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COMMUNICATION

Human and mouse macrophage-inducible C-type lectin (Mincle) bind *Candida albicans*

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Candida albicans **is a causative agent in mycoses of the skin, oral cavity, and gastrointestinal tract. Identification of receptors, and their respective ligands, that are engaged by immune cells when in contact with *C. albicans* is crucial for understanding inflammatory responses leading to invasive candidiasis. Mincle is a recently identified macrophage-expressed receptor that is important for host responses to *C. albicans*. The carbohydrate-recognition domain of human and mouse Mincle were expressed, purified under denaturing conditions, and successfully refolded. In addition to oligomers, there are isolatable monomeric and dimeric forms of the protein that occur under two different buffer solutions. The human and mouse homologues bound yeast extract, and the isolated dimeric and monomeric species also demonstrated the recognition of whole *C. albicans* yeast cells. The data are indicative of several functional states mediating the interaction of Mincle and yeast at the surface of the macrophage.**

Keywords: Candida albicans/C-type lectin/Mincle/receptor-binding assay

Introduction

Innate immunity is the first-line host defence mechanism against invading infectious agents. The mechanism of host response is initiated by the recognition of "pathogen-associated molecular patterns" by pattern recognition receptors expressed on macrophages, natural killer (NK) and dendritic cells (DC) of the innate immune system (Tabona et al. 1995; Mann et al. 2004; Fuse et al. 2007). Macrophages possess a large selection of lectin receptors on their surface that mediate an extensive range of immune functions including phagocytosis and secretion of various cytokines. Other than the members of the Toll-like receptor family, it is the C-type lectin family that is most prevalent in immune responses, with Mincle (also termed Clec4E and Clecsf9) being a key macrophage surface-expressed C-type lectin. Mincle is a highly inducible early response gene in macrophages responding to pathogens and is a transcriptional target of NF-IL6 in LPS-induced macrophages (Matsumoto et al. 1999). Mincle exhibits highest sequence similarity to group II C-type lectin members that have been identified as macrophage/DC-restricted cell surface receptors. Genes encoding these receptors (e.g., DC-SIGN, DCIR, Mcl, and Dectin-2) are highly organized within the mammalian genome into gene clusters (Balch et al. 2002; Arce et al. 2004). Mincle is a 219-amino-acid, type II transmembrane protein with a 22-amino-acid N-terminal intracellular segment, a transmembrane domain, and an extracellular carboxy terminal that comprises a short stalk and a carbohydrate-recognition domain (CRD). A full-length Mincle gene transfected into mammalian cells is transcribed into a 35 kDa protein whereas the calculated molecular weight is 25.4 kDa, suggesting that this protein is highly posttranslationally modified (Matsumoto et al. 1999). The extracellular domain lacks polymorphism in the general population suggesting an important immunological and biological function that has been evolutionary conserved (Flornes et al. 2004). Members of the C-type lectin family are often alternatively spliced with isoforms encoding truncated neck regions that would normally mediate oligomerization and afford flexibility of the carbohydrate-binding extracellular regions (Willment et al. 2001; Ariizumi, Shen, Shikano, Xu et al. 2000; Mummidi et al. 2001). Such isoforms have been identified for other members of the group II C-type lectins including Dectin-2 and DCIR (Ariizumi, Shen, Shikano, Ritter et al. 2000; Richard et al. 2003), but to date have not been recognized for Mincle. In DCIR, the alternative splicing has been linked to its regulation of expression by pro- or anti-inflammatory stimuli (Richard et al. 2003, 2006). Based on the role in the immune response, C-type lectins can be classified as inhibitory or activating molecules. The former possesses tyrosine-based inhibitory motifs whereas activating lectins have a single positively charged amino acid in a transmembrane domain that is able to mediate a noncovalent association with proteins containing tyrosine-based activating motifs. The transmembrane domain of Mincle possesses a positively charged arginine residue indicating a possible role in activating the immune response (Flornes et al. 2004), and indeed functional studies in Mincle knock-out mice indicate a central role in inducing inflammation in macrophages challenged with *Candida albicans* (Wells et al. 2008).

Members of the C-type lectin family were originally defined by the presence of a lectin-like domain that binds glycan ligands in a Ca^{2+}-dependent manner, and now form part of a larger superfamily that includes proteins that share structural homology but often bind nonsugar ligands such as proteins or lipids (Weis et al. 1998; Marshall and Gordon 2004). The basic C-type lectin domain (CTLD) fold comprises two α-helices, five β-strands, and a double-loop structure, with four conserved

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cysteine residues forming disulfide bridges at the bases of the two loops (reviewed in Zelensky and Gready 2005). Four Ca^{2+}-binding sites exist with occupancy dependent on the particular CTLD sequence (Weis et al. 1991, 1992), but it is the Ca^{2+} located within the long loop region ("site 2") that is involved in Ca^{2+}-dependent carbohydrate binding. Some CTLDs contain characteristic sequence motifs such as "EPN" and "WND" that bind Ca^{2+}, with both the protein and ion coordinating the saccharide ligand (Zelensky and Gready 2005). Mincle and other members of the group II C-type lectins such as Dectin-2, DC-SIGN, and Mcl contain, in addition to highly conserved calcium-binding residues, the mannose-binding residue motif EPN that is not present in other C-type lectins such as Dectin-1 (Weis et al. 1991; Feinberg et al. 2001; Flornes et al. 2004; Brown et al. 2007). Nevertheless, the presence of such carbohydrate-recognition motifs and Ca^{2+} occupancy of site 2 within a CTLD does not guarantee lectin activity, as exemplified by tetranectin (Lorentsen et al. 2000). Thus even though group II C-type lectin receptors, including Mincle, possess the evolutionary conserved key saccharide-binding and calcium-binding residues, their specific ligands have not been fully elucidated (Ariizumi, Shen, Shikano, Ritter et al. 2000; Fernandes et al. 2000). The interaction between *C. albicans* and host immune cells, such as macrophages, could be characterized with the availability of the receptor or a domain that retains functional binding activity. We present the cloning, purification, and characterization of such a domain of Mincle mouse and human homologues. The proteins were purified to high yields under denaturing conditions, refolded, and shown to exist in both dimeric and monomeric states. Significantly, our investigation demonstrates that purified Mincle proteins bind to yeast extract and that both dimer and monomer species of Mincle CRD bind also to heat-killed whole *C. albicans* yeast cells.

Results and discussion

Human and mouse Mincle CRD proteins can form monomers and dimers in solution

Refolding of denatured and purified human and mouse Mincle CRD using the FoldIT Screen (Hampton Research; "FP-1" method; see *Materials and methods*) exhibited clear solutions in dialysis buttons, after a 7-day incubation period, for buffers 1, 3, 8, 10, 12, and 15 (Table I). Solution from each dialysis button was assayed using our standard ELISA protocol and showed that all the buffer conditions led to refolding of the CRD into its correct conformation for both the human (Figure 1A) and the mouse homologues (Figure 1B). The same ELISA analysis, but in this case using equivalent human and mouse protein concentrations, was applied to the Mincle homologues that were refolded using an alternative "FP-2" method (Figure 1). The proteins folded correctly. The observed higher absorbance readings for the human than the mouse homologue being likely a consequence of the monoclonal antibody being raised against human recombinant protein.

Analysis of protein samples under each of the buffer conditions by native PAGE showed a varied extent of oligomer formation. Size exclusion chromatography (SEC), initially using Superdex 75 (S75) and Superdex 200 (S200) columns, was used to assess the oligomeric states and determine associated molecular weights of refolded CRD proteins. Fractions of human and mouse homologues refolded using the FP-1 buffers, particularly 10, revealed a high degree of aggregation shown also by silver-stained native PAGE (pH 5.5), where low protein migration into the gel and smearing is indicative of aggregation. However, components of a fraction of both human and mouse proteins refolded using the FP-1 buffer 8 could be separated by the S200 and S75 columns. Column fractions containing protein (confirmed by Western blot analysis) along with column calibration enabled correlation of the derived molecular weights to the fractions. Specific molecular weight species were identified, with also a high degree of oligomerization. Molecular weight estimations of 19.5 kDa and 20.5 kDa were obtained for human and mouse Mincle, respectively. The calculated molecular weights of the cloned CRD fragments correspond each to ~22 kDa, and thus the SEC analysis of protein refolded via FP-1 buffer 8 indicates the isolation of a monomeric species for both homologues. Using the same analyses, a fraction of human and mouse Mincle proteins refolded using the FP-2 method was identified that coincided with molecular weight estimations of ~38 kDa and ~42 kDa, respectively, in accord with the isolation of a homodimer. Improved ability to accurately isolate protein species was attained using an alternative approach involving denaturing and refolding via a pulse renaturation method (Vallejo and Rinas 2004) and incorporating a longer chromatographic column. Though some aggregation of the refolded proteins was still observed (denoted by 'A' in Figure 2A–D) dimeric and monomeric species were more easily identified and could be subsequently isolated as a homogeneous preparation. Interestingly, the pulse renaturation method led to the isolation of both dimeric and monomeric species for either refolding buffer, except in the case of mouse CRD refolded using the FP-2 buffer where only monomeric species was identified (Figure 2D). Our results demonstrate that not only the buffer composition but also the manipulation of refolding kinetics coupled to the use of longer chromatographic columns influences the extent of aggregate formation as well as the successful isolation of monomeric or dimeric states of the Mincle CRD protein. This ability of the Mincle CRD proteins to exhibit isolatable monomeric and dimeric forms, shown consistent to both homologues, could be anticipated to have importance for its biological function. Our results provide the first evidence that Mincle CRD can adopt stable monomeric and dimeric forms, and add a significant dimension to these lectins given that the CRDs of other members of this group, such as Dectin-1 and macrophage mannose receptor, have only been reported as forming monomers in solution (Feinberg et al. 2000; Brown et al. 2007).

Mincle proteins bind soluble C. albicans yeast extract and whole, heat-killed yeast cells

The ability of the refolded human and mouse proteins to bind to yeast extract (*C. albicans* isolate SC5314) was examined via a receptor ELISA assay. Equivalent sample volumes to those used in the standard ELISA, and from the same dialysis buttons, were assayed for binding to yeast extract (Figure 3A, B). Mincle refolded CRD proteins showed some interaction under most conditions, with buffers 8 and 10 of the FP-1 method being the most consistent between the human and mouse, and with both homologues refolded using the FP-2 method recognizing a component of the yeast extract. Our results provide the first evidence that human Mincle CRD interacts with yeast extract

Table I. Composition of the buffers used in refolding protocols FP-1 and FP-2

		Buffer	Salt	Denaturant	Additives	Redox
FP-1[a]	1	55 mM Tris, pH 8.2	250 mM NaCl, 10 mM KCl	None	0.055% PEG 4000	1 mM DTT
	3	55 mM MES, 6.5	10 mM NaCl, 0.5 mM KCl	550 mM Guanidine-HCl	400 mM sucrose, 500 mM L-arginine	1 mM GSH, 0.1 mM GSSG
	8	55 mM MES, 6.5	250 mM NaCl, 10 mM KCl	–	500 mM L-arginine, 0.055% PEG 4000	1 mM GSH, 0.1 mM GSSG
	10	55 mM Tris, pH 8.2	10 mM NaCl, 0.5 mM KCl	–	400 mM sucrose	1 mM GSH, 0.1 mM GSSG
	12	55 mM MES, 6.5	250 mM NaCl, 10 mM KCl	550 mM Guanidine-HCl	500 mM L-arginine	1 mM DTT
	15	55 mM MES, 6.5	10 mM NaCl, 0.5 mM KCl	–	400 mM sucrose, 500 mM L-arginine	1 mM DTT
FP-2[a]		200 mM Tris, pH 8	–	–	1 M L-arginine	1 mM GSH, 0.1 mM GSSG

[a]Buffer FP-1 contains 1 mM EDTA chelator and buffer FP-2 10 mM EDTA.

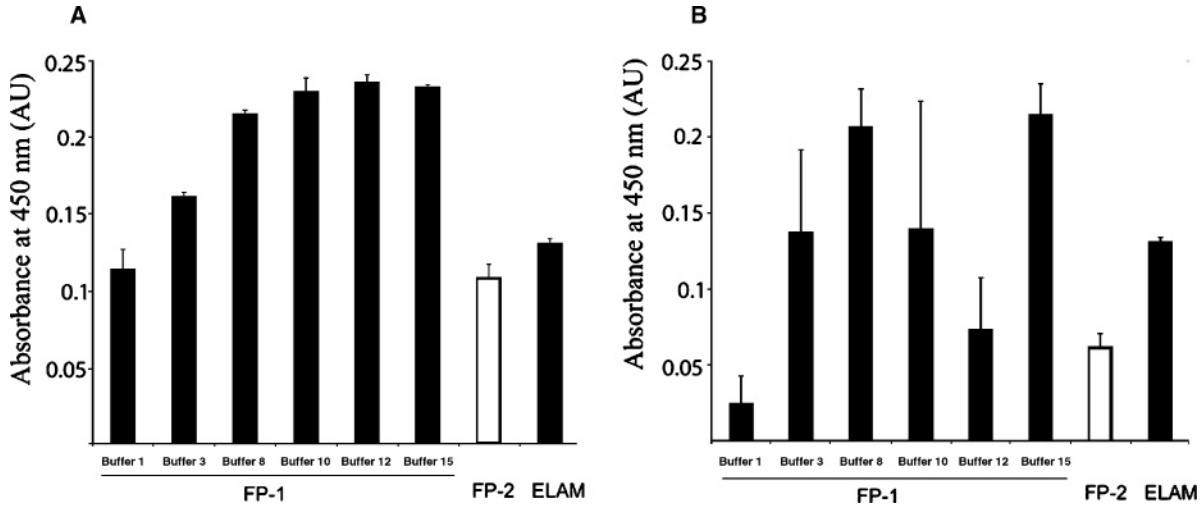

Fig. 1. ELISA of refolded human and mouse Mincle proteins using FP-1 or FP-2 methods. (**A**) Human and (**B**) mouse refolded CRD protein solution (10 μL, protein, concentration was not quantitated) recovered from dialysis buttons was diluted into 100 μL of carbonate buffer and added to a well of a microtiter ELISA plate. In parallel, 1 μg/mL of human and mouse Mincle CRD protein refolded using the FP-2 protocol was analyzed and 1 μg/mL of ELAM total cell lysate (a cell line known to express Mincle) was added to different wells as a positive control. Refolded and native Mincle CRD protein was recognized by the addition of an α-Mincle monoclonal antibody. All values represent an average of readings obtained from triplicate wells.

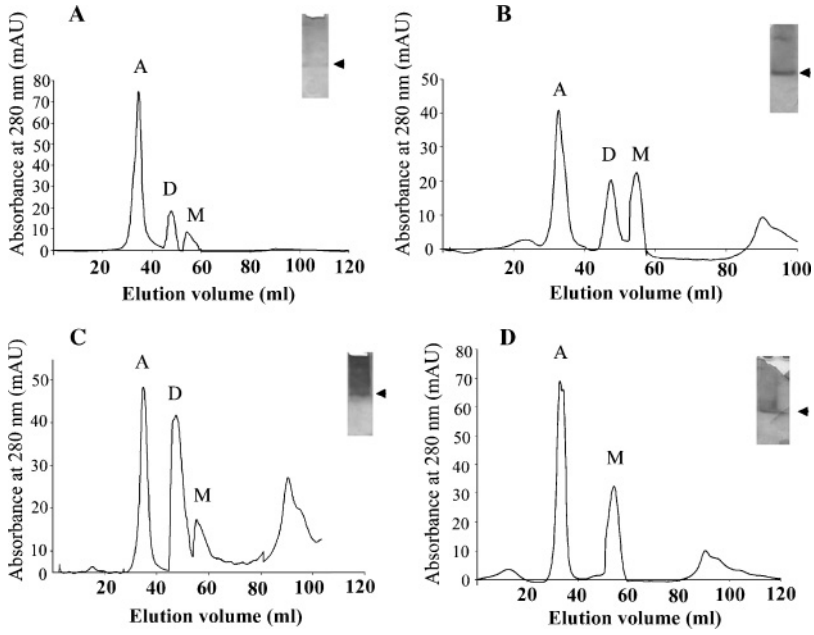

Fig. 2. Analysis of proteins refolded using the FP-1 buffer 8 (human (**A**), mouse (**B**)) and refolded using the FP-2 buffer (human (**C**), mouse (**D**)). All separations were done using the Sephacryl 100 column. Chromatograms for each of the SEC elutions are represented. The inset in each chromatogram is a silver-stained 12% continuous native PAGE loaded with 10 μL of the refolded protein solution, with the arrowhead representing migration of each refolded protein into the gel. Chromatogram labels: A represents elution of aggregates, whereas D and M denote elution of dimeric and monomeric species, respectively.

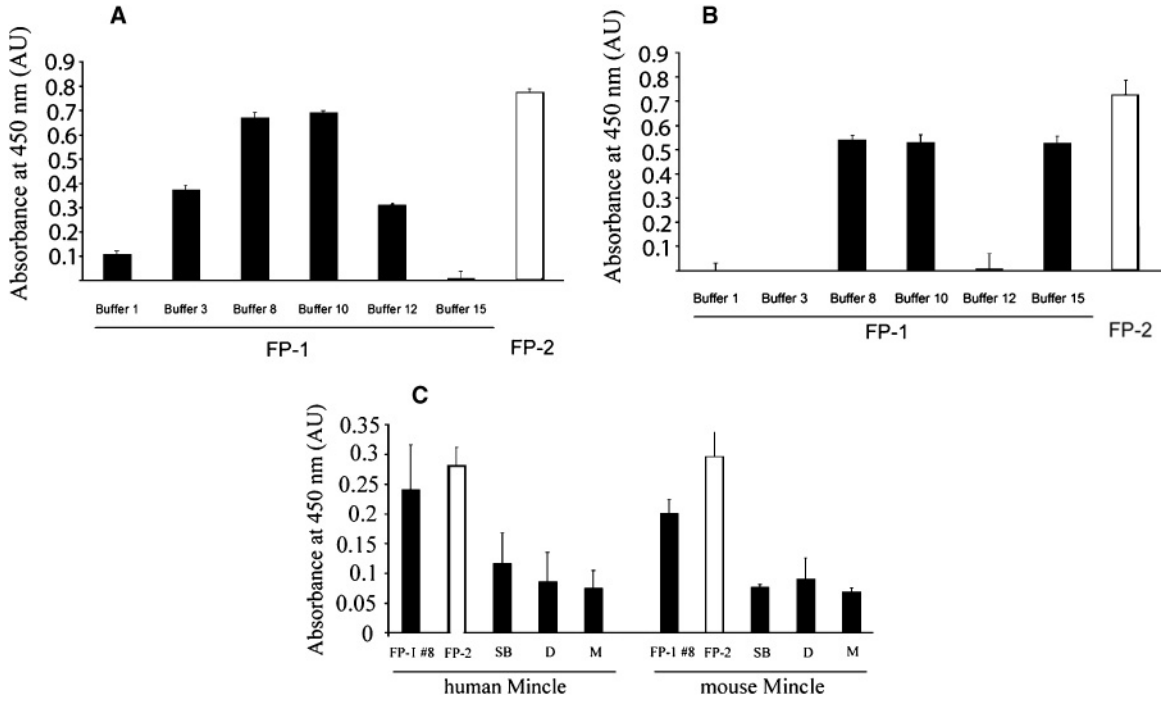

Fig. 3. Binding of the FP-1 and FP-2 refolded recombinant Mincle CRD protein to soluble extracts of yeast from *C. albicans* (SC5314). (**A**) Human and (**B**) mouse refolded protein solution (10 μL) recovered from dialysis buttons. All values represent an average of readings obtained from duplicate wells in two separate experiments. (**C**) Binding of refolded recombinant human and mouse Mincle CRD proteins (5 μg/mL per well) to whole, heat-killed *C. albicans* yeast cells (isolate SC5314). Isolated dimer and monomer CRD Mincle proteins were in the storage buffer comprising 50 mM Tris ± HCl, pH 8, and 150 mM NaCl. "SB" represents a positive control as this analysis is of human and mouse dialyzed into a storage buffer prior to ELISA analysis. All values represent an average of readings obtained from duplicate wells in two separate experiments.

(Figure 3A), adding to comparable evidence for the mouse Mincle CRD, herein and also in our recent report of mouse Mincle CRD to recognize three strains of *C. albicans* yeast extract as well as *S. cerevisiae* (Wells et al. 2008). These in vitro findings of Mincle CRD recognition of yeast component(s) are supported as being the representative of Mincle's biological function in vivo, by our work showing colocalization of endogenous Mincle and *C. albicans* in the early phagosome of the yeast-ingesting RAW264.7 macrophages (Wells et al. 2008).

The investigation provides evidence that Mincle CRD interacts with a component of the yeast; however, the nature, either proteinaceous or saccharide, of the ligand or binding partner is not identified. In order to address this we developed an alternative receptor ELISA assay using whole, heat-killed, pathogenic *C. albicans* yeast cells (strain SC5314). Refolded human and mouse Mincle CRD showed specific binding to the whole yeast cells for proteins refolded using the FP-1 buffer 8 and FP-2 buffer (Figure 3C). The human and mouse Mincle dimeric species as well as the refolded FP-2 proteins dialyzed against the storage buffer (SB; 50 mM Tris ± HCl, pH 8, and 150 mM NaCl; the same protein quantities were applied to the ELISAs before and after this dialysis) showed attenuated binding levels (Figure 3C). This attenuation may be a direct consequence of observed oligomer precipitation during dialysis, leaving a lower proportion of oligomers compared to dimers in the solution. Such oligomers would be expected to exhibit greater binding overall due to multivalency (Drickamer 1999). Whether in the biological system it is a monomeric, homodimeric, or even a higher oligomeric state of Mincle, that is, the receptor form, is as yet unknown. However, it is clear that solutions containing Mincle CRD as a mixture of protein states can bind both soluble yeast extract and whole yeast cells and that both isolated stable monomeric and dimeric protein forms are able to bind to whole yeast particles.

This first demonstration that refolded human and mouse Mincle CRD protein recognize whole yeast cells proves that the ligand for the Mincle homologues is presented on the surface of the *C. albicans* yeast particle, and thus is a constituent of the cell wall. Members of several different C-type lectin groups have been identified as receptors for *Candida* and/or *Saccharomyces* (Fradin et al. 2000; Cambi et al. 2003; Kohatsu et al. 2006; Lillegard et al. 2006). DC-SIGN is one such receptor. There is ∼45% amino acid sequence identity between the CRD of human Mincle and that of DC-SIGN and our homology modeling (MODELLER (Sali and Blundell 1993)) using the DC-SIGN crystal structure (PDB code 1SL5; (Guo et al. 2004)) as a template (*unpublished*) shows good alignment of their EPN mannose-binding motifs. This and the fact that mannose-containing oligosaccharides dominate the surface of *C. albicans* (Fradin et al. 2000) are suggestive of mannose-containing oligosaccharides being the natural ligand for Mincle. Pattern-recognition receptors necessarily exist as large protein complexes. Our data indicate the likelihood of several functional states for Mincle, which we predict, is important in providing a dynamic repertoire in the recognition of complex pathogens such as *C. albicans*.

Materials and methods

Macrophage isolation and RNA extraction

Monocyte-derived macrophages were derived from human buffy coat samples (Australian Red Cross Blood Service, Brisbane, Australia) via centrifugation over a Ficoll Hypaque™ Premium gradient (200 × *g* for 45 min; GE Healthcare, Sweden). Purification of the human monocytes was done following that reported previously (Schroder et al. 2007). Bone marrow-derived mouse macrophages (BMMs) were isolated from 6- to 8-week-old male mice and differentiated using recombinant CSF-1 (10 ng/mL Chiron Corp) as previously described (Wells et al. 2003). Total RNA was extracted from harvested cells using QIAGEN RNeasy columns and quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE). cDNA synthesis was performed using SuperScript III (Invitrogen) as per the manufacturer's protocol.

Cloning, expression, purification, and refolding of the extracellular domain of human and mouse Mincle

A human Mincle cDNA fragment corresponding to the published extracellular domain of the human CLEC4E sequence (GenBank™ accession number NM_014358) was cloned from cDNA generated (outlined above) using the forward primer 5′-GCATCATATGTGTGATGAGA- AAAAGTTTCAGC-3′ and the reverse primer 5′-GCATCTCGAGTTAAAGAGATTTTCC-TTTGTTCAAAGG-3′. The resulting fragment (representing amino acids 52–219 of Mincle) was digested with restriction enzymes NdeI and XhoI and ligated into the corresponding sites within the pET-14b vector (EMD Biosciences Novagen, WI). The mouse extracellular domain was cloned from the corresponding cDNA sequence of Clec4e (GenBank™ accession number NM_019948) using the forward primer 5′-ATGACT-ACATATGACATATCGCAGCTCTCAAATT-3′ and reverse primer 5′-TGAGGATCCTTAGTCCAGAGGACTTATTTC-3′. The fragment (corresponding to amino acids 46–214) was digested with restriction enzymes NdeI and BamHI and ligated to the corresponding sites within the pET-14b vector (Novagen) in frame with a His_6 tag. Resultant human and mouse clones were sequenced using BigDye Terminators™ and a 3130xl Genetic Analyzer (ABI Biosystems).

Protein expression trials using several different bacterial strains (BL21 (DE3) pLysS and codon-supplemented Rosettagami (DE3) pLysS) and various growth conditions (varying temperatures and IPTG concentrations) revealed that both mouse and human Mincle CRD proteins were completely restricted to the insoluble fraction. Consequently, recombinant proteins were expressed using optimal conditions of 25°C using 1 mM IPTG, purified under denaturing conditions, and subsequently refolded. Purification of insoluble Mincle CRD was done via a protocol modified from that described for the rotavirus spike protein VP5* (Denisova et al. 1999). Specifically, 500 mL of LB was inoculated using an overnight culture (1/100) and incubated at 25°C until OD_{600} reached 0.6. The culture was then induced for 3 h at 25°C using 1 mM IPTG, centrifuged at 4000 × *g* in 12256-H rotor (Sigma-Aldrich, St. Louis, MO) for 10 min, washed in PBS, and frozen at −80°C. The bacterial pellet was incubated in the lysis buffer (100 mM sodium phosphate, 150 mM NaCl, 1 mM PMSF), sonicated twice for 30 s using a tampered microtip at 20% (Vibra Cell, Sonic), and centrifuged at 20,000 × *g* for 30 min to isolate the insoluble fraction. The resulting pellet was incubated in the denaturing buffer (100 mM sodium phosphate, 8 M urea, 1 mM βME) at room temperature rotating overnight and centrifuged at 9000 rpm for 30 min in 12169-H rotor (Sigma-Aldrich). Ensuing supernatant was incubated with 5 mL of His-Select™ HF Nickel Affinity Gel (Sigma-Aldrich) pre-equilibrated in the denaturing buffer. The bound protein was washed (100 mM sodium phosphate, 10 mM imidazole, 8 M urea, 1 mM βME) and subsequently eluted using the elution buffer (100 mM sodium phosphate, 500 mM imidazole, 8 M urea, 1 mM βME). The concentration of the resulting eluate was estimated by measuring absorbance at 280 nm and aliquots stored at −80°C.

Refolding of the human and mouse CRD proteins initially purified under denaturing condition was performed via two parallel approaches. Firstly, using the FoldIT Screen (Hampton Research) where the proteins were dialyzed against respective refolding buffers using 100 μl dialysis buttons in Linbro® 24-well plates (Hampton Research) over a 7 day period; and secondly using the protocol that has been previously used for dectin-1 (Brown et al. 2007) herein termed FP-1 and FP-2, respectively. The latter method involved rapid dilution (1/200) of denatured protein (∼20 mg/mL) into a refolding buffer (Table I) and further incubation at 4°C for 48 h with gentle stirring. Alternatively, denatured human and mouse CRD proteins were refolded using a pulse renaturation method (Vallejo and Rinas 2004). This involved the addition of the denatured protein (0.15 mg/mL per pulse) to the refolding buffer every 12 h. After 10 additions, refolding was allowed to proceed for a further 48 h when the mixture was centrifuged using 10,000 × *g* at 4°C for 30 min to remove precipitate and applied to a Sephacryl 100 16/60 column.

Protein analysis and assays

Proteins were resolved on 15% SDS–polyacrylamide gels (Mini-PROTEAN 3 system; BioRad). Protein was mixed directly with a sample buffer or, if necessary, precipitated with trichloroacetic acid (TCA). Samples were denatured immediately prior to use by incubation at 95°C for 5 min in the presence of β-mercaptoethanol. Electrophoresis was done at 200 V and resolved proteins were either visualized using Coomassie Blue or transferred onto a polyvinylidene diflouride membrane (Millipore). The membrane was probed with an α-his monoclonal antibody (cell signaling) followed by a goat α-mouse antibody coupled to a horseradish peroxidase. Reactive bands were visualized by overlaying the membrane onto a chemiluminescent substrate (SuperSignal® West Pico; Pierce) after mixing the components 1:1 as outlined by the manufacturer. After a 5-min incubation, the membrane was positioned between two sheets of transparency film, exposed to light-sensitive film (CL-XPosure™ Film, Pierce), and processed manually using developer and fixer solutions (Kodak™).

Standard ELISA. The refolded protein was assayed using a standard ELISA protocol. Samples (50–100 μL) were applied to wells of microtiter dishes (Nunc, Thermo Fisher Scientific, NY) and left to adsorb for 1 h. Wells were washed with PBS/0.1% Tween 20 and blocked for 1 h with 1% BSA/0.1% Tween 20/PBS. Mincle CRD refolded protein was detected by the addition of a mouse α-Mincle antibody raised against a full-length recombinant Mincle (Clec4E, Abnova) followed

by peroxidase-conjugated goat α-mouse IgG and substrate ō-phenylenediamine (Sigma-Aldrich, St. Louis, USA).

ELISA Assay for Receptor Activity. C. albicans yeast extract (0.5 mg/mL) was applied to the wells of microtiter dishes (Nunc) and left to adsorb for 2 h. For heat-killed *C. albicans* whole yeast (OD_{600} of 0.1) incubation was at 4°C overnight. The wells were washed with PBS/0.1% Tween 20 and then blocked for a further hour by the addition of 1% BSA/0.1% Tween 20/PBS or with 5% BSA/0.1% Tween 20/PBS for yeast extract and whole yeast cells, respectively, before the addition of purified, refolded protein. The Mincle CRD protein that bound to yeast extract or to whole yeast was detected using mouse α-Mincle or α-his antibodies followed by a peroxidase-conjugated goat α-mouse antibody and substrate ō-phenylenediamine (Sigma-Aldrich). Absorbance measurements at 450 nm were taken using a Wallac Victor3 microplate reader (PerkinElmer).

Size exclusion chromatography (SEC) was carried out on purified and refolded Mincle proteins using an ÄKTAPurifier perfusion chromatography workstation and Superdex 200 or 75 (S200 and S75, respectively) or alternatively with Sephacryl S-100 16/60 columns (S100) (Applied Biosystems). All solutions were filtered through a 0.2 μm nitrocellulose membrane (Supor®-200, PALL Life Sciences) and degassed prior to use. The sample was centrifuged at 10,000 × *g* for 10 min prior to loading to remove any particulate matter. The S100 column was equilibrated in 1–2 column volumes of storage buffer (SB; 50 mM Tris ± HCl, pH 8, 150 mM NaCl) before the sample application. The sample was injected at 0.2 mL/min and allowed to elute with fractions (1 mL) collected for further analysis. The column was calibrated by the injection of a mixture of protein standards of known molecular weight (Ovalbumin, Chymotrypsinogen, and bovine serum albumin) prior to the sample run, by plotting K_{av} versus log MW (molecular weight). $K_{av} = V_e - V_o / V_t - V_o$, where V_e is the elution volume of the protein, V_o is the void volume of the column obtained from the elution of dextran blue, and V_t is the column volume (120 mL).

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Conflict of interest statement

None declared.

Abbreviations

CRD, carbohydrate recognition domain; CTLD, C-type lectin domain; DC, dendritic cells; FP, folding protocol; Mincle, macrophage-inducible C-type lectin; NK, natural killer cells; SB, storage buffer; SEC, size exclusion chromatography.

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