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Research paper

A platform for complementation and characterization of familial haemophagocytic lymphohistiocytosis 3 mutations

Edo Elstak, Arjan de Jong, Peter van der Sluijs *

Department of Cell Biology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands

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ABSTRACT

Mutations in *UNC13D* cause the severe immune disorder familial haemophagocytic lymphohistiocytosis type 3 (FHL3). The gene product munc13-4 is expressed in hematopoietic cells and is essential for degranulation. Little information is available on genotype–phenotype relationships of *UNC13D* mutations. Some mutants may have residual functionality which qualifies them as promising targets for attempts to enhance function pharmacologically. A problem for such analysis is the scarcity of patient material. We established assays in the RBL-2H3 cell line to assess functionality of lentivirally transduced munc13-4 mutants. The basic principle of which is to silence endogenous rat munc13-4 and replace it with siRNA resistant YFP-tagged human variants. Localization, degranulation, and membrane binding kinetics can now easily be analyzed quantitatively. Such a system might also be useful to screen small molecular weight compounds for their ability to rescue degranulation in cells with reduced functional munc13-4.

1. Introduction

FHL is a genetically heterogeneous disorder, characterized by defective cytotoxic T lymphocyte (CTL) and natural killer (NK) cell activity, and hypercytokinaemia (Arico et al., 1988; Henter et al., 1991; Filipovich, 2006). FHL is fatal unless treated by immune suppression and hematopoietic stem cell transplantation (Cesaro et al., 2008; Ohga et al., 2010). FHL links to five genetic origins: chromosome 9, *PRF1*, *UNC13D*, *STX11* and *STX11BP2* (Stepp et al., 1999; Feldmann et al., 2003; zur Stadt et al., 2005, 2009). Mutations in *UNC13D* are classified as FHL3 and the protein munc13-4 is necessary for maturation of lytic granules and for their docking at the immunological synapse (Feldmann et al., 2003; Menager et al., 2007).

Munc13-4 involvement in secretory lysosome release has been established in neutrophils, platelets, NK cells, CTL, and RBL-2H3 cells (Feldmann et al., 2003; Shirakawa et al., 2004; Neeft et al., 2005; Pivot-Pajot et al., 2008). The RBL-2H3 cell line has been used extensively as a (mast cell) model for degranulation (Kapp-Barnea et al., 2003; Nomura et al., 2009; Tadokoro et al., 2010), and the functional analysis of ectopically expressed perforin mutations (Risma et al., 2006). The cells exhibit properties common to basophils and mast cells; both degranulate after dimerization of the IgE bound FcεRI by multivalent antigens (Kepley et al., 1998; Gilfillan and Tkaczyk, 2006). Proximal signalling of the receptor leads to activation of PKC and elevated levels of Ca^{2+}. Secretory lysosomes release their content by non-polarized compound exocytosis that is microtubule dependent and regulated by rab27a/b (Rohlich et al., 1971; Roa et al., 1997; Smith et al., 2003; Nomura et al., 2009). Many components of the signalling and fusion machinery are shared with CTL. The degranulation pathway of RBL-2H3 cells therefore provides a relevant immunological model system to study the functionality of munc13-4 and FHL3 mutants.

Mutations in *UNC13D* are scattered over the entire gene sequence and do not seem to cluster in specific areas (Santoro et al., 2006; Rudd et al., 2008). They cause single amino acid substitutions, frame shifts, deletions and premature stop codons and might be responsible for the different onset and outcome of FHL3. This diversity impairs prediction of disease severity by gene analysis. Thorough analysis of patient material is often hampered

Abbreviations: FHL, familial haemophagocytic lymphohistiocytosis.

* Corresponding author. Dept of Cell Biology, UMC Utrecht, NL-3584 CX Utrecht, The Netherlands. Tel.: +31 88 7557578; fax: +31 30 2541797.
E-mail address: p.vandersluijs@umcutrecht.nl (P. van der Sluijs).

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by availability. Limited data exists on munc13-4 mutants that goes beyond expression at mRNA and protein levels, and derives mainly from the *JINX* mouse (Crozat et al., 2007). We therefore established a robust knock-in system in which silencing of endogenous munc13-4 is combined with stable lentiviral transduction of YFP-tagged munc13-4 constructs. Advantages of this platform include the application of versatile cell assays to quantitative (residual) munc13-4 function, and to rank severity of FHL3 mutations. It also offers the possibility to test small molecular weight compounds for their ability to restore secretory lysosome degranulation in FHL3.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies were obtained from the indicated sources: p80 mouse hybridoma 5G10 (J. Bonifacino, NIH Bethesda), mouse anti serotonin (DAKO), mouse anti rat CD63 (BD Biosciences), mouse IgE anti DNP clone SPE-7 and corresponding antigen HSA-DNP (Sigma), mouse anti GFP (Roche), conjugated secondary antibodies (Jackson Immuno Research Laboratories). The rabbit antibody against munc13-4 has been described (Neeft et al., 2005). Following plasmids were generously provided by indicated colleagues: peYFP-N1-Munc13-4 cDNA (V.Gerke, ZMBE Münster), pLNT-SFFV-WPRE-Gateway (G. Griffiths, CIMR Cambridge), lentiviral helper plasmids psPAX2 and pMD2.G (A. Mertens UMC Utrecht). cDNAs encoding human munc13-4 and munc13-4-Δ608-611 were generated previously in this lab (Neeft et al., 2005).

2.2. Cell culture

The RBL-2H3 cell line was generously provided by Ronit Sagi Eisenberg (Tel Aviv University) and cloned by limited dilution to obtain clones in which β-hexosaminidase secretion could be stimulated to yield at least 30% release. The cells were cultured in DMEM containing 1 mg ml^{-1} glucose, 10% heat-inactivated FCS (Bodinco), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2 mM L-glutamine, 50 nM 2-mercaptoethanol and passaged 3 times a week 1/20. Degranulation of RBL-2H3 cells is influenced by FCS and care was taken to obtain a batch that did not affect degranulation. HEK293T were cultured in DMEM (Invitrogen) 5 mg ml^{-1} glucose, 10% heat-inactivated fetal calf serum (Sigma), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine.

2.3. Lentivirus preparation

YFP-tagged munc13-4 cDNAs were extended with attB sites for cloning in pDONr201 (Invitrogen). After amplification inserts were transferred to the lentiviral plasmid pLNT–SFFV–WPRE–Gateway (Demaison et al., 2002) using LR Clonase II (Invitrogen). This lentiviral plasmid was developed to induce gene expression in hematopoietic cells using a spleen focus forming virus (SFFV) promoter, Woodchuck hepatitis virus posttranscriptional regulatory element (WPRE), central polypurine tract (cPPT) and appended with a gateway cloning cassette between the long terminal repeats (LTR). Primers GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTGAGCAAGGGCGAGGAGCTG (f), GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACGGTGCCGGCCGCAAGGC (r) were used for YFP on the N-terminus, and GGGGACAAGTTTGTACAAAAAAGCA GGC TTCATGGCGA CACTCCTCTCCCATCC (f), and GGGGACCACTTTGTACAAG AAAGCTGGGTCTTACTTGTACAGCTCGTCCATGC for YFP at the C-terminus. All constructs obtained through PCR were verified by sequencing. VSV-G pseudotyped lentivirus was prepared in low passage number HEK293T cells. To this aim 3×10^6 cells were seeded in a 10 cm dish and transfected the next day with 16 μg 1/1.1 ratio of pLNT–SFFV–WPRE–cDNA, the packaging plasmid psPAX2 and pMD2.G using calcium phosphate transfection. After 18 h medium was replaced with 6 ml fresh medium. Twenty four hours later, virus was harvested and new medium added for a final period of 24 h. Virus containing supernatants were stored at 4 °C, pooled and filtered through 0.2 μm. Virus stocks were concentrated 100 fold by centrifugation for 2 h at 25,000 rpm in a SW-28 rotor. The concentration procedure removed HEK293T medium which was detrimental to normal RBL-2H3 growth. Virus was resuspended in 0.01 volume RBL-2H3 medium supplemented with 20 mM Hepes pH 7.6, aliquotted and stored at −80 °C. Virus efficiency was determined by titration on HEK293T and RBL-2H3 cells through fluorescence microscopy and western blot detection.

2.4. Generation and maintenance of stable RBL-2H3 lines

RBL-2H3 cells stably expressing lentiviral constructs were made by infecting a 50% confluent 10 cm dish with 10 μl of concentrated virus in the presence of 6 μg ml^{-1} polybrene in 10 ml medium. Medium was replaced after 24 h and cells were allowed to expand for another 24 h. Cells were then cultured and evaluated for expression. Typical transduction efficiencies were 70%. Cells were collected after two weeks for FACS sorting and we sorted a 99% positive cell population of at least 5×10^5 cells using a FACSAria (BD). Cells were then transferred to 10 cm dishes for maintenance culture. Lentiviruses reverse transcribe their RNA and integrate the newly made DNA into the chromatin (Bukrinsky et al., 1993). In our hands selection or resorting was not needed within 2 months after transduction, since expression levels remained constant during this period. If expression drops however, cells can easily be sorted as above to enrich for transfectants with higher expression.

2.5. Western blot and siRNA

For Western blot, 1×10^6 RBL-2H3 cells were lysed in 250 μl 1% Triton X-100, 100 mM NaCl, 50 mM Tris–HCl pH 7.6. Lysates were cleared by centrifugation and collected in 5× Laemmli buffer containing 10% SDS, 50% glycerol, 0.625 M Tris–HCl pH 6.8, 250 mM DTT 0.01% Bromophenol blue. Samples were electrophoresed through 7.5% SDS-PAA gels and blotted on Immobilon-FL PVDF (Millipore). Blots were analyzed for munc13-4, YFP and actin using primary antibodies described above. Secondary alexa680 or IrDye800 fluorescent antibodies were used for detection in the Odyssey imaging system (Li-Cor). Antibody incubations were typically 45 min, followed by three washing steps in blocking buffer (Li-Cor). siRNA depletion of endogenous rat munc13-4 in RBL-2H3 cells was done through a sequence targeting the ORF of rat munc13-4; GGAACAAGAUUUUUCACAAtt (Applied Biosystems). The siRNA was introduced using AMAXA nucleofection (Lonza), 100 μl buffer T, protocol X-001 according to manufacturers' instructions.