suggest that 2F1-Ag is not expressed significantly in most T and B cells, nor in most non-hematopoietic cells.

In the rat, MAFA expression was detected on a mucosal mast cell line, RBL-2H3, as well as on mast cells of the lung [17, 18]. Therefore, we investigated expression of 2F1-Ag on mouse mast cells. Neither the mastocytoma cell line P815 (Fig. 5A) nor the immature mast cell line 10P12 (Fig. 5B) expressed the 2F1-Ag at detectable levels on the cell surface. Furthermore, 2F1-Ag was not detected on the surface of *ex vivo* mast cell preparations in the mouse. We failed to detect staining with 2F1 mAb of bone marrow-derived mast cells (BMMC) from B6 mice (Fig. 5C) or BALB.B mice (data not shown). Similarly, c-kit-positive peritoneal mast cells isolated *ex vivo* through peritoneal lavage and differential density centrifugation did not react with mAb 2F1 (Fig. 5D). In cytospin preparations, both bone marrow-derived and peritoneal mast cell preparations stained uniformly positive for toluidine blue, supporting their identity as mast cells (data not shown). 2F1-Ag was also not detected on splenic granulocytes expressing the marker Gr-1 (Fig. 5E) while it was readily detectable on the surface of a subpopulation of splenic NK1.1^{+}CD3^{-} cells that were stained in parallel (Fig. 5F).

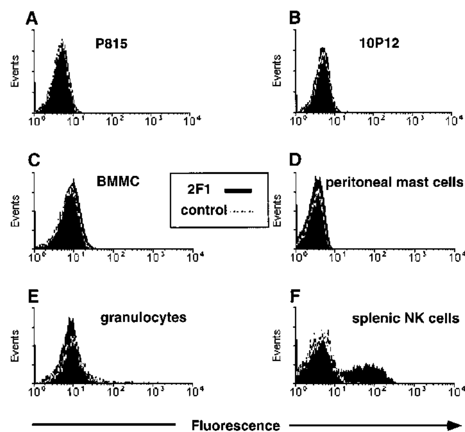

Figure 5. Mouse 2F1-Ag is expressed on the surface of NK cells, but not mast cells. (A–F) Cells were stained with 2F1 (filled curve) or an isotype control (dotted line) and FITC-conjugated anti-hamster Ig. (C–F) Mast cells, granulocytes and NK cells were from B6 mice. (C, D) BMMC and peritoneal mast cells were generated as described in Sect. 4.6 and gated for c-kit^{+} cells indicative of mast cells. (E) Granulocytes were identified as Gr-1^{+} nylon wool-passaged spleen cells with a high degree of forward and side light scatter. (F) Gated NK1.1^{+}CD3^{-} nylon wool-passed splenocytes were analyzed. All samples were preincubated with mAb 2.4G2 to prevent FcγRII/III-mediated staining. The results shown are representative of at least three independent experiments for each cell population.

It remained possible that 2F1-Ag is synthesized in mast cells, but in a form that is unreactive with 2F1-mAb, or inaccessible to the antibody. Therefore, we employed RT-PCR to assay 2F1-Ag mRNA levels in BMMC, unseparated splenocytes, and sorted 2F1^{+} or 2F1^{-} LAK cells as controls. Fig. 6 shows that 2F1-Ag mRNA was completely absent in two different preparations of BMMC while it was readily detectable in unseparated spleen cells from C.B-17 or B6 mice. As expected, enrichment of NK cells expressing the 2F1-Ag on the cell surface (sorted 2F1^{+} LAK cells, 97 % pure) greatly increased the amount of 2F1-Ag mRNA while depletion of 2F1-positive cells among the NK cell population (sorted 2F1^{-} LAK cells, 98 % pure) strongly reduced the presence of 2F1-Ag transcripts. The very weak 2F1-Ag-specific signal found for this population was most likely due to the presence of a small number of contaminating 2F1^{+} LAK cells. Amplifications performed in parallel with β-tubulin-specific primers demonstrated similar amounts of cDNA in all preparations. Therefore, 2F1-Ag mRNA expression is readily detectable in NK cells but absent in BMMC as determined by this very sensitive method.

Taken together, these results show that 2F1-Ag is expressed predominantly by NK cells in mice, and is probably not a functionally important receptor on murine mast cells.

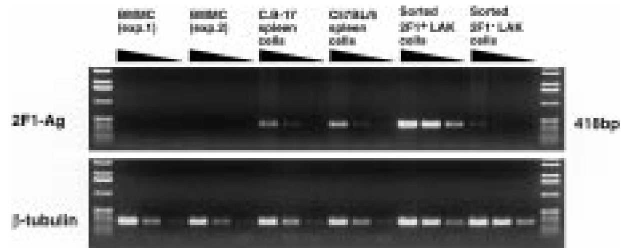

Figure 6. Mouse 2F1-Ag mRNA is not expressed in BMMC. RT-PCR was performed with RNA preparations from two different preparations of BMMC, unseparated spleen cells from C.B-17 or B6 mice and sorted 2F1^{+} (97 % pure) or 2F1^{-} (98 % pure) LAK cells. PCR results from tenfold dilutions of similar amounts of cDNA are shown.

3 Discussion

Using the novel NK cell-reactive mAb 2F1, we describe the expression cloning and tissue distribution of the 2F1-Ag, a lectin-like type II transmembrane cell surface receptor. The evidence suggests that 2F1-Ag is the mouse homolog of rat MAFA, an inhibitory receptor expressed by rat mast cells. First, 2F1-Ag exhibits a high degree of sequence identity to rat MAFA, 86 % at the nucleotide level, and 80 % at the amino acid level. Second, the simple Southern hybridization pattern suggests that the gene encoding 2F1-Ag belongs to a "single-gene family", which would argue against the possibility that another highly related gene is the actual homolog of rat MAFA. Although 2F1-Ag is likely the mouse homolog of rat MAFA, the absence of the molecule from mouse mast cells (see below) has led us to hesitate in referring to the molecule as "MAFA".

We observed three bands in EcoRI-digested rat DNA hybridizing with the mouse 2F1-Ag probe, compared to one major band reported previously [18]. This discrepancy is probably due to the fact that our probe included the 3' UTR sequences in addition to coding sequences, while the previous study employed a probe containing only coding sequences. Indeed, we only observed one strongly hybridizing band in BamHI digests of rat DNA corresponding to the size reported by Bocek et al. [18]. Hence, while it has not been ruled out that multiple genes are present in bands of the same size, it seems likely that the 2F1-Ag is the mouse homolog of rat MAFA. We also report the sequence of a putative human homolog, which also exhibits a simple Southern hybridization pattern (data not shown). Recently, the sequence of another putative human MAFA homolog was entered into the databases ([26], Genbank accession number AF034952). This sequence is 99.6 % (nucleotide level) and 98.4 % (amino acid level) identical to the human sequence we report here (data not shown), arguing for limited polymorphism of the human MAFA gene. In all three species we failed to observe any RFLP, suggesting that the genes are relatively nonpolymorphic.

Surprisingly, our data show that 2F1-Ag is expressed primarily in NK cells, rather than in mast cells. Whether MAFA is expressed by rat NK cells has not been directly addressed in the published studies. Although it was reported that MAFA transcripts could not be detected by RT-PCR in rat spleen cells or PBL [18], it remains possible that the negative results reflect the low frequency of NK cells in these cell populations. Therefore, it is possible that MAFA/2F1-Ag functions in NK cells in all species. On the other hand, we observed no cell surface expression of 2F1-Ag in mouse mast cell lines, BMMC, or peritoneal mast cells (Fig. 5). 2F1-Ag mRNA was readily detectable in LAK or A-LAK cell preparations while it was completely absent in BMMC (Figs. 4 and 6). Attempts to address whether the 2F1-Ag is also expressed on lung mast cells of the mouse have been hampered by the difficulty in preparing *ex vivo* populations of mouse pulmonary mast cells, and the failure of mAb 2F1 to stain tissue sections (data not shown). Although it remains possible that 2F1-Ag is expressed by pulmonary mast cells in the mouse, the failure to observe expression of 2F1-Ag in other mouse mast cell populations may indicate that the receptor does not exert its primary function in mast cells. Alternatively, the receptor may function in both types of cells. Interestingly, other inhibitory receptors, such as the IgSF member gp49B1, are expressed in common by NK cells and mast cells [16, 21, 22].

Little is known concerning the human cell types that express the MAFA homolog. The putative homolog published by Lamers et al. [26] was derived from a pulmonary cell cDNA library, while the EST library that yielded the sequence reported herein was derived from an endothelial cell cDNA library. It cannot be concluded from these observations that endothelial cells or pulmonary mast cells actually express the human MAFA gene, because the mRNA could have come from other cell types contaminating these preparations. Lamers et al. [26] also identified MAFA mRNA in the basophilic cell line KU 812, in line with data by Geller-Bernstein et al. [27] who suggest that human MAFA can be expressed by basophils. After submission of this manuscript, we learned that Butcher et al. [28] have detected human MAFA transcripts not only in basophils, but also in NK cells. Therefore, MAFA seems to have a broader expression pattern in humans than in rats and mice, possibly reflecting more than one immunological function of this molecule.

Bocek et al. [18] identified at least two distinct rat MAFA transcripts, suggesting that the primary transcript is subject to alternative mRNA splicing. The two transcripts were similar except that one lacked the transmembrane and cytoplasmic domains, raising the possibility that it encodes a secreted form of the protein. In contrast, we detected only a single transcript for 2F1-Ag by Northern blotting (Fig. 4), the size of which (1.4 kb) was close to the size of the cDNA we cloned. The apparent lack of a potentially secreted form of 2F1-Ag in A-LAK cells could reflect evolutionary divergence, or could reflect differences in RNA splicing between NK cells and mast cells. The truncated form of MAFA may also be too infrequent in A-LAK cells to be detected by Northern blotting.

Rat MAFA inhibits proximal stimulatory signal transduction and the release of inflammatory mediators upon cotriggering with FcεRI on RBL-2H3 cells [20]. This inhibi-

tory signaling is most likely mediated by the ITIM-like cytoplasmic IYSxLxLP motif which is also present in the cytoplasmic tail of the inhibitory, lectin-like human NKG2A NK cell receptor [24]. For NKG2A, inhibitory signal transduction is relayed by the phosphatase SHP-1, which associates with the cytoplasmic portion of NKG2A upon ligand binding [28]. Whether SHP-1 is also the critical signal transducer for rat or mouse MAFA remains to be established, but studies by Guthmann et al. [17] indicate that cytoplasmic tyrosine and serine phosphorylation of rat MAFA are likely to be involved in MAFA signaling.

The inhibitory function reported for rat MAFA on RBL-2H3 cells and predicted from the 2F1-Ag protein sequence (Fig. 2) prompted us to look for an inhibitory function of the protein on mouse NK cells. As effector cells, we used unseparated or sorted 2F1-positive and -negative NK cells activated by poly-I:C *in vivo* or IL-2 *in vitro.* The cells were tested in various cytotoxicity activation or inhibition assays against a large number of different tumor or lymphoblast target cells. We observed no effects that could be attributed to the 2F1 mAb (data not shown). However, since it has also proven difficult to demonstrate inhibition caused by other antibodies against known inhibitory receptors, including various Ly49 receptors (our unpublished data), we can draw no conclusion. Further studies, perhaps involving 2F1-Ag gene knockouts, will be required to determine whether 2F1-Ag mediates inhibition of NK cell functions.

The analysis of 2F1-Ag-mediated NK cell effector function has been hampered by the failure to identify a physiological ligand for the receptor. Binsack et al. [30] have shown calcium-dependent binding of N-terminal mannose moieties of neoglycans to recombinant rat MAFA. However, the fact that the extracellular domain of MAFA/2F1-Ag is similar to the NKD found in Ly49 receptors (Fig. 2, [25]) raises the possibility that MAFA/2F1-Ag, like Ly49 receptors, interacts with both protein and carbohydrate ligands. Ly49A, for example, functionally interacts with H-2D^d molecules lacking N-glycans [31], but also binds certain oligosaccharides [31, 32]. Therefore, 2F1-Ag may also bind a protein ligand, possibly in addition to carbohydrate residues.

Interestingly, the expression of the 2F1-Ag on mouse NK cells is substantially reduced in MHC class I-deficient mice (L. C. and D. H. R., in preparation). We considered the possibility that MHC class I is a counter-receptor for 2F1-Ag and tested this hypothesis utilizing a semiquantitative cell adhesion assay that is adequate to detect Ly49-MHC class I interactions (T. Hanke and D. Raulet, unpublished). We detected no interaction between 2F1-Ag and MHC class I, suggesting that the effect of MHC class I on 2F1-Ag expression is indirect. However, considering the important role of class I MHC recognition in regulating NK cell functional activity, the down-regulation of 2F1-Ag on NK cells from class I-deficient mice is likely to reflect a biologically significant role for this receptor. The identification of physiological ligands for the MAFA/2F1-Ag proteins should provide clues to their biological function on NK cells or mast cells.

4 Materials and methods

4.1 Animals and cell lines

C57BL/6NCr (B6) and BALB/cByJ (BALB/c) mice were bred in the animal facilities of the University of California, Berkeley. BALB.B mice were kindly provided by I. Weismann. 129/SvJ (129) and DBA/2J (DBA/2) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). C.B-17/lcrTacfDF (C.B-17) and C.B-17/lcrTac-scidfDF (C.B-17 SCID) mice were purchased from Taconic (Germantown, NY). LEW/SsNHsd (Lewis) and BN/SsNHsd (BN) rats were purchased from HSD (Indianapolis, IN). COS-7 (CRL1651), 10P12 (CRL2036) and P815 (TIB64) cell lines were purchased from ATCC (Rockville, MD).

4.2 Expression cloning

COS-7 cells were transfected with a pME18S expression vector-based cDNA library derived from mouse C.B-17 SCID LAK cells (kindly provided by V. Kumar and P. Mathew) using the Lipofectamine reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. The cells were passaged after 1 day and harvested after another day with PBS/0.02 % EDTA. Incubations with antibodies were performed for 20 min at 4 °C using saturating amounts of reagents in PBS/3 % FCS. The transfectants were incubated with mAb 2F1, washed twice, and incubated with biotin-conjugated goat-anti-hamster Ig (Caltag, Burlingame, CA). After extensive washing, the cells were incubated with SA-conjugated magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's instructions. Labeled cells were enriched by magnetic adhesion (Dynal) and washed four times. The extrachromosomal DNA was extracted as described by Hirt et al. [33] and amplified in the *E. coli* DH5α strain. After three rounds of transfection and enrichment, COS-7 cells were transfected with individual plasmids and stained with mAb 2F1. Three plasmid clones (clones # 3, # 20, # 34) were found to encode the 2F1-Ag. The cDNA inserts of the positive clones were subcloned into pBluescriptSK$^-$ (Stratagene, La Jolla, CA). Automated sequence analysis with T3 and T7 primers revealed identical cDNA for the three clones analyzed (Genbank accession number AF097357).

4.3 Southern and Northern blot analysis, RT-PCR

Southern and Northern blot analyses were performed essentially as described by Sambrook et al. [34]. For Southern blot analysis, genomic mouse or rat tail DNA or genomic DNA from human PBL was analyzed. For the Northern blot, total cellular RNA from various cells and organs was examined. The blots were hybridized with a random-primed (Boehringer Mannheim, Mannheim, Germany), ^{32}P-labeled 1.1-kb EcoRI/XbaI fragment of the clone # 3 cDNA. The membranes were washed three times at RT in 2 × SSC, 0.1 % SDS and once in 0.5 × SSC, 1 % SDS at 65 °C. The blots were exposed for 1 to 3 days at –70 °C and developed automatically. After hybridization with the clone # 3 probe, the Northern blot was stripped by incubation for 2 h in 94 % formamide/1 % SDS followed by 30 min 0.1 × SSC/1 % SDS at 68 °C, and was subsequently reprobed with a β-tubulin cDNA probe.

For RT-PCR, 1 μg of total RNA from BMMC, RBC depleted spleen cells or sorted LAK cells was reverse transcribed using an oligo-$(dT)_{15}$ primer (Boehringer Mannheim) and M-MLV reverse transcriptase (Gibco BRL). Subsequently, tenfold dilutions of samples were amplified in parallel using Taq polymerase (Promega, Madison, WI) and 2F1-Ag-specific (5' primer 5'-GTGCAGACAAAGGCTCACATCTC-3', 3' primer 5'-AACCCCCAGAAAGGTATGCTAATC-3') or β-tubulin-specific primers.

4.4 Identification of a human MAFA homolog

The rat MAFA cDNA sequence (Genbank accession number X79812) was used to search the EST database for homologous sequences, using the XREFdb website (http://www.nci.nlm.nih.gov/XRFdb). A human EST (Genbank accession number AA188327) was identified that displays high homology to the rat MAFA cDNA sequence. The corresponding clone (I.M.A.G.E. Consortium Clone ID 625662 [35]) was subcloned into pBluescriptSK$^-$ (Stratagene) and sequenced using T3 and T7 primers. For sequence analysis and alignment, MacVector® and ClustalW® software was used.

4.5 Antibodies, staining and flow cytometry

Cells (5×10^5–2×10^6) were stained in PBS/3 % FCS/0.02 % sodium azide at 4 °C with saturating amounts of reagents. Where necessary, nonspecific FcγRII/III-mediated binding was blocked by pre-incubation with mAb 2.4G2 [36]. Staining of unconjugated mAb 2F1 was visualized by secondary FITC-conjugated goat anti-hamster Ig (Caltag). For multicolor analysis, we employed 2F1-FITC (conjugated using the Boehringer Mannheim FITC conjugation kit), and the following reagents purchased from Pharmingen (San Diego, CA): anti-NK1.1-PE, anti-NK1.1-biotin, anti-CD3-PE, anti-c-kit-PE. Anti-Gr-1-biotin was purchased from Caltag. Biotin-conjugated mAb were developed with streptavidin (SA)-PE (Molecular Probes Inc., Eugene, OR), or SA-Tricolor (Caltag). Stained cells were washed two or three times between reactions and once before analysis. The samples were analyzed on an EPICS XL flow cytometer (Coulter, Hialeah, FL) with a live gate set based on the scatter profile. WinMDI® software was used for data display. Results are shown as $\log_{10}$ fluorescence intensities on a four-decade scale.

For sorting, LAK cells from B6 mice were incubated on ice with saturating amounts of mAb 2F1 for 20 min, washed twice and stained with FITC-conjugated goat anti-hamster Ig (Caltag). Live lymphocytes were sorted on an ELITE cytometer (Coulter) into 2F1$^+$ and 2F1$^-$ fractions.

4.6 Isolation of bone marrow-derived and peritoneal mast cells

BMMC were generated essentially as described by Bender et al. [37]. In short, unfractionated bone marrow cells from B6 or BALB.B mice were cultured at 2.5×10^5 cells/ml in supplemented RPMI 1640 medium containing 25 U/ml IL-3 (Pharmingen). Nonadherent cells were passaged weekly. After 4 weeks, the nonadherent cells were $>$ 85 % large c-kit$^+$ cells, with a high degree of side scatter, indicative of mast cells. Essentially all of the cells stained with toluidine blue in cytospin preparations, confirming their identity as mast cells.

Peritoneal mast cells were isolated from peritoneal lavage cells from 12-month-old B6 mice. The cells were isolated and washed in TG buffer (0.8 % NaCl, 0.1 % glucose, 0.1 % $NaHCO_3$, 0.02 % KCl, 0.005 % Na_2HPO_4, 0.1 % gelatin in H_2O) and subjected to centrifugation for 15 min at 2500 rpm through a 22 % (w/v) Metrizamide (Sigma, St. Louis, MO)/TG layer. The majority of pelleted cells stained strongly positive with toluidine blue in cytospin preparations. Flow cytometry revealed a high percentage ($>$ 60 %) of large c-kit$^+$ cells with high degree of side scatter, characteristics indicative of mast cells.

4.7 LAK and A-LAK cells

LAK cells were generated by culturing RBC depleted, nylon wool-passed splenocytes from B6 mice for 4 days in RPMI/FCS medium containing 7500 U/ml recombinant human IL-2 (Chiron, Emeryville, CA). A-LAK cells were generated essentially as described by Karlhofer et al. [1] and contained $>$ 95 % NK1.1$^+$ TCRαβ$^-$ cells.

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