Published in final edited form as:

Biochem Biophys Res Commun. 2009 November 13; 389(2): 284–289. doi:10.1016/j.bbrc.2009.08.140.

Identification of X-DING-CD4, a new member of human DING protein family that is secreted by HIV-1 resistant CD4+ T cells and has antiviral activity

Adam Lesner¹, Rasheda Shilpi¹, Anna Ivanova¹, Mary Ann Gawinowicz², Jacob Lesniak³, Dimitar Nikolov³, and Malgorzata Simm^{1,*}

¹Protein Chemistry Laboratory, St. Luke's/Roosevelt Institute for Health Sciences, Columbia University, New York, NY 10019, USA

²Protein Chemistry Core Facility, Columbia University, New York, NY 10032, USA

³Laboratory of Structural Biology and Neuroscience, Memorial Sloan-Kettering Cancer Center, New York, NY 10065, USA

Abstract

We reported previously the antiviral activity named HRF (HIV-1 Resistance Factor) secreted by HIV-1 resistant cells. This work describes the identification of HRF from cell culture supernatant of HRF producing cells (HRF(+) cells). Employing the proteomics and cell based activity assay we recovered ten peptides sharing 80-93% sequence homology with other eukaryotic DING proteins; discrete amino acid characteristics found in our material suggested that HRF is a new member of DING proteins family and consequently we designated it as X-DING-CD4 (extracellular DING from CD4⁺T cells). The presence of X-DING-CD4 in the extracellular compartment of HRF(+) but not control HRF(-) cells was confirmed by specific anti-X-DING-CD4 antibody. Similar as the unfractionated HRF(+) cell culture supernatant, the purified X-DING-CD4 blocked transcription of HIV-1 LTR promoted expression of luciferase gene and replication of HIV-1 in MAGI cells. The X-DING-CD4 mediated antiviral activity in MAGI cells could be blocked by specific antibody.

Keywords

HIV; resistance; CD4⁺T cells; DING protein

Introduction

The first report of a novel protein equipped with characteristic DINGG N'terminal sequence emerged several years ago [1]. Since then several groups reported isolation of similar proteins expressed in cells of prokaryotic and eukaryotic organisms [2;3;4;5]. All of these proteins had highly conserved N'terminal sequence and based on this unique amino acid alignment they have been clustered into a new DING protein family [6]. Despite a notable sequence homology,

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* Address correspondence to: Malgorzata Simm, Ph.D. St.Luke's/Roosevelt Institute for Health Sciences, Molecular Virology Division, 432 West 58th St. Rm. 709, New York, NY 10019 ms130@columbia.edu

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DING proteins acquired diverse regulatory functions [2]. For example, prokaryotic DING proteins are related to the superfamily of phosphate-binding proteins [2] while in plants, DING proteins associate with germin-like proteins [6;7]. Although there is no specific function designated to plant DING proteins, it has been suggested that they could regulate cell signaling between intra- and extracellular spaces [7]. DING homologue, p27SJ isolated from *Hyperforicum perforatum* was found to block HIV-1 in human cells [8]. The recent report suggested that the phosphatase activity of p27SJ protein has an impact on cell growth and function [9].

Human DING proteins have been detected in a variety of tissues and frequently were linked to various organ pathologies [1;10;11]. A review of biological functions of DING proteins as a group revealed wide array of their activities such as neuronal [12] and skeletal muscle cell signaling [13], lymphocyte proliferation [1], or blocking adhesion of crystals to renal epithelial cells [14]. The X-ray structure of another DING member, a human plasma phosphate binding protein (HPBP), revealed its strong similarity to prokaryotic phosphate solute binding proteins (SBPs) and suggested potential role in transport of phosphate ions in human plasma [4].

Our group studied novel mechanisms of antiviral activity in CD4 + T cells that is maintained by soluble factors secreted by HIV target cells. We established a cellular model based on transformed CD4⁺ T cells called HRF(+) (HIV-1 Resistance Factor +) [15;16;17;18;19;20;21]. The HRF(+) cells were resistant to infection by HIV-1 [18] and the activity secreted by HRF(+) cells blocked transcription of virus in susceptible primary and transformed CD4⁺ T cells [17;22]. Using two known characteristics of HRF - the solubility and its LTR transcription inhibitory properties we combined proteomics and 1G5 cell [23] based rapid suppression assay (RSA) to isolate and identify HRF from HRF(+) cell culture supernatant. During this procedure we identified the active moiety from HRF(+) cell culture supernatants as 40 KDa protein matching the HPBP amino acid sequence [24]; however some differences in amino acid composition suggested that the identified protein is not HPBP and consequently, we designated this new DING protein as X-DING-CD4 (extracellular DING from CD4 cells).

Materials and Methods

Cell cultures, viruses and reagents

HRF(+) and HRF(-) cells distinguished by HIV-1 resistance and HIV-1 susceptibility have been described previously [16;17;18;20;21;25;26]. 1G5 cells stably expressing luciferase gene driven by HIV-1 long terminal promoter (LTR) [23] and Magi-CCR-5 cells [27] were obtained through NIH AIDS Research and Reagent Program Division of AIDS, NIAID; human monocytes from healthy, HIV-1 negative volunteers were obtained by elutriation from the whole blood. Monocytes were allowed to differentiate in 12-well plates (Corning Scientific) under culture conditions as described before [25;26;28].

Continuous cultures of 1G5, HRF(+), and HRF(-) cell lines were maintained in RPMI 1640 (Sigma) supplemented with 5% fetal bovine serum, antibiotics and glutamate; MAGI-CCR-5 cells were cultured in DMEM supplemented with 10% fetal bovine serum, selective antibiotics and glutamate at 37°C in a 5% CO₂ 95% air-humidified incubator.

The rabbit polyclonal antibody to X-DING-CD4 was raised against a synthetic 23 amino acid X-DING-CD4 peptide derivative coupled to KLH (keyhole limpet hemacyanin) (Genmed Synthesis, Inc).

To prepare biologically active supernatants HRF(+) and control HRF(-) cells were cultured in protein-free hybridoma medium (Sigma) as described before [16;17]. Briefly; 2×10⁶/ml HRF(+) and control HRF(-) cells were cultured overnight in protein free Hybridoma medium

(Sigma) supplemented with glutamate. Subsequently, cells were removed by centrifugation and supernatants were filtered through 0.45µm membranes (Millipore) and concentrated by lyophilization from 30-ml aliquots using Labconco Lyophilizer. Protein powder was resuspended in 10 ml distilled water and subjected to dialysis against 10mM Tris-Cl, pH 7.4 using benzoylated cellulose tubing with MW cut-off of 1.2KDa (Sigma). Dialyzed material was concentrated again by lyophilization and stored at 4°C. For testing the biological activity by rapid suppression assay (RSA) [25] lyophilizates were resuspended in sterile distilled water to 100x concentrate followed by second dilution in Hybridoma medium (Sigma) to 1x concentrate.

The purification and identification of X-DING-CD4

The starting material for purification of HRF was clarified, filtered supernatant from overnight cultures of HRF-producing cells in protein free hybridoma medium prepared as described above. This material was separated during 40min. 0-100% linear gradient of B (Tris-Cl (pH 8.1) +1M NaCl) at flow of 3.0ml/min through 5ml ion exchange High Trap Q XL column by Acta Prime chromatography system (all from Pharmacia). Eluted protein peaks were dialyzed and tested by rapid suppression assay (RSA) to identify the HRF fraction. RSA measures the expression of HIV-1 LTR promoted luciferase gene and has been our standard assay to detect the transcription inhibitory activity of HRF [16;17]. Subsequently, the multiple HRF fractions from IXC representing total of 1.5L HRF(+) cell culture supernatant were pooled and subjected to further separation by fast performance liquid chromatography (FPLC) Superdex G-75 GF column (all from Pharmacia) in GF-1 buffer (40mM Tris-Cl, pH8.0, 2mM MgCl₂, 200mM KCl).

To confirm the purity of the isolated HRF, the biologically active fraction was resolved through 7-10% step gradient PAGE and proteins were visualized by MS compatible EZBlue gel staining reagent (Sigma). This final purification stage produced only one visible protein band migrating with the mobility of approximately 40KDa protein; this protein band was excised from the gel and was reduced with DTT and alkylated with iodoacetamide, then washed several times with 30% acetonitrile/ 0.05 M Tris-HCl, pH 8.5 to remove residual stain and alkylating reagent. The protein sample was digested overnight with 100ng of modified endoproteinase Lys-C (sequencing grade, Roche Molecular Biochemicals, IN) in 0.025M Tris, pH 8.5 at 32°C. Peptides were extracted from the gel with 50% acetonitrile/2% TFA and subjected to LC-MS/MS analysis on a Micromass Q-ToF hybrid quadrupole/time-of-flight mass spectrometer with a nanoelectrospray source. Capillary voltage was set at 1.8kV and cone voltage 32V; collision energy was set according to mass and charge of the ion, from 14eV to 50eV. Chromatography was performed on an LC Packings HPLC with a C18 PepMap column using a linear acetonitrile gradient with flow rate of 200 nl/ min. Raw data files were processed using the MassLynx ProteinLynx software with the MaxEnt algorithm and the resulting .pkl files were submitted to a Mascot search (www.matrixscience.com).

Testing the antiviral activity of X-DING-CD4

The antiviral activity of X-DING-CD4 and its synthetic peptide derivatives was tested by Magi assay [27]. Briefly: MAGI-CCR-5 cells were seeded 24 hour prior to assay in a 96-well plate (Costar) at 6.2×10^3 cells per well in DMEM supplemented with 10% fetal bovine serum, antibiotics and glutamate. Subsequently, cells were exposed to treatments in the presence of DMEM supplemented with 5% fetal bovine serum, antibiotics, glutamate and 20µg/ml DEAE dextran. Twenty four hours later cells were infected with 0.1pg/cell NL4-3 HIV-1 isolate [29]. Replication of virus was evaluated forty eight hours later after fixation of cells with 1% formaldehyde and 0.2% glutaraldehyde in PBS. Expression of β-galactosidase was visualized by 50 min exposure to X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 0.4mg/ml 2mM MgCl₂, 4mM potassium ferricyanide at 37°C. After enumeration of the infected (blue)

cells, all cells were lysed and subjected to the protein assay (Bio-Rad). All data were adjusted by total protein concentration and anti-viral activity of X-DING-CD4 or its peptide derivatives was calculated as percent inhibition.

Results

The IXC chromatography separated the HRF(+) material into seven fractions (Fig. 1A). Based on RSA measurements the biological activity defined for the un-fractionated HRF(+) cell culture supernatant eluted after 30 minutes gradient at high salt concentration (Fig. 1A & C). The subsequent separation of biologically active material eluted from IXC column by FPLC produced three protein peaks with biological activity detected in fraction 1 (Fig. 1B & C). To confirm the purity of the isolated HRF, this material was resolved through 7-10% step gradient PAGE and proteins were visualized by MS compatible EZBlue gel staining reagent (Sigma) (Fig. 1B insert). This final purification stage produced only one visible protein band migrating with the mobility of approximately 40KDa protein.

Mass spectrometry analysis identified four peptides matching to one partial protein sequence in the NCBI database, GLP-binding protein 1a, gi:29373999, from *Arabidopsis thaliana* with a combined score of 133 (Table 1). Except for the four peptides which matched to a known fragmentary plant sequence, much of the tandem mass spectral data remained unassigned and unmatched. We attempted to manually interpret as much sequence as possible from the MS/MS spectra as well as verify the peptide sequences identified through the Mascot search program. In addition to the four known sequences (AAFLTNDYTK, FVAGVSNK, NVHWAGSDSK, LTATELSTYATNK), five more stretches of sequence were identified as shown in Table 1: DINGGGATLPQK, VLTAGFAPYLGVGSGNGK, QVPSVATSVAL, FAASTLAGLDDATK, and TNVCNGLGRPL which showed high homology to other members of DING protein family [8;24] (Fig. 2).

We compared peptides found in our material to two most complete eukaryotic DING proteins: p27SJ - a plant DING protein that has antiviral activity [8] and HPBP [24] (Fig. 2). This alignment showed that DING protein isolated from HRF material is related closer to human HPBP than to a plant derived p27SJ; discrete differences detected in our material suggested however, that protein secreted by HRF(+) cells is a new member of the DING family, and we designated it as X-DING-CD4 (extracellular DING from CD4+ T cells).

The unique feature of X-DING-CD4 extends between amino acids in position 41-52 where the X-DING-CD4 peptide TNDYK FVAGVSN does not align to any known eukaryotic DING proteins. Other unique features found in X-DING-CD4 are: (1) the substitution of (218) proline by polar serine; (2) substitution of (372) lysine by leucine and (4) substitution of (376) glutamine with leucine. Also intriguing is the presence of a small fraction of X-DING-CD4 64-76 peptides distinguished by methylated glutamic acid in position 68. This modification of X-DING-CD4 was found in 10% of 64-76 peptides. Methyl esterification of glutamic acid was first reported by Hoelz et al [30] as a novel posttranslational modification distinguishing diverse isoforms of proliferating cell nuclear antigen (PCNA). We believe that presence of methylated glutamic acid in fraction of X-DING-CD4 indicates presence of various isoforms of this protein and might be related to its function as inhibitor of HIV infection.

To confirm the presence of X-DING-CD4 in the extracellular material of HRF(+) cells we synthesized a 21AA peptide (XD-1: N'-GTKNVHWAGSDSKLTATELSTY(C)-C') coupled with KLH (keyhole limpet hemacyanin) through C' cysteine and used it for induction of specific anti-X-DING-CD4 antibodies in rabbits (Genmed Synthesis, Inc). This antibody was used for WB evaluations upon the IXC purified X-DING-CD4 and control HRF(+) and HRF(-) cell culture supernatants and detected a protein band migrating with similar mobility to

protein separated in Fr.# 1 during FPLC purification. This protein band was present only in material harvested from HRF(+) but not HRF(-) cells (Fig. 3A).

Subsequently, we tested the capacity of the IXC purified X-DING-CD4 to block replication of HIV-1 in a single cycle infection employing Magi assay. As shown in Fig. 3B both the un-fractionated HRF(+) cell culture supernatant and IXC purified X-DING-CD4 inhibited replication of HIV-1 by up to 62% and 66% respectively; while control HRF(-) material or hybridoma medium blocked virus by 29% and 17% correspondingly. Incubation of X-DING-CD4 with specific antibody but not control isogenic IgG reduced the antiviral activity of this material by 65% (Fig. 3C).

The two known characteristics of HRF: solubility and inhibition of LTR transcription were tested throughout the identification of antiviral activity secreted by HIV-1 resistant cells. The WB analysis confirmed that extracellular material of HRF(+) cells contains a soluble protein recognized by specific antibody; while RSA showed that purified X-DING-CD4 blocked HIV-1 LTR transcription with higher efficiency than the un-fractionated material. The third characteristic of HRF activity, protection of HIV-1 susceptible cells from virus infection was confirmed by Magi assay and this antiviral activity could be depleted by specific antibody. Based on this evidence we conclude that the soluble antiviral activity secreted by HRF(+) cells is X-DING-CD4.

Discussion

We have identified a human DING protein that has potential to block HIV transcription. This potent anti-viral activity carried by product of human gene indicates that our cells have potential to block HIV invasion. So what is the reason that this protective response to virus is not rapidly activated in human CD4⁺ T cells upon HIV infection? One explanation points to presence of restrictive mechanism regulating transcription of human DING proteins. In particular, difficulty in retrieving the complete cDNA sequences from human cells suggests very low/restricted transcription activity or short life-time of its mRNA. It has been suggested that expression of DING proteins in human cells could be temporally or evolutionary restricted by yet unknown mechanisms [31]. Our findings support this hypothesis. The isolation of X-DING-CD4 from CD4⁺ T cells that are resistant to HIV infection indicates the induction of a novel protective pathway that enables constitutive expression of this protein. This pathway could be induced by prolonged cell exposure to replication-defective HIV-1 [18]. We believe that during this exposure to the attenuated form of virus, restrictive mechanism regulating expression of DING protein was shut down resulting in the induction of protective cellular responses involving actions of X-DING-CD4 and CTCF transcription regulator [22].

Similar like several other human DING proteins [4;14;32], X-DING-CD4 was isolated from an extracellular compartment indicating that this is a soluble protein. This observation was predicted in our previous reports showing that exposure of HIV susceptible primary and transformed cells to un-fractionated HRF(+) cell culture media and IXC purified X-DING-CD4 resulted in the protection of these cells from virus infection [17;22;25]. We hypothesize that the X-DING-CD4 could act upon HIV target cells through the ligation to a cell surface receptor which led to activation of downstream events including induction of CTCF transcription. The signaling function of other eukaryotic DING proteins have been described for neuronal [12] and skeletal muscle cells [13].

Human DING proteins have been detected in a variety of pathological and non-pathological tissues [1;10;11] but gene coding sequences remain unknown. Up to date several partial cDNA sequences of eukaryotic DING protein have been deposited in data banks but the complete amino acid sequence derived from coding cDNA is unknown; and only complete human DING

protein, the HPBP amino acid sequence, was assigned from the electronic density map [24]. Our attempts to define X-DING-CD4 coding sequences resulted in recovery of 387bp fragment homologous to partial genomic DNA sequence (CW626261) published in GenBank (data not shown).

Failure to isolate human DING gene sequences promoted hypothesis that these proteins found in human tissues could originate from symbiotic bacteria. Both pros and cons to this proposition have been discussed in recently published review by Berna et al [31]. We argue this theory based on following observations. Almost all human DING proteins isolated from human tissues have diverse physiological functions depending on the compartment they have been isolated from. It would be unlikely that one bacterial protein could exert so diverse activity upon various tissues. Also most of human DING proteins were found in tissues such as synovial fluid, renal epithelium, serum or brain. These body compartments are not a physiological environment for bacteria or bacterial products and their presence in blood or brain most likely would have triggered immune responses followed by accumulation of specific antibodies. The final argument supporting the human origin of X-DING-CD4 is presence of two isoforms distinguished by rare methyl esterification of glutamic acid in peptide 64-76. Methylation in mammalian cells is an irreversible post-translational modification; presence of 10% methylated X-DING-CD4 in our material indicates active mechanism regulating function of this protein in HIV resistant CD4⁺ T cells and could be linked to antiviral activity.

Identification of X-DING-CD4 characterized by potent antiviral activity implies yet another function of DING proteins - their role in the innate responses to HIV-1 infection. Further investigation of X-DING-CD4 mediated non-canonical mechanism to block virus replication in CD4⁺ T cells will define this novel pathway and its translational potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

IG5 cells were obtained through the NIH AIDS Research and Reagent Program, Division of AIDS, NIAID, NIH. This work was supported by AI48388 AI061286 and from the National Institutes of Health.

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Fig. 1. Purification of HRF from cell culture supernatant
Cell culture supernatant was separated through (A) ion exchange High Trap QXL column; active fraction harvested from IXC purification was separated by FPLC using Superdex G-75 GF column. Arrows indicate fractions with HRF activity; insert shows 7-20% step gradient SDS-PAGE analysis of HRF(+) peak. (C) RSA on pooled protein fractions from the subsequent purification steps. RSA values show percent inhibition of HIV-1 LTR-promoted expression of luciferase gene by 0.5%/Vol of the cell culture supernatants or 70ng/ml purified X-DING-CD4.

Fig. 2. The alignment of X-DING-CD4 to known eukaryotic DING proteins
HPBP-human phosphate binding apoloipoprotein; p27^{SJ}-*Hyperforicum perforatum* truncated DING protein.

Fig. 3. Detection of X-DING-CD4 in the extracellular compartment of HRF(+) cells by anti-X-DING-CD4 antibody and testing its antiviral activity by MAGI assay

(A) Lanes HRF(+/-) represent 30ml of concentrated cell culture supernatants; lane X-DING-CD4^{IXC} represents 500ng of IXC purified X-DING-CD4; (B) Magi-CCR5 cells were treated with 70ng/ml of IXC purified X-DING-CD4 or 10%/Vol of HRF(+) and control HRF(-) filtered supernatant from overnight cultures in protein free hybridoma medium; (C) 100ng/ml of IXC purified X-DING-CD4 was treated with anti-X-DING-CD4 antibody or control rabbit IgG fraction for 2 hours; subsequently Magi cells were exposed to antibody treated or naïve X-DING-CD4 protein.

Table 1
Sequences of X-DING-CD4 peptides determined *de novo* from MS/MS spectra

Position		m/z			Sequence
Start	End	Observe	Mr(expt)	Mr(calc)	
1	12	586.0172	1170.019	1169.604	DINGGGATLPQK
19	28	854.7460	1707.477		VLTAGFAPYL/
46	53	411.3689	820.7232	820.4443	FVAGVSNK
54	63	550.9430	1099.871	1099.504	NVHWAGSDSK
64	76	707.0663	1412.118	1411.719	LTATELSTYATNK
64	76	714.1161	1426.217	1425.735	LTAT[methyl-Glu]LSTYATNK
85	95	800.7472	1599.479		QVPSVATSVALL/
215	228	691.0853	1380.156	1379.693	FAASTLAGLDDATK
366	376	601.0222	1200.029	1199.608	TNVCNGLGRPL
366	376	608.0193	1214.020	1213.623	TNVCNGLGRPL[C-terminal

Full and partial sequences from Lyc-C peptides are listed with putative positions based on the alignment with known DING proteins (Fig.2). Cys residues are modified by iodoacetamide. The symbol / indicates a partial sequence with C-terminal residues undetermined. Ile and Leu were indistinguishable and written as Leu.