Molecular cloning and characterization of pig immunoreceptor \textit{DAP10} and \textit{NKG2D}

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\textit{Original Paper}

\textbf{Abstract} Pig immunoreceptor \textit{DAP10} cDNA was cloned from a peripheral blood lymphocyte (PBL) cDNA library using human \textit{DAP10} cDNA as a probe. The length of the pig \textit{DAP10} cDNA is 465 bp and it contains an open reading frame of 237 bp. The predicted polypeptide sequence is 79 amino acids, consisting of an 18-amino acid leader, a 16-amino acid extracellular domain, a 24-amino acid transmembrane segment, and a 21-amino acid cytoplasmic domain. The amino acid sequence of pig \textit{DAP10} has 68\% and 78\% sequence identity with human \textit{DAP10} and mouse \textit{DAP10}, respectively. Pig \textit{DAP10} has a conserved aspartic acid in the transmembrane domain, two cysteines in the extracellular domain, and a phosphatidylinositol-3 kinase-binding site (YxxM) in the cytoplasmic region. Genomic organization reveals that pig \textit{DAP10} comprises four exons and three introns. Pig \textit{DAP10} and \textit{DAP12} are genetically linked on Chromosome (Chr) 6 at 6q21 in opposite transcriptional orientation, separated by 152 bp. In Northern blot analysis, \textit{DAP10} transcripts were detected predominantly in lymphohematopoietic tissues. Pig \textit{NKG2D} cDNA has an open reading frame of 642 bp. Its expected polypeptide sequence is 214 amino acids. Pig \textit{NKG2D} has 66\% sequence identity with human \textit{NKG2D} and 56\% identity with mouse \textit{NKG2D}. The \textit{NKG2D} gene maps to pig Chr 5q25. RT-PCR analysis reveals that pig \textit{NKG2D} transcripts are expressed in PBLs, NK cells, macrophages, and monocytes. When transiently transfected into COS-7 cells, pig \textit{NKG2D} requires \textit{DAP10} for cell surface expression.

\textbf{Keywords} \textit{DAP10} · \textit{NKG2D} · Natural killer cells · Phagocytes · Pig

\section*{Introduction}

Natural killer (NK) cells play an important role in immune surveillance, including tumor elimination, control of infectious diseases, and cytokine production (Bancroft 1993; Trinchieri 1989). These functions can be mediated by many membrane receptors, which are divided into two groups: the C-type lectin family and the immunoglobulin superfamily (Lauring 1998). NK-cell killing is one such function involving membrane receptors. NK-cell cytotoxicity can be inhibited by many MHC class I receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs); these motifs are defined as I/VxYxxL/I (Blery et al. 1997; Daeron et al. 1995; Fry et al. 1996; Vely and Vivier 1997). Inhibitory NK-cell receptor ligation induces the phosphorylation of the tyrosine residues within the ITIM, resulting in the recruitment of the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase, SHP-1, which mediates the inhibition (Binstadt et al. 1996, 1997; Burshtyn et al. 1996; McVicar et al. 1998). However, certain subgroups of NK receptors (such as Ly49D, CD94/NKG2C, and KIR2DS) lack ITIMs and, furthermore, contain a positively charged amino acid residue in the transmembrane (TM) region (Lauring 1997; Vely and Vivier 1997). These receptors associate with \textit{DAP12}, which...
contains an immunoreceptor tyrosine-based activation motif. DAP12 is a type I membrane phosphoprotein that can bind to both ZAP-70 and Syk kinases, leading to a cascade of NK-cell activation (Lanier et al. 1998a, 1998b; McVicar et al. 1998).

Recently, a new membrane adaptor molecule DAP10 was reported to contain a phophatidylinositol-3 (PI-3) kinase-binding site (YxxM) in the cytoplasmic domain (Wu et al. 1999). DAP10 forms a complex with NKGD2 which is expressed on NK cells, CD8αβ T cells, and γδ T cells (Bauer et al. 1999; Wu et al. 1999). The human NKGD2 receptor binds to MICA or MICB (MHC class I chain-related A or B), which are expressed on epithelial-derived tumor cells (Bauer et al. 1999; Groh et al. 1999). Thus, tumor cells expressing MICA/B are sensitive to lysis by NK cells and γδ T cells (Bauer et al. 1999).

In this paper, we describe the cloning, gene structure, and expression pattern of the pig DAP10 and NKGD2 genes.

Materials and methods

Experimental animal and blood cell isolation

Young specific pathogen-free (SPF) Minnesota miniature swine maintained at FUHS/The Chicago Medical School under the absolute barrier-sustained SPF miniswine facility with sterile feed, water and air (Kim et al. 1980) were used for all the experiments. After peripheral blood mononuclear cells (PBMCs) had been harvested through a Ficoll-Hypaque gradient, monocytes were recovered by plastic adherence, and peripheral blood lymphocytes (PBLs) were recovered from the media (Kim et al. 1980). Polymorphonuclear granulocytes (PMNs) were obtained through dextran sedimentation (Wierda et al. 1993). Pulmonary alveolar macrophages (PAMs) were collected by lung lavage (Rothlein et al. 1981). NK cells were isolated from PBLs using G7 (anti-CD16) monoclonal antibody (mAb)-coated plates by panning. For cell activation, PAMs and PMNs were cultured with human interferon (IFN)-γ (300 ng/ml) for 18 h, and then the culture medium was replaced with fresh medium containing lipopolysaccharide (LPS, 200 ng/ml). The cells were harvested from the culture 48 h after LPS stimulation.

cDNA library construction

Total RNA from pig PBLs was isolated using STAT-60 (TEL-Test, Friendswood, Tex.). Poly(A)+ RNA was purified from total RNA using a Fast Track 2.0 kit (Invitrogen, Carlsbad, Calif.). Four micrograms of pig PBL poly(A)+ RNA was used to synthesize cDNA using the Universal Ribolclone cDNA synthesis system (Promega, Madison, Wis.). Pig PBL cDNA whose size was 350–500 bp was eluted from the gel for ligation to EcoRI adapters. The pig PBL cDNA library was constructed in Lambda ZAPII (Stratagene, La Jolla, Calif.), packaged in vitro using Gigapack extract (Stratagene), and then transfected into XL-1 Blue MRF’ (Stratagene).

Cloning of human DAP10

Human DAP10 cDNA was amplified from human PBMC mRNA by RT-PCR using human DAP10-specific primers and used as a probe (Wu et al. 1999). The human DAP10 cDNA PCR product was cloned into the pGEM-T easy plasmid (Promega). (Note: The sequence data are available from GenBank (accession number AF285447).)

Library screening and DNA sequencing

The screening of the library was performed as described previously (Yim et al. 2000). Human DAP10 cDNA was labeled with [α-32P]dCTP and then used as a probe. The plasmids from colonies were isolated and subjected to Southern blot analysis to confirm that the plasmid contained pig DAP10 cDNA. DNA sequencing was performed with an automated sequencer at The University of Chicago by PCR amplification using a fluorescent dideoxynucleotide dye terminator.

Northern blot analysis

Total RNA was extracted from PBLs, PMNs, PAMs, monocytes, spleen, liver, thymus, lymph nodes, muscle, and mammary gland using STAT-60 (TEL-Test). Twenty micrograms of total RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde. Northern hybridization was performed with [α-32P]dCTP-labeled pig DAP10 cDNA in hybridization solution [0.25 M Na2HPO4, 7 ml/L, 1 mM EDTA, 0.5% blocking reagent (NEN Life Science Products), 5% SDS]. Hybridization was performed at 65°C overnight. The membrane was washed in washing solution I [2× saline–sodium phosphate–EDTA (SSPE), 2% SDS] at 65°C and then in washing solution II (0.2×SSPE, 0.1% SDS) at room temperature. As a control, pig β-actin PCR product was labeled with [α-32P]dCTP and used as a probe.

Cloning of the pig genomic DAP10 gene

A total of 200 ng of pig genomic DNA was used as a template to amplify the pig genomic DAP10 gene using a forward primer (CTTCTGTGGGACCCAGACCTCCCTCC) and a reverse primer (TTGCAGGTTAAGCGCTTATAGCGG). PCR was performed for 35 cycles as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. PCR products were isolated from the gel and ligated into the pGEM-T easy plasmid (Promega) for sequencing.

Cloning of pig NKGD2 cDNA and RT-PCR analysis

Pig NKGD2 cDNA was cloned by RT-PCR using degenerate primers based on human and mouse NKGD2 sequences. Pig PBL cDNA was amplified using forward primer T(G/A)AT GG(G/C)(G/ATG(T/G)GATTCCGTG(G/A)TCG(G/A)AG TCTC and reverse primer T(C/T)TTTACACA(C/G)(C/T) CCTT(G/T)CTAGCGATGTA for 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min. One microgram of total RNA from PBLs, NK cells, PAMs, LPS-stimulated PAMs, PMNs, LPS-stimulated PMNs, and monocytes was used to synthesize cDNA using the Advantage RT for PCR kit (Clontech, Palo Alto, Calif.). PCR was performed with cDNAs using forward primer (GTCCATGCCTAAAAACTGGAATG) and the degenerate reverse primer mentioned previously.

Chromosomal localization of pig DAP10 and NKGD2

Physical mapping was done by PCR amplification on a somatic cell hybrid panel for pig gene mapping (Yerle et al. 1996). The primer sequences were as follows for DAP10 and DAP12: CAAAATCTACAATCAACATCAGCC (forward), GATGTGTCACAGCGGACTCTCACAC (reverse); and for NKGD2: TAAATGAGACGAAGACTCCTGCC (forward), GTTGTAGTGA GAGGATGAA (reverse). The temperature profile included 1 cycle at 95°C for 2 min, followed by 35 cycles at 94°C for 30 s,