Identification of CLEC12B, an Inhibitory Receptor on Myeloid Cells*

Received for publication, May 23, 2007 Published, JBC Papers in Press, June 11, 2007, DOI 10.1074/jbc.M704250200

Sabrina C. Hoffmann1,3, Carola Schellack2, Sonja Textor5, Stephanie Konold6, Debora Schmitz5, Adelheid Cerwenka5,2, Stefan Pflanz5, and Carsten Watzl1,3

From the 1Institute for Immunology, University of Heidelberg, Im Neuenheimer Feld 305, 69120 Heidelberg, 2Division of Innate Immunity, German Cancer Research Center/D080, Im Neuenheimer Feld 280, 69120 Heidelberg and 3Micromet AG, Staffelseestrasse 2, 81477 München, Germany

Activation of immune cells has to be tightly controlled to prevent detrimental hyperactivation. In this regulatory process molecules of the C-type lectin-like family play a central role. Here we describe a new member of this family, CLEC12B. The extracellular domain of CLEC12B shows considerable homology to the activating natural killer cell receptor NKG2D, but unlike NKG2D, CLEC12B contains an immunoreceptor tyrosine-based inhibition motif in its intracellular domain. Despite the homology, CLEC12B does not appear to bind NKG2D ligands and therefore does not represent the inhibitory counterpart of NKG2D. However, CLEC12B has the ability to counteract NKG2D-mediated signaling, and we show that this function is dependent on the immunoreceptor tyrosine-based inhibition motif and the recruitment of the phosphatases SHP-1 and SHP-2. Using monoclonal anti-CLEC12B antibodies we found de novo expression of this receptor on in vitro generated human macrophages and on the human myelomonocytic cell line U937 upon phorbol 12-myristate 13-acetate treatment, suggesting that this receptor plays a role in myeloid cell function.

Activation of the immune system has to be tightly regulated to prevent the attack of self-antigens and the development of autoimmunity (1). This is in part accomplished by a sophisticated system of various receptors that create a delicate balance of activating and inhibitory signals (2, 3). These receptors are present on different immune cells but play a major role for the innate immune system.

Many receptors share common signaling motifs to transmit their signal into the cell. Activating receptors usually signal via the immunoreceptor tyrosine-based activation motif containing tyrosine residues that can be phosphorylated upon receptor engagement and can recruit Syk family kinases (4). Inhibitory receptors often possess an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM), which can recruit phosphatases upon phosphorylation (5). These phosphatases, like SHP-1 or SHP-2, are able to de-phosphorylate and therefore inhibit intracellular factors that otherwise would promote cellular activation (6).

Interestingly, many activating and inhibitory receptors come in pairs, with a strong homology of the extracellular domains and partially overlapping ligand specificity but opposite signal capacities (2). One reason for the existence of these receptor pairs might be to avoid extreme immune reactions to minor threats. Examples for these paired receptors are killer cell Ig-like receptors on natural killer (NK) cells, which comprise inhibitory and activating receptors, both recognizing the same major histocompatibility complex class I ligand (5, 7). Also in the family of lectin-like receptors can be found antithetic pairs that are specific for the same ligand but transmit opposing signals (8). For example, the NKG2A-CD94 heterodimer transmits an inhibitory signal and NKG2C-CD94 signals in an activating fashion while both receptors recognize the same ligand, HLA-E.

NKG2D is a C-type lectin-like receptor and plays an important role for the activation of natural killer cells (9). NKG2D has no intracellular signaling domain but instead pairs with the adaptor molecules DAP10 and DAP12 to mediate an activating signal. So far, no inhibitory counterpart for NKG2D has been described. Therefore we conducted a data base search for molecules with a high homology to the NKG2D extracellular domain. With this approach we identified a novel cell surface receptor encoded within the same chromosomal region as NKG2D.

Here we describe the molecular cloning, biochemical and initial functional characterization of CLEC12B, a new inhibitory receptor of the lectin-like family.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Cell lines used in this study were human embryonic kidney 293T, Ba/F3 transduced with NKG2D ligands, P815, and NKL. Human monocytes were isolated from peripheral blood mononuclear cells using anti-CD14-positive selection beads (Miltenyi) and used for the in vitro generation of macrophages as described (10). Antibodies used were anti-histidine tag (Serotec, Oxford, UK), anti-SHP-1,
Identification of CLEC12B

RESULTS

In an attempt to identify NKG2D-related molecules, we identified a mouse cDNA clone (AK016908) coding for an unnamed C-type lectin protein product of 275 amino acids (BAB30491). Using this sequence as bait, we identified a related human sequence in the database designated human macrophage antigen-H (AY358810). Comparing the sequences, we found that human macrophage antigen-H (232 amino acids) was missing exon 6, resulting in a premature stop codon and an incomplete C-type lectin domain. Searching the genomic DNA data base, we identified the missing exon 6, which showed homology to the murine sequence. Using primers spanning exons 1 to 6 we identified the complete cDNA coding for a 276-amino acid type II transmembrane protein (DQ368812) belonging to the C-type lectin family of recep-
tors (14, 15). The extracellular domain of the putative protein showed highest homology to human NKG2D (36% similarity). However, unlike NKG2D it did not contain a charged amino acid in its transmembrane region but an ITIM sequence (VTYATL) in the cytoplasmic tail (Fig. 1A).

The genetic locus of this receptor was mapped to human chromosome 12p13.2 and mouse chromosome 6qF3 in the vicinity of other C-type lectin-like receptors (Fig. 1B). Overall, the receptor showed a high similarity to CLEC12A (16–18% similarity), which was located on the same chromosomal locus. We therefore named this new receptor CLEC12B. Using reverse transcription PCR we found expression of CLEC12B in human cDNA libraries prepared from various tissues except brain (Fig. 1C). Interestingly, in mammary gland and ovary we only found a truncated splice variant lacking exon 4. As this exon encodes part of the C-type lectin-like domain, this alternative transcript would yield a non-functional protein.

Because of its high similarity to the NKG2D extracellular domain we speculated that this receptor could be an inhibitory counterpart of NKG2D. Similar to NKG2D, surface expression of this receptor was independent of CD94 expression and the receptor formed disulfide-linked homodimers in transfected cells (data not shown). We produced an Ig fusion protein of the extracellular domain of human CLEC12B to test whether it might recognize the same ligands as NKG2D. Ba/F3 cells stably expressing the human NKG2D ligands MICA, MICB, ULBP1, ULBP2, or ULBP3 were stained at high levels with a NKG2D-Ig fusion protein. However, we detected no binding of the Ig fusion protein containing the extracellular domain of human CLEC12B (Fig. 2), demonstrating no apparent overlap in ligand specificity with NKG2D.

To examine CLEC12B expression and biochemical properties, we produced mouse monoclonal antibodies against human CLEC12B. We selected three subclones that specifically recognized the receptor in transfected cells either in Western blot (clones 12 and 16, Fig. 3A and data not shown) or in flow cytometry (clones 30 and 16, Fig. 3B and data not shown). CLEC12B was detected as a double band in Western blot analysis of transfected cells, possibly due to differential glycosylation of the
Using the antibody clone 30 in flow cytometry, we detected strong expression of CLEC12B on the human pro-myelocytic cell line U937 after PMA stimulation but not in non-stimulated cells (Fig. 4A). The induction of CLEC12B protein expression was confirmed by Western blot analysis using another anti-CLEC12B-specific mAb (clone 12) (Fig. 4B). Interestingly, the endogenous CLEC12B protein was detected as a single band as compared with the double band of the transfected protein. The reason for this difference is unknown so far.

This indicates that CLEC12B can be expressed on monocytic cells after a cell differentiation-inducing stimulus. To further characterize this we purified CD14-positive monocytes from human peripheral blood mononuclear cells. Although we detected no surface expression on freshly isolated monocytes or any other leukocyte population (data not shown), we observed the surface expression of CLEC12B on in vitro differentiated human macrophages (Fig. 4C). This expression was confirmed by Western blot analysis using three independent anti-CLEC12B mAbs (Fig. 4D and data not shown).

To characterize receptor function, we generated a mutant of human CLEC12B by exchanging the ITIM tyrosine with phenylalanine. The His-tagged wild type (WT) or ITIM tyrosine-to-phenylalanine mutant (mutant) expression on NKL transductants as determined by anti-His antibody staining for detection of CLEC12B. The use of the anti-CLEC12B mAb clone 30 gave comparable results. C and D, redirected lysis assay of NKL cells stably expressing His-tagged CLEC12B WT (C) or ITIM mutant CLEC12B (D) against P815 cells. Redirected lysis was stimulated by the indicated antibodies (IgG, isotype control antibody). Shown is an effector-to-target ratio of 10:1. Similar results were obtained at an effector-to-target ratio of 5:1 (data not shown).
Identification of CLEC12B

FIGURE 6. CLEC12B can recruit SHP-1 and SHP-2. 293T cells were transfected with His-tagged WT or ITIM mutant (m) CLEC12B in combination with SHP-1 (A) or SHP-2 (B). Cells were treated with (+) or without (−) pervanadate (PV). Lysates were immunoprecipitated using control IgG or anti-His antibodies, and samples were analyzed by Western blotting for co-immunoprecipitated SHP-1 or SHP-2 (top panel), phosphorylated CLEC12B (anti-phosphotyrosine (P-Tyr), second panel), and total immunoprecipitated CLEC12B (anti-CLEC12B mAb clone 12, third panel). To demonstrate comparable expression of SHP-1 and SHP-2, lysates were analyzed by Western blotting (bottom panel).

in the WT receptor. Interestingly, the lower band of the transfected CLEC12B detected by Western blotting was preferentially phosphorylated (Fig. 6, A and B). Upon phosphorylation the WT, but not the mutant receptor, could recruit SHP-1 and SHP-2 as detected by co-immunoprecipitation (Fig. 6, A and B). This indicates that the inhibitory function of CLEC12B can be mediated by the recruitment of SHP-1 or SHP-2 to its ITIM upon receptor phosphorylation.

DISCUSSION

The cDNA sequence for CLEC12B was found during a data base search for sequences with homology to NKG2D. Because of the high homology of CLEC12B’s extracellular domain with NKG2D and its ITIM motif, this new lectin-like receptor appeared to be a potential inhibitory counterpart. Yet binding of CLEC12B Ig fusion proteins to NKG2D ligands could not be detected. Additionally we did not find any expression of CLEC12B on primary T cells, NK cells, or on NK cell lines. This makes it very improbable that CLEC12B is an inhibitory counterpart to NKG2D, at least with respect to expression and competitive binding to common NK cell ligands.

Functional analysis of full-length CLEC12B revealed that this receptor is able to signal in an inhibiting fashion via recruitment of the phosphatases SHP-1 and SHP-2. Although CLEC12B triggering could inhibit NKG2D-mediated NK cell activation (Fig. 4), its inhibitory activity was not limited to NKG2D. Other activating NK cell receptors such as 2B4 (20) were sensitive to the inhibitory effect of CLEC12B (data not shown), demonstrating that CLEC12B can inhibit a broad range of receptor-mediated activating signals. In preliminary experiments we also investigated the function of endogenous CLEC12B on PMA-stimulated U937 cells. Stimulating these cells with lipopolysaccharide induced the production of tumor necrosis factor α. However, engagement of CLEC12B by plate-bound anti-CLEC12B antibody did not significantly inhibit lipopolysaccharide-induced tumor necrosis factor α production (data not shown). Plate-bound antibodies only stimulate their respective receptor at the site of contact between the cell and the plastic dish. In contrast, lipopolysaccharide is a soluble mediator and can therefore exert its effect over the entire surface of the cell. It is therefore likely that the local inhibitory effect of CLEC12B engagement was not sufficient to counteract the more global lipopolysaccharide signal. This demonstrates that CLEC12B is only potent in blocking cellular activation when it is co-engaged with an activating receptor in a defined region of the plasma membrane of a cell.

We found CLEC12B mRNA expression in almost all tissues tested. In mammary gland and ovary only a truncated variant of CLEC12B lacking exon 4 could be detected. As this exon encodes a part of the extracellular domain essential for formation of a functional C-type lectin-like domain, the resulting receptor will be nonfunctional. It will be interesting to investigate the regulation of this differential splicing of CLEC12B in different tissues.

We found protein expression of CLEC12B on in vitro differentiated macrophages and on PMA-stimulated U937 cells. We did not detect CLEC12B expression on in vitro generated dendritic cells (generated from CD14+ cells with granulocyte macrophage colony-stimulating factor and interleukin 4 for 6 days) or on any freshly isolated peripheral blood leukocyte population (data not shown). The expression on macrophages could explain why we found CLEC12B expression in various tissues using reverse transcription PCR, as macrophages and related immune cells are spread throughout the body.

CLEC12B may be involved in limiting the activity of monocyte-derived immune cells after cell differentiation and possibly during inflammatory diseases. It will therefore be interesting to investigate CLEC12B expression during various infectious or chronic diseases. Together with the identification of its ligand, this might give more insight into the biological function of this novel receptor.

Acknowledgments—We acknowledge the help of Zlatko Radev, Ewelina Miczka, Birgitta Messmer, and Carmen Mader.

REFERENCES

1. Jabri, B., and Meresse, B. (2006) Curr. Top. Microbiol. Immunol. 298, 139–156
2. Lanier, L. L. (2001) Curr. Opin. Immunol. 13, 326–331
3. Taylor, L. S., Paul, S. P., and McVicar, D. W. (2000) Rev. Immunogenet. 2, 204–219
4. Humphrey, M. B., Lanier, L. L., and Nakamura, M. C. (2005) Immunol. Rev. 208, 50–65
5. Moretta, L., and Moretta, A. (2004) Curr. Opin. Immunol. 16, 626–633
6. Stębniak, C. C., Watzl, C., Billadeau, D. D., Leibson, P. J., Burshtyn, D. N., and Long, E. O. (2003) Mol. Cell. Biol. 23, 6291–6299
7. Stewart, C. A., Laugier-Anfossi, F., Vely, F., Saudquin, X., Riedmuller, J., Tissotant, A., Gauthier, L., Romagne, F., Ferracci, G., Arora, F. A., Moretta, A., Sun, P. D., Ugolini, S., and Vivier, E. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 13224–13229
8. Lopez-Botet, M., Bellon, T., Llano, M., Navarro, F., Garcia, P., and de Miguel, M. (2000) Hum. Immunol. 61, 7–17
9. Cerwenka, A., and Lanier, L. L. (2001) Nat. Rev. Immunol. 1, 41–49
10. Nedvetzki, S., Sowinski, S., Eagle, R. A., Harris, J., Vely, F., Pende, D., Trowsdale, J., Vivier, E., Gordon, S., and Davis, D. M. (2007) Blood 109, 3776–3785
11. Watzl, C., Peterson, M., and Long, E. O. (2000) Tissue Antigens 56, 240–247
12. Eissmann, P., and Watzl, C. (2006) J. Immunol. 177, 3170–3177
13. Eissmann, P., Beauchamp, L., Wooters, J., Tilton, J. C., Long, E. O., and
14. Sobanov, Y., Bernreiter, A., Derdak, S., Mechtcheriakova, D., Schweighofer, B., Duchler, M., Kalthoff, F., and Hofer, E. (2001) Eur. J. Immunol. 31, 3493–3503
15. Robinson, M. J., Sancho, D., Slack, E. C., Leibundgut-Landmann, S., and Sousa, C. R. (2006) Nat. Immunol. 7, 1258–1265
16. Marshall, A. S., Willment, J. A., Lin, H. H., Williams, D. L., Gordon, S., and Brown, G. D. (2004) J. Biol. Chem. 279, 14792–14802
17. Bakker, A. B., van den Oudenrijn, S., Bakker, A. Q., Feller, N., van Meijer, M., Bia, J. A., Jongeneelen, M. A., Visser, T. J., Bijl, N., Geuijen, C. A., Marissen, W. E., Radosevic, K., Throsby, M., Schuurhuis, G. J., Ossenkopppele, G. J., de Kruijf, J., Goudsmit, J., and Kruisbeek, A. M. (2004) Cancer Res. 64, 8443–8450
18. Han, Y., Zhang, M., Li, N., Chen, T., Zhang, Y., Wan, T., and Cao, X. (2004) Blood 104, 2858–2866
19. Long, E. O., Barber, D. F., Burshtyn, D. N., Faure, M., Peterson, M., Rajagopalan, S., Renard, V., Sandusky, M., Stebbins, C. C., Wagtman, N., and Watzl, C. (2001) Immunol. Rev. 181, 223–233
20. Bhat, R., Eisssmann, P., Endt, J., Hoffmann, S., and Watzl, C. (2006) J. Leukocyte Biol. 79, 417–424