ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 7, pp. 716-727. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 7, pp. 883-896.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM07-034, May 21, 2007.

Proteomic Analysis of Human NK-92 Cells after NK Cell-Mediated Cytotoxicity against K562 Cells

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Received February 7, 2007
Revision received March 24, 2007

Abstract—To better understand the natural killer (NK) cell cytotoxicity mechanism at the proteome level, we comparatively analyzed the proteome of the human NK-92 cells which participate in NK cell-mediated cytotoxicity assay and that of control cells. Soluble proteins were separated by two-dimensional gel electrophoresis (2-DE), 75 protein spots were found to be reproducibly differentially expressed between control and cytotoxic human NK-92 cells. A total of 60 different proteins were unequivocally identified by MALDI-TOF MS coupled with database interrogation; 37 proteins were up-regulated, whereas 23 proteins were down-regulated. Western blotting analysis of heat shock protein 60 (HSP60) and cathepsin W verified their proteome results. Some of identified proteins are involved in NK-92 cytotoxicity, which is consistent with the literature. In addition, we modeled the pathway networks between differentially expressed proteins and cellular processes of secretion and exocytosis through PathwayStudio software. The results of this study help to provide insight into the molecular mechanism of NK cell cytotoxicity.

DOI: 10.1134/S000629790707005X

Key words: cytotoxicity, mass spectrometry, NK cells, proteome, two-dimensional gel electrophoresis

The innate immune responses provide the first line of defense against different infectious agents. Natural killer (NK) cells are part of innate immunity and thought to represent important effectors of the innate immune response [1]. NK cells are distinct from T cells or B cells and have distinct morphologic, phenotypic, and functional properties, and they do not require prior sensitization for the expression of their activity. NK cells play a major role in the immune response against tumor cells, viral infections, and allografts [2] as well as in inflammatory diseases such as arthritis [3], chronic obstructive pulmonary disease [4], and inflammatory bowel disease [5]. NK cells mediate cell killing through a variety of mechanisms, including perforin/granzyme granule-mediated exocytosis pathway or producing a large array of cytokines and chemokines [6]. The granule-mediated exocytosis pathway is the major molecular mechanism for NK cell cytotoxicity, the pathway powerfully activates cell-death pathways of target cells that operate through the activation of apoptotic cysteine proteases (caspases), but it also leads to target cell death in the absence of activated caspases [7]. NK cells also rapidly secrete a number of cytokines and chemokines including interferon (IFN)-γ, TNF-α, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-5 and IL-13, which promptly promote NK cytolytic activity [8, 9]. NK cell activation is regulated by the fine balance of positive and negative signaling pathways initiated by multiple receptors displaying activating, costimulatory, or inhibitory activity, whose expression and/or functional capability can be modulated during NK cell activation [10].

A variety of techniques have been used to explore the molecular mechanisms associated with NK cell cytotoxi-

Abbreviations: 2-DE) two-dimensional gel electrophoresis; caspases) apoptotic cysteine proteases; ERK) extracellular signal regulated kinase; FBS) fetal bovine serum; GM-CSF) granulocyte-macrophage colony stimulating factor; HRP) horseradish peroxidase; HSP60) heat shock protein 60; IFN) interferon; IL) interleukin; IPG) immobilized pH gradient; IEF) isoelectrofocusing; ITAM) immunoreceptor tyrosine-based activation motif; ITIM) immunoreceptor tyrosine-based inhibition motif; MAPK) mitogen-activated protein kinase; MEM) minimum essential medium; Mr) molecular mass; NK) natural killer; PBS) phosphate buffered saline; PI3K) phosphoinositide 3-kinase; PMF) peptide mass fingerprint; PTK) protein tyrosine kinase; PTPs) protein tyrosine phosphatases; WASp) Wiskott–Aldrich syndrome protein.

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city function, and many key regulators and signaling pathway molecules involved in NK cell cytolytic function have also been reported [11-16]. However, these strategies depend on an assumption that was made about the upstream or downstream molecules, and identifies individual molecules on the basis of direct interaction, which only have the ability to evaluate a single protein component at a time. So they cannot form comprehensive information about NK cell function.

In contrast, proteomic analysis is unbiased and does not require direct interaction of searching objects [17], and allows simultaneous monitoring of the expression of hundreds and even thousands of proteins in a sample. Then unbiased and large-scale proteomic technologies for the assessment of NK cell cytotoxicity are needed, and these should bring new challenges into this area of research and provide a comprehensive understanding of NK cell-mediated cytotoxicity against NK-sensitive target cells. Up to now, those proteomic studies only focused on analyzing membrane proteins in different NK cell populations [18, 19], and differentially expressed proteins in activated versus non-activated NK cells [20]. To our knowledge, no studies have been published that analyze the molecular mechanisms of NK cytolysis against target cells at the proteome level.

NK-92 cells were developed and characterized by Gong et al. [21]. These cells, which have been activated by human IL-2, have the characteristics of activated NK cells, and therefore they are a useful model to study NK cell biology. The assay of NK cell-mediated cytotoxicity against K562 cells has been widely used to study functional characteristics of activated NK cells.

The aim of this study was to determine which proteins play a critical role in NK-92 cells cytotoxicity. To achieve this purpose, we have used 2-DE combined with MS to identify proteins whose expression is changed after NK cell-mediated cytotoxic assay in human NK-92 cells. With this approach, we identified 60 different proteins, most of which have important cellular functions, and some may play a critical role in NK92 cytotoxicity. Furthermore, several of the identified proteins have been shown to participate in NK cell cytotoxicity signaling pathways. The results will no doubt provide new information to elucidate the mechanisms of NK-mediated cytotoxicity.

MATERIALS AND METHODS

Materials. Human NK-92 cell line was purchased from the American Type Culture Collection (Rockville, USA). Medium and other cell culture reagents were obtained from Gibco BRL (USA). Monoclonal antibodies were obtained from Axxora LLC (USA). Supersignal West Pico Trial Kit was purchased from Pierce (USA). IPG strips, IPG buffers, and protein assay kit were purchased from Bio-Rad Laboratories (USA). Proteomic grade trypsin and all chemical reagents were of the highest purity available and purchased either from Sigma-Aldrich (USA) or Fisher Scientific (USA).

Cell culture. The human NK-92 cells were cultivated in α-minimum essential medium (MEM) containing 2 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, 0.02 mM folic acid, antibiotics, 100 U/ml recombinant IL-2, 12.5% horse serum, and 12.5% fetal bovine serum (FBS). K562 cells were cultivated in RPMI-1640 containing antibiotics and 10% FBS.

Assay of NK cell-mediated cytotoxicity against target K562 cells. Cytotoxic attack assay was performed using K562 as target cells as previously described [22]. Briefly, K562 cells were washed with phosphate buffered saline (PBS) once and incubated with 1% paraformaldehyde (methanol-free) in PBS, pH 7.4, on ice for 30 min. Then the cells were washed four times with PBS to remove all paraformaldehyde. Effector cells (NK-92) and target cells (K562) were incubated at a certain effector/target ratios (5 : 1) as well as effector cells only. Cells were rapidly pelleted at 1000 rpm in a microcentrifuge at 4°C to allow cells to form contacts and incubated for 15 min at 37°C and 5% CO_2 in a cell culture incubator. After incubation, cells were centrifuged and the pellet was ready to lyse.

Sample preparation. Cell pellet was lysed using lysis buffer (10 mM Tris-HCl, pH 7.4, containing 1% NP-40, 140 mM NaCl, 1 mM EDTA, 50 mM NaF, 10 mM iodoacetamide, 1 mM sodium orthovanadate, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), and 10 μg/ml leupeptin, pepstatin, and aprotinin) for 1 h at 4°C, then centrifuged for 15 min at 3000 rpm and subsequently for 30 min at 13,000 rpm at 4°C to remove all cell debris. The total protein concentration of the samples was determined using a Protein Assay Kit. The supernatants were stored at −70°C or used immediately.

2-DE. For isoelectrofocusing (IEF), cell extracts (about 200 μg of protein) were solubilized in a rehydration sample buffer containing 6 M urea, 2 M thiourea, 2% CHAPS, 0.3% (w/v) dithiothreitol (DTT), 1.3% (w/v) IPG buffer, pH 5-8, and a trace of bromophenol blue dye. Immobilized pH gradient (IPG) strip, 17 cm, covering a pH range of 5-8, was allowed to rehydrate under active rehydration (50 V) in 300 μl of this protein solution for 12 h with a mineral oil overlay. IPG strips containing proteins (sample loading by rehydration) were subjected to IEF on a Protean IEF Cell apparatus (Bio-Rad). The setting for step 1 was 500 V for 1000 V·h; step 2 was 1000 V for 1000 V·h, and step 3 at 8000 V for 60,000 V·h; limit/gel 50 mA, focus temperature 20°C. Vertical SDS-PAGE was used for the second dimension using 12.5% polyacrylamide slab gels. The gel strips were removed from the Protean IEF cell system. They were equilibrated with gentle rocking for 15 min with buffer A (6 M urea,

0.375 M Tris, pH 8.8, 20% glycerol, 2% (w/v) SDS, 2% (w/v) DTT) and then with buffer B (6 M urea, 0.375 M Tris, pH 8.8, 20% glycerol, 2% (w/v) SDS, 2% (w/v) iodoacetamide). After equilibration, proteins were separated in the second dimension electrophoresis on 12.5% SDS-polyacrylamide gels in a Protean II Xi Cell apparatus (Bio-Rad), the gel being run at 30 mA/gel constant current. The run was continued until the bromophenol blue front reached the bottom of the gel. Gels were silver-stained using a MS-compatible silver staining procedure. All 2-DE experiments were performed at least in triplicate. Gels were scanned with UMAX Power Look III and Magiscan software (UMAX Technologies).

Image analysis. For the image analysis, cytotoxic attack assay was performed in three experimental replicates, and the 2-DE experiment was performed at least in triplicate for each experimental replicate. Gel comparison was done both manually and with the PDQuest 7.0 software (Bio-Rad). Background was subtracted, followed by protein spot localization and quantification. The quantity of each spot was normalized by total valid spot intensity. Protein spots were identified as being significantly different ($p < 0.05$ by Student's *t*-test) if the intensity was changed more than 2-fold in at least two independent experiments.

In-gel trypsin digestion. Enzymatic digestion was performed as previously described [23]. Briefly, differentially expressed protein spots were excised from the gels and cut into pieces. Each spot was destained in 100 μl of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 10 min. The sample was washed three times with 30 mM ammonium bicarbonate/acetonitrile (1 : 1) and dehydrated with acetonitrile. The spots were reduced with 20 mM DTT, followed by alkylation with 55 mM iodoacetamide, and washed three times. The gel pieces were dried in a vacuum centrifuge for 30 min (Savant Speed-Vac). The gel pieces were swollen in 10 μl of digestion buffer (30 mM ammonium bicarbonate containing 10 ng/μl of trypsin) in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 20 μl of the same buffer without trypsin, and the samples were incubated overnight at 37°C. Following enzymatic digestion, the resultant peptides were extracted three times with 10-20 μl of 5% TFA in 50% acetonitrile and concentrated to 4-5 μl in the same solvent.

MALDI-TOF mass spectrometric analysis and database search. The peptide mixtures from the tryptic digests were desalted using Millipore ZIP plate (Millipore, USA) according to the manufacturer's instructions. Typically, peptides obtained after tryptic digestion were eluted in 1 μl of 50% (v/v) acetonitrile/0.1% TFA and mixed 1 : 1 with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile/0.3% TFA. Samples (0.5 μl) were spotted on a stainless steel target plate and air-dried before analysis in the mass spectrometer. Peptide masses were analyzed using MALDI-TOF MS (Voyager DE-Pro; Applied Biosystems, Germany) and then using Voyager software for data collection and analysis. Spectra data were processed using Data Explorer v.4.0 (Applied Biosystems). External mass calibration with a standard peptide mixture and internal calibration of samples using Sar1-angiotensin (1002.5525 daltons), ACTH (2093.0867 daltons), and insulin B-chain (3494.6513 daltons) were also performed *prior to* the database search. The resulting peptide mass fingerprints (PMFs) were used to match protein candidates in the NCBInr human protein databases using the ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe) search engine. Parameter setting: a maximum of one missed cleavage for trypsin hydrolysis, a fragment ion mass tolerance set to 0.2 dalton, oxidized at methionine residues, and iodoacetamidated at cysteine residues. The search was restricted to human proteins. Proteins were identified on the basis of minimum sequence coverage of more than 15% and *Z* score >1.65.

Western blot analysis. For Western immunoblotting, control and cytotoxic attack assay treatment NK-92 cell lysates were cleared by centrifugation at 13,000 rpm at 4°C for 10 min. Total protein lysate was resolved on 12.5% SDS-PAGE and electroblotted onto Immuno-Blot PVDF membrane and blocked with 1% BSA in TTBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 30 min. The blocked membranes were incubated initially with monoclonal anti-cathepsin W precursor antibody (CW40-1B1) and monoclonal anti-HSP60 antibody and secondarily with the appropriate horseradish peroxidase (HRP)-coupled secondary antibodies. Bound antibodies were detected by chemiluminescence using the Supersignal West Pico Trial Kit.

PathwayStudio database analysis. The possible functional relationships among the differentially expressed proteins were identified by using the PathwayStudio 4.0 software (Ariadne Genomics). This software explores gene interaction networks represented in the ResNet database. The database contains more than 100,000 events of regulation, interaction, and modification between 15,000 proteins, cellular processes, and small molecules. Data analyzed through this technique can then be resolved into cogent models of the specific biological pathways activated under the experimental conditions used in the microarray analyses.

RESULTS

2-DE maps and image analysis of NK-92 cells. Based on previous data indicating that NK cell-mediated cytotoxicity is a rapid event that can be completed within 22 min [24] and that tyrosine phosphorylation in NK cells increases rapidly and reaches its peak at 15 min after stimulation with K562 cells [25], the proteome was analyzed at 15 min during NK cell-mediated cytotoxicity

assay. In this study, we modified a protein extraction method of NK-92 cells to be suitable for 2-DE as previously described [26], which only lysed NK-92 cells, but did not lyse the paraformaldehyde fixed K562 cells. The control and participated cytotoxic attack assay NK cell extracts were applied to the pH 3-10 immobilized IPG strips and displayed in 12.5% acrylamide gels. The results showed that the major protein spots of the two samples were clustered in the pH 5-8 range; however, it was difficult to analyze the differential expression pattern in the range of pH 5-8. Therefore, the subsequent experiments were carried out using pH 5-8 immobilized IPG strips to facilitate not only the analysis of protein expression pattern but also the improved resolution in the narrow pH range.

Representative silver-stained 2-DE gel images from control and cytotoxic attack assay NK cells are shown in Fig. 1. Similar results, with minor variations in intensity, were obtained in the three experiments performed. After the image analyses of two samples using PDQuest 7.0 software, a total of 75 protein spots were found to be differentially expressed with intensity changes greater than 2-fold with confidence intervals at least 95% (Student's *t*-test, $p < 0.05$) in at least two independent experiments. From the 75 differentially expressed spots found from the 2-DE gels, 43 spots were at least 2-fold up-regulated after cytotoxic attack assay, and 32 spots were 2-fold down-regulated. The differentially expressed protein spots are marked on the images in Fig. 1.

Identification of proteins from 2-D gel spots. The differentially expressed protein spots were cut out, analyzed by MALDI-TOF MS after tryptic lysis, and identified in databases. Among the 75 protein spots analyzed, 37 up- and 23 down-regulated proteins were unambiguously identified. The remaining 12 differentially expressed spots were not identified because of their relative low concentrations. Tables 1 and 2 list the individual identified proteins, p*I* and molecular mass (Mr), sequence coverage, and *Z* scores, respectively. In Fig. 1, the identified spots were marked with the numbers corresponding to the spot number and the unidentified were indicated with circles. Three proteins were identified from two different spots, namely enolase 1 (spot 31); triosephosphate isomerase 1 (spot 53); hypothetical protein (spot 58). Further studies are required to confirm whether these are isoforms of the same protein resulting from posttranslational modifications (phosphorylation, glycosylation, etc.).

To classify identified proteins, each of the proteins was classified by molecular function based on information from the ResNet database using PathwayStudio software and Gene Ontology (GO) annotation (Tables 1 and 2). Most of the proteins were related to signal transduction, cell skeleton, cell metabolism, cell proliferation/adhesion, enzymes, and immune response, which were linked to NK functions.

Confirmation of differentially expressed proteins by Western blotting. To confirm and validate our proteomics results, Western blotting was employed to assess the

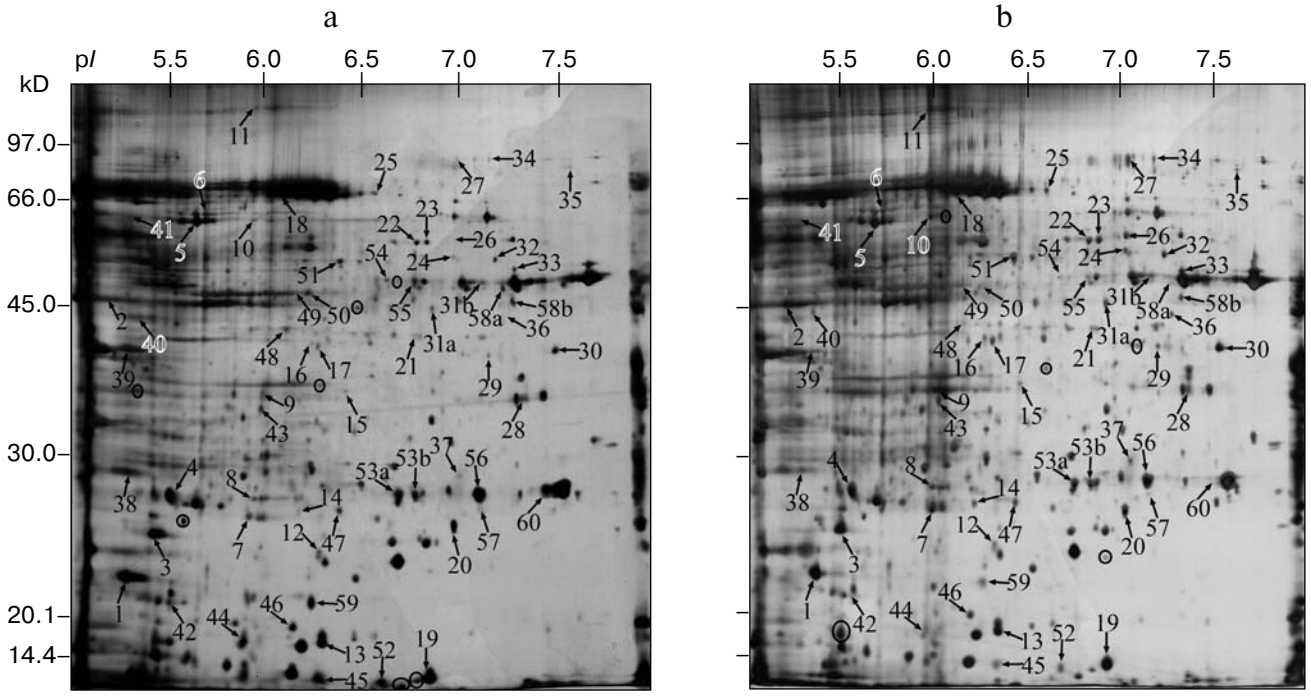

Fig. 1. Representative 2-DE gel image of control (a) and cytotoxic attack assay (b) human NK-92 cells. Soluble proteins were separated on pH 5-8 linear IPG strips followed by 12.5% SDS-PAGE gels. Proteins were silver stained after electrophoresis. Solid arrows indicate the differentially expressed protein spots that were identified by PMF. Proteins that have not been identified are indicated with a circle.

Table 1. Up-regulated proteins identified by PMF after cytotoxic attack assay

Protein name	Spot number	Gene name	NCBI accession	pI/M_r		Sequence coverage, %	Z score
				theoretical	experimental		
1	2	3	4	5	6	7	8
Signal transduction*							
Suppressor of fused variant 3	2	*SUFU*	AAF35866	5.4/53.4	5.2/45.1	15	2.40
Eukaryotic translation initiation factor 3, subunit 2 β	4	*GNAI2*	NP_003748	5.4/36.9	5.5/27.2	18	2.18
Cdc42 isoform 1	12	*CDC42*	NP_001782	6.2/21.6	6.2/23.9	51	1.96
MPP3 protein	25	*MPP3*	Q13368	6.3/66.6	6.6/66.5	31	2.02
CAMP-regulated guanine nucleotide exchange factor I	26	*RAPGEF3*	AAD02890	7.0/62.5	6.9/61.0	20	2.08
MAP kinase-activated protein kinase 5	29	*MAPKAPK5*	NP_003659	7.4/54.5	7.2/38.6	22	2.35
MAPK kinase 3	36	*MAP2K3*	NP_659732	7.1/40.7	7.5/44.8	51	1.86
Cell metabolism							
Chain A, human glyoxalase I with benzyl-glutathione inhibitor	1	*GLO1*	1FROA	5.1/20.9	5.3/22.7	42	1.86
3-Oxoacid CoA transferase 1	23	*OXCT1*	NP_000427	7.2/56.6	6.8/55.2	33	1.97
Succinate-CoA ligase, ADP-forming, β subunit	28	*SUCLA2*	NP_003841	7.1/50.6	7.4/34.4	19	2.11
Leucine aminopeptidase	33	*LAP3*	NP_056991	7.6/56.4	7.3/50.2	24	2.04
Aconitase	34	*ACO2*	AAD19351	7.2/86.3	7.2/81.6	24	1.75
Enzymes							
Alkaline phosphatase	5	*ALPPL2*	P10696	5.8/57.6	5.6/59.4	22	2.35
Poly(ADP-ribose)glycohydrolase	11	*PARG*	AAH52966	6.0/112.5	5.9/114.1	37	2.21
Inositol polyphosphate-5-phosphatase, 40 kD	37	*INPP5A*	AAK13252	7.2/35.9	7.0/28.2	17	1.85
Molecular chaperones							
Heat shock protein 60 (HSP60)	6	*HSPD1*	AAA36022	5.7/61.2	5.6/62.2	36	1.78
58 kD glucose-regulated protein	10	*GRP58*	BAA03759	6.2/57.1	5.9/59.7	20	1.86
Cell proliferation/adhesion							
E74-like factor 5	7	*ELF5*	CAC48256	5.8/31.9	5.9/25.8	31	1.81
ENO1 protein	9	*ENO1*	AAH04325	5.6/30.2	5.9/34.7	31	2.13
Cyclin-dependent kinase inhibitor 1B	13	*CDKN1B*	AAH01971	6.5/22.2	6.3/16.5	34	2.11
HNT protein	15	*HNT*	AAH50716	6.5/35.5	6.4/35.0	55	2.08
Enolase 1	31a	*ENO1B*	NP_001419	7.0/47.5	6.9/43.9	35	1.74

Table 1 (Contd.)

1	2	3	4	5	6	7	8
Enolase 1	31b	*ENO1B*	NP_001419	7.0/47.5	7.2/47.9	29	2.13
Immune response							
MHC class I antigen B*53	14	*HLA-B*	P30491	6.0/40.8	6.3/28.0	37	1.70
Pulmonary surfactant-associated protein D	17	*SFTPD*	P35247	6.3/38.0	6.2/39.6	26	2.26
Protein binding							
FK506-binding protein 7	8	*FKBP7*	NP_057189	6.1/30.3	5.9/26.8	59	1.98
Dihydropyrimidinase-like 4	22	*DPYSL4*	NP_006417	6.8/62.5	6.8/55.1	23	2.18
Transport							
Amyloid β precursor protein-binding protein 2	18	*APPBP2*	NP_006371	6.4/67.7	6.1/67.0	48	2.28
Cytochrome *b*	19	*MTCYB*	CAD29712	7.0/13.9	6.9/11.6	23	1.94
Biosynthesis							
HMG-1L10	20	*HMG1L10*	Q9UGV6	7.0/24.4	7.0/25.2	18	1.74
ATP-dependent RNA helicase DDX25	21	*DDX25*	Q9UHL0	6.7/42.5	6.8/40.8	20	2.09
Xeroderma pigmentosum D complementing protein	27	*ERCC2*	AAM45142	7.1/84.7	7.0/77.3	17	2.12
60S acidic ribosomal protein PO	30	*MRT4*	AAD52608	7.8/27.6	7.6/38.7	31	1.90
Apoptosis							
Tumor necrosis factor receptor superfamily member 10D	16	*TNFRSF10D*	Q9UBN6	6.2/42.9	6.1/39.9	26	2.33
Cell skeleton							
Tektin 3	24	*TEKT3*	NP_114104	6.9/57.1	7.0/52.4	37	2.24
DNA replication							
Origin recognition complex, subunit 3	35	*ORC3L*	NP_862820	7.8/83.4	7.6/77.7	16	2.16
Miscellaneous							
Annexin A8	3	*ANXA8*	AAH04376	5.6/37.1	5.4/24.7	15	2.39
Glycylpeptide N-tetradecanoyl-transferase 2	32	*NMT2*	NP_004799	7.3/57.3	7.2/51.9	35	2.10

* The proteins were classified into different functional classes according to their known functions.

expression of HSP60 (up-regulated protein) and cathepsin W (down-regulated protein) in control and cytotoxic attack assay human NK-92 cells. Consistent with the proteomics results, these two proteins were found to be up-regulated and down-regulated in cytotoxic attack assay of human NK-92 cells (Fig. 2).

Pathway prediction analysis. Because the relationships among nodes are thought to determine cell behavior, mapping the altered connections among nodes in a signaling network could indicate cytotoxicity mechanisms of NK cells. In this paper, we have attempted to leverage what little information is available about the pro-

Table 2. Down-regulated proteins identified by PMF after cytotoxic attack assay

Protein name	Spot number	Gene name	NCBI accession	p*I*/ M_r		Sequence coverage, %	*Z* score
				theoretical	experimental		
1	2	3	4	5	6	7	8
Signal transduction*							
Ran-binding protein 1	38	*RANBP1*	BAA07269	5.2/23.4	5.3/28.4	60	2.35
USH1C protein	41	*USH1C*	AAH16057	5.3/60.5	5.2/60.5	26	2.03
Rho GDP dissociation inhibitor (GDI) β	42	*ARHGDIB*	NP_001166	5.1/23.0	5.5/21.3	63	2.26
Oncogene DJ1	44	*PARK7*	NP_009193	6.3/20.0	5.9/17.4	63	1.88
CHRNB2	50	*CHRNB2*	CAA05108	6.7/57.9	6.2/46.2	44	1.97
Killer cell lectin-like receptor subfamily C, member 1	60	*KLRC1*	NP_998822	7.9/24.7	7.7/28.9	20	2.41
Enzymes							
NADH dehydrogenase subunit 1	47	*mt-Nd1*	AAL54553	6.1/35.4	6.4/26.1	61	2.43
Serine/threonine protein phosphatase 2B catalytic subunit, γ isoform	54	*PPP3CC*	P48454	6.8/57.8	6.6/48.2	26	1.65
Cathepsin W	56	*CTSW*	AAB82449	7.2/42.8	7.1/27.1	20	1.71
Biosynthesis							
CTP:phosphocholine cytidyltransferase *b*	48	*PCYT1B*	NP_004836	6.0/42.2	6.1/41.5	21	1.63
Phenylalanine hydroxylase-stimulating protein	52	*PCBD*	AAB25581	6.3/11.9	6.6/9.6	24	1.46
Triosephosphate isomerase 1	53a	*TPI1*	AAH17917	6.4/26.9	6.7/27.0	67	2.15
Triosephosphate isomerase 1	53b	*TPI1*	AAH17917	6.4/26.9	6.8/27.0	46	1.76
Cell metabolism							
HPHRP	43	*PTER*	AAK14923	6.1/39.8	6.0/33.4	21	2.33
dUTP pyrophosphatase	46	*DUT*	NP_001939	6.2/17.9	6.1/20.2	40	2.09
2-Hydroxyphytanoyl-CoA lyase	55	*HPCL2*	Q9UJ83	7.1/64.5	6.7/48.3	25	1.93
Cell skeleton							
α1 actin	39	*ACTA1*	NP_001091	5.2/42.9	5.3/39.3	40	1.81
Cell proliferation							
Secretagogin precursor	40	*SCGN*	NP_008929	5.2/32.2	5.3/42.9	29	1.73
Transport							
HSV-1 stimulation-related 1	49	*HSRG1*	NP_055755	5.9/59.7	6.2/46.2	25	2.26
Chloride intracellular channel 5	57	*CLIC5*	CAC36880	7.1/26.1	7.2/26.2	30	2.02

Table 2 (Contd.)

1	2	3	4	5	6	7	8
mRNA cleavage							
Cleavage stimulation factor subunit 2	51	*CSTF2*	NP_001316	6.2/61.0	6.4/51.6	25	2.11
Immune response							
HLA-B52 variant	59	*HLA-B52v*	BAB18306	5.6/21.2	6.2/21.4	70	2.30
Miscellaneous							
COMM domain containing 2	45	*COMMD2*	AAH46131	6.2/22.8	6.3/11.4	28	1.97
Hypothetical protein	58a		CAD97642	7.7/47.4	7.3/46.8	36	1.96
Hypothetical protein	58b		CAD97642	7.7/47.4	7.3/44.9	33	2.01

* The proteins were classified into different functional classes according to their known functions.

teins that may contribute to NK-mediated cytotoxicity in order to identify additional candidate proteins for cytotoxicity based on the results from our proteome study. Our hypothesis was that by constructing pathways between the cellular processes (exocytosis and secretion) that are already suspected to be involved in NK cytolysis function and our positional candidate proteins, we could identify a subset of those positional candidates more likely to be involved in cytotoxicity. We constructed a model pathway using PathwayStudio 4.0 software for direct regulators of the identified proteins and the cell processes (Fig. 3a), and built a direct interaction pathway showing the direct interaction between them (Fig. 3b). The validity of the connections was confirmed by inspection of the supporting literature. We observed through these analyses that 18 of the differentially expressed proteins found in our experiment have been directly regulated by other function groups (Fig. 3a), and seven proteins have been shown to directly regulate cell processes (Fig. 3b). The variation of protein expression could have an influence on exocytosis and secretion, either directly or through regulatory effects, etc.

DISCUSSION

NK cells express an array of activating receptors that can trigger cytolytic programs, as well as cytokine or chemokine secretion. Some of these activating receptors initiate protein tyrosine kinase (PTK)-dependent pathways through noncovalent associations with transmembrane signaling adaptors that harbor intracytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). The signaling pathways leading to NK cytotoxicity have been under intensive investigation, and currently available data indicate the involvement of mitogen-

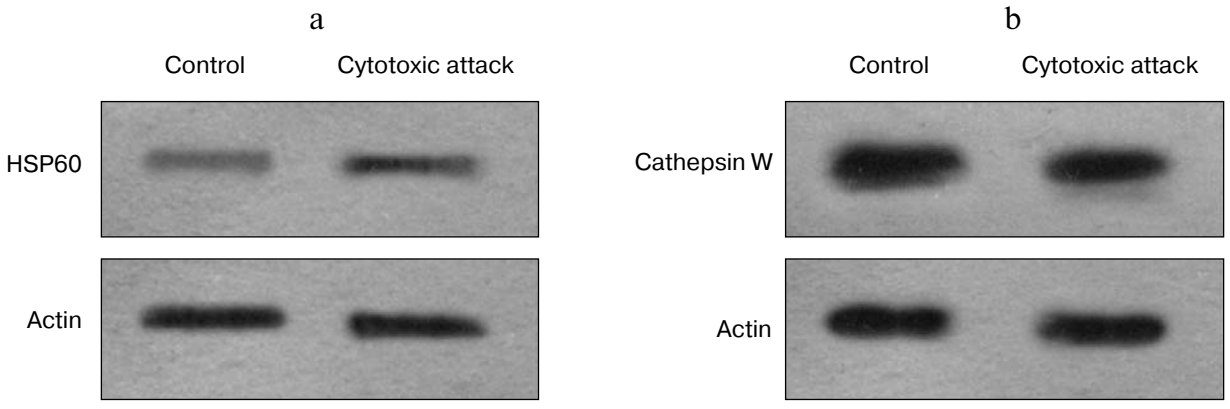

Fig. 2. Western blot analysis of HSP60 (a) and cathepsin W (b) in control and cytotoxic attack assay NK-92 cells. Total cellular proteins of control and cytotoxic attack assay NK-92 cells were separated by SDS-PAGE and detected with antibody against HSP60 and cathepsin W. Actin was used as loading control. Results are representative of three independent experiments.

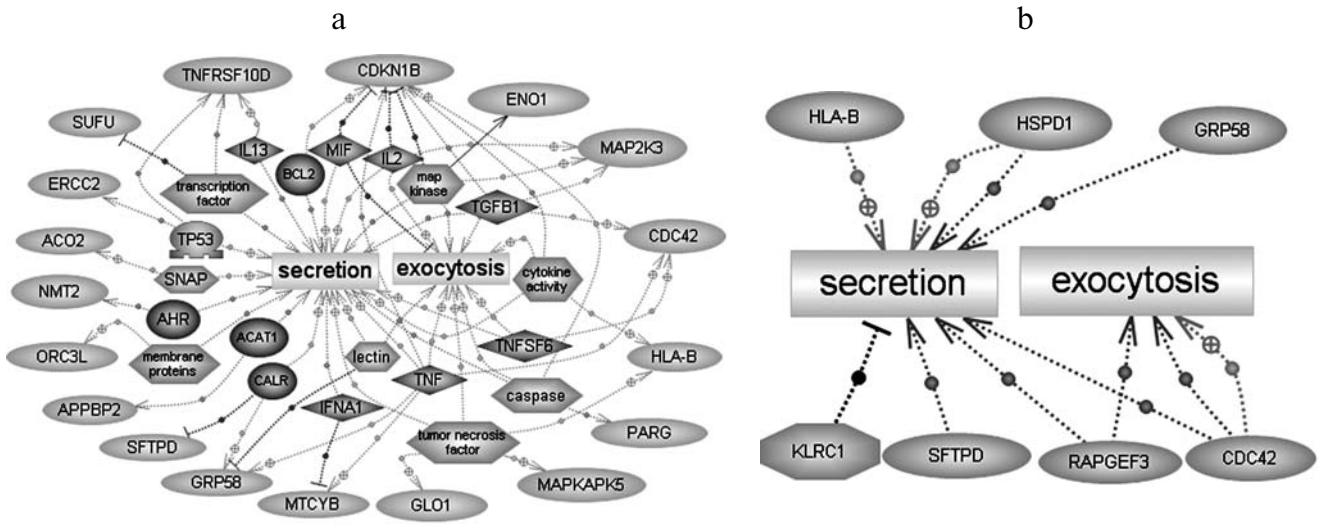

Fig. 3. Correlation pathway networks between cellular processes (secretion and exocytosis) and the identified proteins of NK-92 cells. a) Direct regulatory pathway showing the common regulators of those proteins and cell processes; b) direct interaction pathway showing the interaction between the cell processes and the identified proteins. These pathways were built using the PathwayStudio software. Arrows with plus sign indicate positive regulation, blunt ended lines indicate negative regulation, and gray arrows indicate unknown regulation. The meaning of the different shapes surrounding the entities is the following: ellipse, identified up-regulated protein; octagon, identified down-regulated protein; rectangle, cellular process; circle, protein added by software; rhomb, ligand added by software; o-vertex, transcription factor added by software; hexagon, functional class added by software.

activated protein kinase (MAPK) 4/extracellular signal regulated kinase (ERK), p38, VAV, Pyk2, Rac1, Syk, phosphoinositide 3-kinase (PI3K), etc. [16]. NK cells also express inhibitory receptors that antagonize activating pathways through protein tyrosine phosphatases (PTPs). These inhibitory receptors are characterized by intracytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs), which can recruit the protein tyrosine phosphatase SHP-1 or/and SHP-2, and dephosphorylate some signal molecules [27]. Therefore, the tyrosine-phosphorylation status of several signaling components that are substrates for both PTKs and PTPs is thus key to the propagation of the NK cell effector pathways. In addition, some studies have demonstrated that expression change of several proteins (i.e. cathepsin W) play key roles in NK-mediated cytotoxicity [28]. However, at present little is known about the functional roles of differential expression of proteins in the NK cytotoxic response. Therefore, understanding the integration of these factors at a comprehensive level is central to the understanding and manipulation of NK cell functions.

Proteomics opens new insights in many research areas of life sciences, allowing comprehensive understanding of the mechanism of life from the indirect gene to the functional executioner protein. In this study, we performed comparative proteomics to find differentially expressed proteins in control and cytotoxic attack assay human NK-92 cells. The comparative analysis of paired samples indicated that 75 protein spots showed significant differences in expression level of control and cytotoxic attack assay cells. Among the 75 protein spots analyzed, 63 spots corresponding to 60 different proteins were identified, 37 proteins were up-regulated, 23 proteins were down-regulated, whereas the remaining 12 spots contained too little material for identification. The majority of the identified proteins are implicated in various cellular processes, such as signal transduction, cellular metabolism, cytokine production, transport functions, etc. Figure 4 highlights the involvement of some of the proteins identified in our study in NK function pathways. Here we discuss briefly the differentially expressed proteins related to NK cell cytotoxicity.

One of the most interesting down-regulated proteins was killer cell lectin-like receptor subfamily C, member 1 (KLRC1) (also named NKG2A). The protein belongs to the killer cell lectin-like receptor family, also called NKG2 family, which is a group of transmembrane proteins preferentially expressed in NK cells. This family of proteins is characterized by the type II membrane orientation and the presence of a C-type lectin domain. This protein forms a complex with another family member, KLRD1/CD94 (NKG2A/CD94), has been implicated in the recognition of the MHC class I HLA-E molecules in NK cells [29], and NKG2A/CD94 heterodimers in humans negatively control NK direct lysis of target cells [30]. The inhibitory effects of human NKG2A are thought to be mediated by phosphorylation at the tyrosine residue of the ITIM in the cytoplasmic domain and subsequent recruitment of the protein tyrosine phosphatase SHP-1 [31]. Human NKG2A has two ITIMs, and the

presence of two ITIMs in NKG2A is thought to be important for the transduction of inhibitory signals for NK cells [32]. Therefore, down-production of NKG2A in NK cells may be associated with increasing cytolysis function of NK cells.

Cathepsin W is a cysteine protease that belongs to the C1A-family of papain-like proteases [33] and was found to be expressed in cytotoxic lymphocytes [34]. Cathepsin W, which is predominantly expressed in NK cells, was found to be up-regulated by IL-2 [35]. However, the endogenous protein expression was decrease during the process of NK cell-mediated cytotoxicity against K562 cells [28]. Our data confirmed the change in the protein. The protein plays an important role in cellular cytotoxic processes mediated by NK cells [28]. The cathepsin family is associated with lysosomes and shares sequence homology; cathepsin B provides self-protection for degranulating cytotoxic lymphocytes. Since the proteolytic activity of cathepsin W has not been identified, the underlying mechanism of its functional involvement in the cytotoxic process is currently unknown. Further studies will be required to understand the mechanism of the changes observed here in expression levels of this protein during NK cell-mediated cytotoxicity.

In this report, we have found out that Cdc42 isoform 1 protein expression is up-regulated in NK92 cells that participated in the cytotoxic attack assay. The protein is a small GTPase of the Rho family, which plays important roles in a variety of cellular functions. RhoA, Rac1, and Cdc42, the three different subfamilies of this family, are involved in the control of crucial cellular processes including cell growth and development, apoptosis, lipid metabolism, cytoarchitecture, membrane trafficking, and transcriptional regulation [36]. RhoA, Cdc42, and Rac1 are also involved in neuropeptide substance P signaling pathways, which can directly stimulate production of cytokines including IL-1, IL-6, and IL-8, and TNF-α [37]. A previous report indicates that Cdc42 is a critical upstream event regulating Wiskott–Aldrich syndrome protein (WASp) activity in generating NK cell cytotoxicity [38]. Interestingly, we find that Rho GDP dissociation inhibitor (GDI) β protein expression is down-regulated in this report. Rho GDP dissociation inhibitor was identified as a down-regulator of Rho family GTPases typified by its ability to prevent nucleotide exchange and membrane association [39]. RhoA [40] and Rac1 [16] are pivotal regulators of adhesion, granule exocytosis, and NK cell cytotoxicity. Therefore, the results indicate that the changes in the expression of the two proteins are pivotal regulators of granule exocytosis and cytotoxicity.

Interestingly, our data show a significant increase in MAPK-activated protein kinase 5 and MAPK kinase 3 in cytotoxic attack assay human NK-92 cells. MAPK-activated protein kinase 5 is strongly activated in response to cellular stress and cytokines, and its activation is mediated by p38 kinase and participates in the p38 MAPK signaling pathway [41]. MAPK kinase 3 is a dual specificity protein kinase that belongs to the MAPK kinase family, and this kinase is activated by mitogenic and environmental stress and participates in the MAP kinase-mediated signaling cascade. It phosphorylates and thus activates p38 MAPK. The p38 kinase and MAPK have been widely reported to be crucially involved in the proliferation, activation, and natural cytotoxicity of NK cells [10, 42]. In addition, it has been shown that the increase in MAPK-activated protein kinase 5 and MAPK kinase 3 can activate cytolysis function of human NK cells.

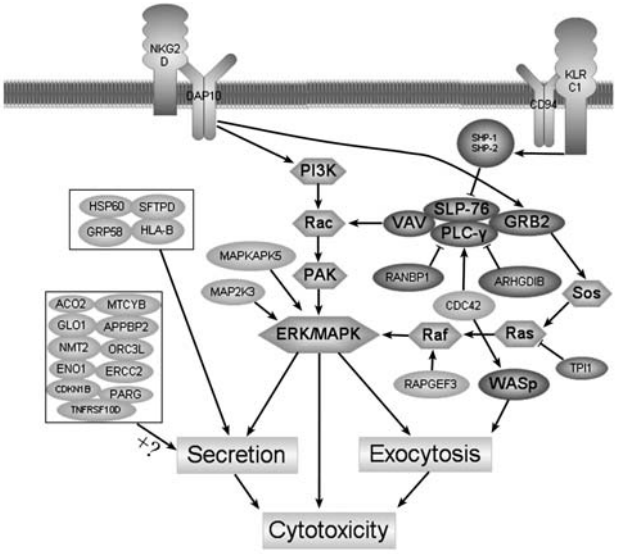

Fig. 4. NK cell effector signaling pathways. The pathways reflect PathwayStudio data, and interactions and protein functions found in the primary literature. MAP2K3, MAPKAPK5, Cdc42, RAPGEF3, and the proteins in panes are the identified up-regulated proteins. KLRC1, RANBP1, ARHGDIB, and TPI1 are the identified down-regulated proteins. The others indicate previously established interactions. Arrows indicate positive regulation or activation and blunt ended lines indicate inhibition.

Another protein of interest among those up-regulated in cytotoxic attack assay human NK-92 cells is HSP60. This mitochondrial protein may function as a signaling molecule in the innate immune system. The protein is essential for the folding and assembly of newly imported proteins in mitochondria. HSP60 directly stimulates cytokine synthesis and enhances the activation of cytotoxic T cells through activated macrophages [43]. Moreover, macrophages have been reported to respond to human HSP60 with increased production of IL-12 and IL-15 [44], which are important activators of an NK cell subset. It is intriguing that this study showed a significant up-regulation of HSP60, probably representing enhancing and accelerating the stimulation of NK cells.

In addition, many of the other identified proteins, which are differentially expressed in control and cytotoxic attack assay human NK-92 cells, are not yet identified as involved in the process of NK cells cytolysis. Further

research is needed to understand the link between these proteins and cytotoxicity of human NK cells.

In this work, using comparative proteomics analysis, we identified a set of proteins that are directly or indirectly involved in the cytolysis process of NK cells, which are consistent with the literature. Using the software PathwayStudio, we found that some of the identified proteins have a clear relationship with the secretion and exocytosis processes. Surprisingly, the proteins that relate through regulatory effects with the secretion and exocytosis processes are all the up-regulated proteins, and the proteins that have a directly positive regulation to the cellular processes are also up-regulated proteins, this suggesting that the variation of protein expression could have a positive influence on secretion and exocytosis, which are important for NK cell cytotoxicity. Furthermore, by searching the known interactions in PathwayStudio data and literature, we embedded our results in NK cell effector signaling pathways, which were reviewed previously [25, 45]. However, confirmation of these links requires further more detailed study. Finally, this work shows that proteome analysis is an efficient method for identifying novel functional proteins and provides new insight into the mechanisms of NK cells cytotoxicity.

This work was partly supported by the Natural Science Foundation of China (No. 90209016).

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