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Nongranular Proteolytic Enzymes of Rat IL-2–Activated Natural Killer Cells. II. Purification and Identification of Rat A-NKP 1 and A-NKP 2 as Constituents of the Multicatalytic Proteinase (Proteasome) Complex

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Abstract We have recently described nongranular, cytosolic, high-molecular-weight trypsin-like (A-NKP 1) and chymotrypsin-like (A-NKP 2) proteases of interleukin-2–activated rat natural killer (A-NK) cells. A functional correlation between the inactivation of A-NKP 2 and the inhibition of rat A-NK cell-mediated cytotoxicity was found. Herein we describe the 6,000-fold purification of A-NKP 2 to apparent homogeneity following: isopycnic sucrose gradient fractionation of postnuclear supernatants, molecular sieve chromatography, and heparin-Sepharose® chromatography. We also report the novel finding that A-NKP 2 as well as A-NKP 1, derived from either rat A-NK cells or the rat NK leukemic cell line CRNK-16, are constituents of the multicatalytic proteinase (MCP/proteasome) complexes of these cells. Characteristic biochemical, biophysical, and electron microscopic/ultrastructural similarity to the rat liver proteasome was observed. However, Western blot analysis using polyclonal antibodies to the rat liver proteasome clearly indicated differences in the rat hepatic proteasome and the CRNK-16–derived proteasomal subunits. The identification, characterization, and purification of A-NKP 1 and A-NKP 2, described herein, now allow for further investigation of the potential role of these proteasome components in NK cell function. Moreover, the proteasome of NK and A-NK cells can now be compared and contrasted to the granzymes of lytic granules with respect to their role in cell-mediated cytotoxicity. © 1994 Wiley-Liss, Inc.

Key words: NK/A-NK cells, multicatalytic proteinase complex, proteasome, proteases, enzyme purification

Natural killer (NK) cells [Herberman and Ortaldo, 1981] are large granular lymphocytes (LGL) [Timonen et al., 1979a,b] that lyse susceptible tumor cells, without apparent prior sensitization, in a major histocompatibility complex unrestricted manner [De Landazuri and Herberman, 1972; Storkus and Dawson, 1991; Trinchieri, 1989]. NK cells are also potent mediators of antibody-dependent cell-mediated cytotoxicity [Timonen et al., 1981; Titus et al., 1987]. Interleukin-2-activated natural killer (A-NK) cells display antitumor cell reactivity against NK-resistant targets [Vujanovic et al., 1988a,b], as well as the ability to infiltrate experimental tumors in vitro [Jääskeläinen et al., 1992], and accumulate within experimental metastases in vivo [Basse et al., 1991a,b].

The findings that NK cells produce extracellular [Goldfarb et al., 1984; Wasserman et al., in press], plasma membrane–associated [Carpén et al., 1986; Goldfarb et al., 1984], lytic granule–associated [Goldfarb, 1985; Griffiths and Muller, 1991; Hameed et al., 1988; Hudig et al., 1987; Kamada et al., 1989; Velotti et al., 1992], and nongranzyme, high-molecular-weight proteases [Goldfarb et al., 1992] has led to the investigation of the role of these enzymes in physiologic functions of NK cells including cell-mediated cytotoxicity and/or killer cell migration and infiltration into disease sites including metastatic

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tumors. We have recently reported a functional correlation between the inactivation of cytosolic, high-molecular-weight proteases (A-NKP 1 and A-NKP 2) and the inhibition of rat A-NK cell-mediated cytotoxicity [Goldfarb et al., 1992]. Therefore, in addition to granzymes, A-NKP 1 and A-NKP 2 apparently represent components of an array of proteolytic enzymes important for A-NK cell function including cell-mediated cytotoxicity. Herein we describe the characterization and purification of high-molecular-weight cytosolic proteinase activities of these cells. Our findings reveal that A-NKP 1 and A-NKP 2 are components of the multicatalytic proteinase (MCP/proteasome) complex [Goldberg and Rock, 1992; Orlowski, 1990; Rivett, 1993] of these cells. Isopycnic sucrose density gradient centrifugation of postnuclear supernatants, molecular sieve chromatography, and heparin-Sepharose® chromatography were used to purify A-NKP 2, as a representative component of the proteasome, to apparent homogeneity from both rat A-NK cells and the rat NK leukemia CRNK-16 cell line [Ward and Reynolds, 1983]. We report that the A-NKP 1 and A-NKP 2 are components of the proteasomes of rat A-NK cells and CRNK-16 cells and have biochemical, immunochemical, biophysical, and electron microscopic/ultrastructural properties similar to those of the rat hepatic proteasome [Rivett, 1985, 1993; Skilton et al., 1991]. The identification, characterization, and enrichment of these proteases now allow for further investigation of their role in NK and A-NK cell function.

MATERIALS AND METHODS

Animals

Male Fischer 344 rats were purchased from Taconic Farms (Germantown, NY) and housed within the pathogen-free facility of the Pittsburgh Cancer Institute and the University of Pittsburgh Medical Center.

Reagents and Chemicals

RPMI-1640 tissue culture medium, nonessential amino acids, and antibiotics were purchased from Gibco (Grand Island, NY). Interleukin-2 was a generous gift from the Cetus corporation (Emeryville, CA). Sucrose (Ultra-pure) was purchased from Beckman (Fullerton, CA). Ethylenedinitrilotetraacetic acid disodium salt (EDTA) was purchased from Fisher Scientific (Fair Lawn, NJ). Dithiothreitol (DTT), 2-mercaptoethanol, fluorogenic protease substrates t-butyloxycarbonyl-Phe-Ser-Arg-7-amido-4-methylcoumarin (Boc-FSR-AMC), N-succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (Suc-AAF-AMC), and the buffers N-[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid] (HEPES) and piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES) were purchased from Sigma (St. Louis, MO). Amicon® nitrogen pressure-based concentration apparatus and Diaflo® 30 kDa cut-off filters were obtained from Amicon® (Beverly, MA). Sephacryl S-400 and heparin-Sepharose® CL-6B chromatography media were purchased from Pharmacia (Piscataway, NJ). Glass columns were purchased from Kimble (Vineland, NJ) and the fraction collection apparatus was purchased from ISCO (Lincoln, NE).

Cells and Cell Lines

YAC-1, a murine (A/J) Moloney virus-induced T lymphoma cell line [Sjogren and Hellstrom, 1965], and P-815, a DBA/2 mastocytoma cell line [Dunn and Potter, 1957], were both maintained in culture medium containing 10% fetal bovine serum, and supplemented as described below. CRNK-16 cells were obtained from Dr. W.H. Chambers of the Pittsburgh Cancer Institute and were maintained in 5% fetal bovine serum containing RPMI-1640 supplemented with L-glutamine, 1% (v/v) MEM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, and 100 U/ml penicillin and 100 μg/ml streptomycin antibiotics.

Preparation of Rat A-NK Cells

Fischer 344 rat-derived interleukin-2 (IL-2)–activated NK (A-NK) cells were generated as previously described [Vujanovic et al., 1988a]. Briefly, rat splenocytes obtained from buffy coat layers via Lympho-paque® centrifugation, 1,000*g* for 25 min at 4°C, were cultured in 1,000 U/ml (Cetus Units) human recombinant IL-2, in RPMI-1640 supplemented with heat-inactivated fetal bovine serum (10% final), 2-mercaptoethanol (5×10^{-5} M final), 1% MEM nonessential amino acids, and 100 μg/ml streptomycin and 100 U/ml penicillin (complete medium). After approximately 48 hr of incubation in a humidified atmosphere containing 5% CO_2 at 37°C, the conditioned medium was decanted and collected via centrifugation at 500*g* for 10 min at 23°C. Residual nonadherent cells were subsequently removed via washing 2× with 37°C complete medium. The conditioned medium was

0.22 μm filtered and returned to the flasks, and the remaining adherent cells were incubated as described above for an additional 3 d.

Preparation of Rat A-NK Cell Postnuclear Supernatant-Associated Proteases

Cellular homogenates served as a source of cell-associated proteolytic activity. All steps subsequent to harvesting were performed at 4°C. Approximately 1–1.5 × 10^9 CRNK-16, or 0.3 × 10^9 5- to 6-d-old rat A-NK cells were harvested from tissue culture flasks using a cell scraper (Falcon Labware), and washed sequentially with RPMI-1640, 0.25 M sucrose in 5 mM HEPES buffer, pH 7.3, and relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, and 10 mM PIPES, pH 7.3). Washes were performed via centrifugation at 600*g* for 10 min. Washed cells were then resuspended in relaxation buffer containing 1 mM ATP at a concentration of 5–10 × 10^7/ml and subjected to nitrogen cavitation at 325–350 psi for 30–40 min. The cells were subsequently monitored for lysis following cavitation and were rehomogenized if whole cells were found. Finally, nuclei were subsequently removed via centrifugation at 450*g* for 10 min, and EDTA was added to the resultant postnuclear supernatant for a 2 mM final concentration.

Isopycnic Density Centrifugation

Postnuclear supernatants from both cell sources were fractionated as described [Kitson and Widnell, 1982]. Briefly, postnuclear supernatants were overlayed within minigradients of 0.0–0.8 M sucrose onto 30 ml of a 0.8–1.8 M linear sucrose gradient containing 1 mM EDTA and 5 mM HEPES buffer (pH 7.3). The gradients were centrifuged in a Sorvall TV-850® (vertical) rotor at 32,000*g* for 16 hr, and 1.1 ml fractions were collected by positive displacement using 2 M sucrose and a 1 ml/min flow rate. Densities of sample fractions were determined by refractive index.

Amicon® Concentration

The CRNK-16–derived sucrose gradient fractions found to contain chymotrypsin-like protease activity were concentrated five- to sixfold under 20 psi nitrogen pressure in a 10 ml capacity Amicon® concentration apparatus containing a 30 kDa exclusion filter.

Gel Filtration Chromatography

Gel filtration columns containing Sephacryl® S-300 and S-400 gels were prepared according to the manufacturer's (Pharmacia) instructions.

Sephacryl S-300 Gel Filtration Chromatography

For purification of A-NK cell-derived material, approximately 3.5 ml samples of pooled sucrose gradient fractions (either neat or concentrated) were applied to a siliconized 1.5 × 35 cm column. For molecular weight determinations, 0.5 ml samples were applied to a 1 × 35 cm column. The elution buffers used were 10 mM ammonium acetate, pH 6.8, and 20 mM HEPES (pH 7.2). The elution of proteins was conducted at 4°C, and fractions were collected at a flow rate of 17 ml/hr.

Sephacryl S-400 Gel Filtration Chromatography

Approximately 3.5 ml samples of CRNK-16–derived pooled sucrose gradient fractions (either neat or concentrated) were applied to a siliconized 1.5 × 110 cm column containing S-400 gel. The elution buffer used routinely was: 10 mM PIPES, 0.5 M sucrose, 1 mM DTT and EDTA, pH 6.5. The elution of proteins was conducted at 4°C, and fractions of approximately 4 ml were collected at a flow rate of 17 ml/hr. Fractions were assayed for protease activity as described below. Fractions containing activity peaks were routinely pooled prior to heparin–Sepharose® chromatography to derive sufficient material for subsequent analysis.

Heparin–Sepharose Chromatography

All steps were performed at 4°C. Pooled S-400 fractions were applied directly to a 1 × 15 cm column containing heparin-Sepharose® CL-6B (Pharmacia), at a flow rate of approximately 1 ml/min. Subsequently, the column was washed with 20% glycerol (v/v), 50 mM KCl, 1 mM DTT and EDTA in 10 mM Tris/HCl, pH 7.4 (H-S elution buffer), at a flow rate of approximately 0.25 ml/min. The proteasome was eluted using a linear gradient of 50–400 mM KCl in H-S elution buffer.

Protein Concentration Determination

The fluorescamine-based protein assay used was as described [Viets et al., 1978]. Briefly, to 25 μl of sample, 250 μl of 50 mM $NaPO_4$ buffer (pH 8.7) was added. The buffered sample was then vortexed vigorously for 3 sec. During vortex-

ing, 100 μl of fluorescamine (0.3 mg/ml acetone) solution was added. This reaction mixture was diluted by adding 625 μl of deionized water, and the fluorescence was measured using an MPF-66 fluorimeter, at 395 nm excitation, and 475 nm emission. Bovine serum albumin was used as a standard.

Assay of Proteolytic Activities

Three protease assay buffers were employed to assay cleavage of 7-amido-4-methylcoumarin (AMC) peptide substrates [Zimmerman et al., 1977, 1978]. To measure Boc-Phe-Ser-Arg-AMC cleaving (trypsin-like) activity: 1 mM dithiothreitol (DTT) and EDTA, 10 μg/ml BSA, 0.02% Triton X-100 (v/v) in 50 mM Tris/HCl buffer, pH 8.7, was used; for Suc-Ala-Ala-Phe-AMC cleaving (chymotrypsin-like) activity 1 mM DTT and EDTA, 10 μg/ml BSA, (0.02%) SDS 10 mM Tris/HCl buffer, pH 7.5, was used and for benzyloxycarbonyl (Z)-Gly-Gly-Leu-AMC cleaving activity 1 mM DTT and EDTA, 10 μg/ml BSA, (0.02%) SDS 10 mM Tris/HCl buffer, pH 8.0, was used. All reaction mixtures contained peptide substrates at 10 μM concentrations unless otherwise noted. The rates of cleavage were determined by taking periodic fluorescent intensity measurements using a Dynatech® microfluor fluorescence plate reader. The amounts of AMC liberated were calculated by normalizing the data relative to the intensity of a 1 μM AMC standard solution.

Analysis by 0.02% SDS-PAGE of Proteasomal Activity

Approximately 5 μg of purified proteasome was applied per lane of a 1.5 mm × 5 cm (resolving section) 4% to 12% gradient polyacrylamide gel. The appropriate volume of a 5× sample buffer stock was added to each sample for a final concentration of (w/v) 0.02% SDS, 10% glycerol, 0.001% bromophenol blue, 1 mM dithiothreitol, 50 mM Tris/HCl (pH 6.8). The 4% stacking gel buffer contained 0.02% SDS, 1 mM DTT, in 15 mM Tris/HCl (pH 6.8). The resolving gel buffer also contained 0.02% SDS, and 1 mM DTT in 50 mM Tris/HCl (pH 8.5). The running buffer used was 0.02% SDS, 250 mM Tris/glycine (pH 8.5).

Detection of Proteolytic Activity Within Gel Slices

Polyacrylamide gel lanes that were analyzed for trypsin-like activity were excised following electrophoresis and incubated in 10 mM Tris/HCl buffer, pH 6.8, containing 2.5% Triton X-100 for 30 min at 23°C. Gel lanes assayed for chymotrypsin-like activity were assayed directly following electrophoresis. Gel lanes were sectioned by manual slicing on a Fakir bed of razor blades; each slice was transferred into a well of a Dynatech® microtiter plate and subsequently sectioned further into cubes. Proteolytic assay was performed as described above with the exception that assays for trypsin-like activity were conducted using 50 μM substrate. Results were expressed as increase in AMC concentration per slice per min.

Immunoblotting and Electron Microscopy

Reagents, apparatus, and affinity-purified rabbit anti-rat hepatic dinitrophenol-proteasome were prepared as described [Rivett, 1989; Rivett and Sweeney, 1991]. Briefly, samples for SDS/PAGE were incubated at 90°C for 2–3 min in the presence of SDS and 2-mercaptoethanol, and applied to a 15% separating gel. Proteasomal subunits resolved by SDS/PAGE were electrophoretically transferred onto nitrocellulose membranes by means of the Bio-Rad Trans-blot apparatus using 25 mM Tris/192 mM glycine/10% (v/v) methanol as a transfer buffer. Transferred proteins were detected using 0.2% Ponceau S stain in 3% (w/v) trichloroacetic acid. To prevent nonspecific binding, the blotted nitrocellulose membranes were incubated in 10% (w/v) Marvel dried milk powder, 10% (v/v) glycerol, 1 M glucose, 0.01% (v/v) Tween 20 in PBS. Following overnight incubation with rabbit anti-proteasome IgG (usually a 1/200 dilution of a 2–3 mg solution), the nitrocellulose was washed and incubated for 1 hr at room temperature with a 1:750 dilution of pig-anti-rabbit antiserum in 3% Marvel dried milk powder, 0.01% (v/v) Tween 20 in PBS. Detection of immunoreactive material was accomplished using 4-chloro-1-napthol (1 mg/ml) in 20% (v/v) methanol/80% (v/v) Tris-buffered saline and 0.01% (v/v) H_2O_2.

Electron microscopic analysis was performed as follows: a molecular suspension of purified rat CRNK-16 proteasomes (10 μg/ml) was spread on formvar/carbon-coated grids, which had been glow discharged to optimize molecular adhesion. Following a 5 min incubation, the excess suspension was removed with 4% uranyl acetate in water. After two further washes, the grids were air dried and examined with a Jeol 100CXII electron microscope.

Rat Liver Proteasomes

The proteasome of rat liver was purified and characterized as previously described [Rivett, 1989].

RESULTS

Biochemical Properties of the Cytosolic Chymotrypsin-Like Activity of Rat A-NK Cells

To test the hypothesis that multiple proteases contribute to physiological functions performed by NK and A-NK cells, we have surveyed the protease content of rat A-NK cells [Goldfarb et al., 1992; Wasserman et al., in press], as well as derived a functional correlation between inactivation of cytotoxicity and the inhibition of a cytosolic chymotrypsin-like activity [Goldfarb et al., 1992]. Thus, efforts to purify this activity were initiated.

The possibility that the chymotrypsin-like activity of interest (A-NKP 2) was a constituent of the proteasome was investigated using the hepatic proteasome as a standard for comparison (Table I, and data not shown). For this chymotrypsin-like protease, the effects of Triton X-100 and SDS detergents were found to be similar to previously published data for effects on MCP complex activities [Wilk and Orlowski, 1980]. Moreover, similar buffer conditions effected the activity of CRNK-16–derived MCP complex in a manner similar to that of A-NK cell-derived material (data not shown). Differences between the rat hepatic and A-NK cell proteasomes regarding the susceptibility of this activity to NaCl and the inhibitor of trypsin-family proteases, antipain, were found. In addition, the trypsin-like activity which accompanied the chymotrypsin-like activity of interest was completely inhibited by SDS (at the concentration indicated) whereas it has been reported that the rat liver proteasomal trypsin-like activity was not.

Fractionation of A-NK Cell Postnuclear Supernatant Protease Activities by Isopycnic Sucrose Gradient Fractionation

The fractionation of rat A-NK cell postnuclear supernatants via sucrose gradient centrifugation revealed the presence of chymotrypsin- and trypsin-like activities that did not comigrate with peak enzymatic activity, indicative of plasma membrane, lysosomes, Golgi, or erythrolytic granules [Goldfarb et al., 1992]. In addition, these activities coeluted in the void volume following G-100 gel permeation chromatography (data not shown). We therefore suspected that these cytosolic protease activities (A-NKP 1 and A-NKP 2) might belong to the proteasome of these cells. Given previously reported findings regarding the multicatalytic nature of cytosolic protease complexes and the ability of the bovine pituitary proteasome to cleave the fluorogenic substrate benzyloxycarbonyl (Z)-Gly-Gly-Leu-AMC [Orlowski and Wilk, 1981; Wilk and Orlowski, 1980, 1983], the distribution of chymotrypsin-, trypsin-like, and Z-Gly-Gly-Leu-AMC cleaving activities was investigated. Following isopycnic sucrose gradient centrifugation, and under conditions found optimal for the detection of chymotrypsin-like activity, the proteolytic activities of both rat A-NK and CRNK-16 cells followed the pattern shown (Fig. 1, and data not shown). The first peak, located at 1.12 gm/ml, contained Z-Gly-Gly-Leu-AMC cleaving and trypsin-like activity as well. These activities were consistently found within a region of the gradient that did not contain peak enzymatic activities indicative of plasma membrane, lysosomes, Golgi, or erythrolytic granules [Goldfarb

TABLE I. Biochemical Properties of Proteasomal Activities: Rat A-NK Cell Versus Rat Liver*

Treatment		A-NK cells		Liver		Reference
		C	T	C	T	
SDS	(0.02%)	214	0	399	150	[Rivett, 1989]
Triton X-100	(0.1%)	37	132	50	100	[Arribas and Castaño, 1990]
	(0.01%)		117	55	110	[Arribas and Castaño, 1990]
NaCl	100 mM	10	50	140		[Yamamoto et al., 1986]
Antipain	0.01 mg/ml	8	0	105	15	[Rivett, 1989]

*Values are averages of at least two experiments and are expressed as % of untreated control activity (approximately 2 pMol AMC/min chymotrypsin-like and 1.5 pMol AMC/min trypsin-like activity was analyzed per test sample). Effects on chymotrypsin- and trypsin-like activities are listed under C and T, respectively. For A-NK cells, postnuclear supernatant material was analyzed. References are listed for published hepatic MCP data.

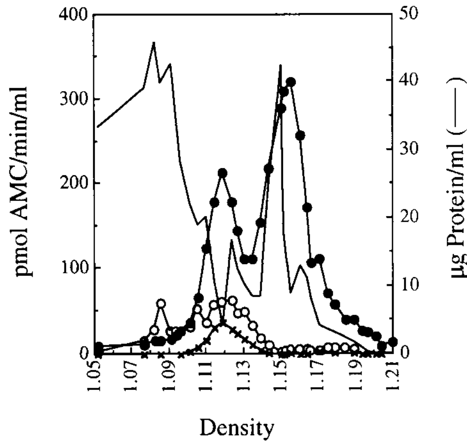

Fig. 1. Isopycnic sucrose density gradient centrifugation of rat A-NK cell postnuclear supernatant proteinase activities. Activities shown are chymotrypsin- (●), trypsin-like (○), and benzyloxycarbonyl-Gly-Gly-Leu-7-amido-4-methylcoumarin (x) cleaving.

et al., 1992]. Under conditions in which 0.02% SDS was included in the assay buffer, a second peak of chymotrypsin-like activity at 1.155 gm/ml was observed, which had not been previously detected during our investigations. Both activity peaks were pooled for attempted resolution by molecular sieve chromatography. The amount of activity recovered at this step was consistently greater than 100%, relative to postnuclear supernatant material. Enrichment of six- to 27-fold overall was achieved following sucrose gradient centrifugation. Fractions containing chymotrypsin-like activity were combined prior to molecular sieve chromatography.

Chromatographic Analysis of A-NK Cell Trypsin-Like and Chymotrypsin-Like Activity

Previous reports of the biochemical properties of proteasomes of other cell types indicated that proteasomal activities were preserved or enhanced in buffers containing 1 mM EDTA and DTT [Tanaka et al., 1986a]. Modification of these buffers such that 0.25 M sucrose was used instead of 20% glycerol resulted in improved recovery of rat A-NK cell-associated chymotrypsin-like activity from 150% to 230% (using S-400 gel).

Typical results of Sephacryl S-300 chromatography of pooled sucrose gradient fractions containing both activity peaks are shown (Fig. 2A). When samples were eluted using 20 mM HEPES buffer (pH 7.2), average recoveries of activity using S-300 gel permeation chromatography for five experiments were 111% and 98% for chymotrypsin- and trypsin-like activity, respectively. Enrichment of the chymotrypsin-like activity was at least 28-fold. Both chymotrypsin- and trypsin-like activities comigrated as a single peak with elution volumes slightly smaller than thyroglobulin (669 kDa), indicating a molecular weight of approximately 700 kDa (data not shown). This value agreed with the reported apparent molecular weight for a variety of proteasomes [Rivett, 1989; Tanaka et al., 1986b].

Pooled sucrose gradient fractions containing chymotrypsin-like activity from 1.0–1.5 × 10^9 CRNK-16 cells were concentrated, using an Amicon® concentration apparatus as described in Materials and Methods, and applied to an S-400 column. A typical chromatography profile of the chymotrypsin-like proteolytic activity is shown (Fig. 2B). A second peak with an approximate apparent molecular weight of approximately 1.4 MDa was observed. Activity peaks from either source were pooled prior to heparin-Sepharose® to obtain sufficient material for analysis.

Given the inhibitory effects of ionic strength upon the chymotrypsin-like activity of the rat A-NK cell proteasome (Table I, and data not shown), low ionic strength buffers were used. The rat liver proteasome was eluted from hepa-

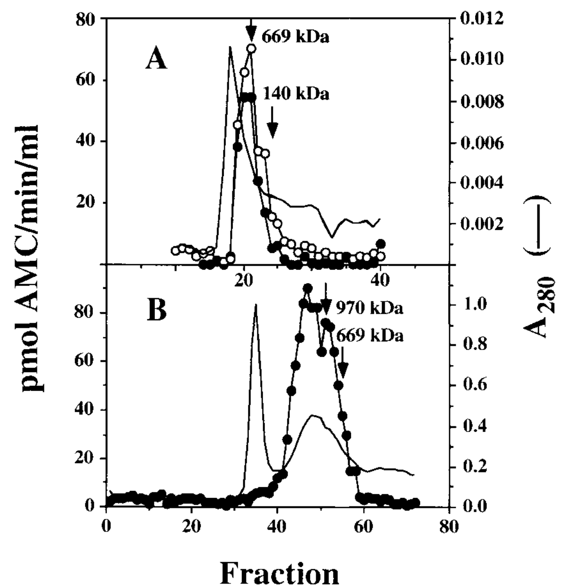

Fig. 2. Gel permeation chromatographic analysis of A-NK cell and CRNK-16–derived MCP complexes. A: Sephacryl S-300 molecular sieve chromatography of rat A-NK cell-derived sucrose gradient fractions containing chymotrypsin-like activity (approximately 134 pMol AMC/min). B: S-400 chromatography of rat CRNK-16–derived proteasomal chymotrypsin-like activity (approximately 860 pmol AMC/min). Symbols are as described in the legend for Figure 1.

rin-Sepharose® columns at approximately 125 mOsM KCl [Tanaka et al., 1986a], and thus this method was used to further purify proteasomes from CRNK-16 and rat A-NK cells. Both rat A-NK cell (Fig. 3A) and CRNK-16 (Fig. 3B) proteasomes were eluted at approximately 125 mM KCl, as shown (Fig. 3). As predicted by previous studies of the proteasome, A-NK cell and CRNK-16–derived Z-Gly-Gly-Leu-AMC cleaving activity co-eluted with both trypsin- and chymotrypsin-like activity peaks; the CRNK-16–derived chymotrypsin-like activities also eluted as a single peak (Fig. 3B). Recovered activity at this step was consistently greater than 200%, and relative to postnuclear starting material, between 3,000- and 8,000-fold enrichment was achieved. The degree of purification achieved for representative batches of the rat A-NK cell and CRNK-16 proteasomes at each stage of enrichment is summarized in Tables II and III, respectively.

Sedimentation Characteristics of Rat Hepatic and Rat A-NK Cell Proteasomes

To confirm the assignment of A-NKP 2 as a constituent of the rat proteasome, we compared the biophysical properties of the rat A-NK cell proteasome to rat liver proteasomes in parallel sucrose gradients (Fig. 4). In addition, as was observed previously for the rat A-NK proteasome, the trypsin-like activity of the rat hepatic proteasome cosedimented with chymotrypsin-like activity. The distribution of trypsin- and chymotrypsin-like activity peaks of the rat hepatic proteasome were more extensive.

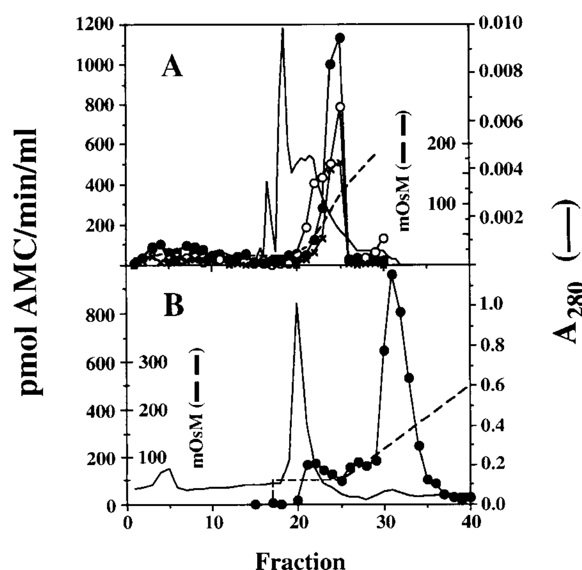

Fig. 3. Heparin-Sepharose® chromatography of pooled S-300 fractions containing A-NK cell-derived chymotrypsin-like activity (A) and pooled S-400 fractions containing CRNK-16–derived chymotrypsin-like activity (B). Symbols are as described in the legend for Figure 1.

TABLE II. Purification of Rat A-NK Cell Proteasomal Chymotrypsin-Like Activity

Purification step:	Specific activity (nmol/min/mg) T[a]	C[b]	Overall fold purification: (C)[b]
PNS[c]	ND	0.31	1.0
Isopycnic SG[d]	1.5	2.3	7.5
Sephacryl S-300	13.3	32.8	107.8
Heparin-Sepharose	ND	1,924.7	6,331.3

[a]T, trypsin-like.
[b]C, chymotrypsin-like.
[c]PNS, postnuclear supernatant (starting material).
[d]Isopycnic SG, isopycnic sucrose gradient centrifugation.

Analysis by 0.02% SDS-PAGE of CRNK-16 Proteasomes

To determine the biochemical characteristics and state of purity of the CRNK-16–derived proteasome, electrophoresis of heparin-Sepharose®–purified material was performed under conditions that maintained detection of trypsin- and chymotrypsin-like activities. Three silver-stained protein bands were found (Fig. 5). All three bands contained trypsin-like activity, and all of the chymotrypsin-like activity was found in the middle band. No silver stain–detectable protein bands were observed in regions of the gel that did not contain either chymotrypsin- or trypsin-like protease activity.

Analysis of Antigenic Crossreactivity by Western Immunoblotting

A previously well characterized polyclonal rabbit antiserum raised against purified hepatic dinitrophenol-proteasome [Rivett and Sweeney, 1991] was used to assess the degree of antigenic crossreactivity between the purified rat hepatic MCP, partially purified rat A-NK cell proteasome, and heparin-Sepharose®–purified CRNK-16 MCP complex subunits. At least two proteins from peak 2 samples (Fig. 1) were crossreactive with antisera directed against rat liver proteasomes (data not shown). Further analysis of CRNK-16–derived proteasomes revealed a series of crossreactive bands ranging from approximately 35 kDa to 20 kDa (Fig. 6, lanes 4 versus 5). In addition, CRNK-16 proteasomal subunits

TABLE III. Purification of the MCP Complex of Rat CRNK-16 Leukemia Cells[a]

Group	Total protein (mg)	Activity (pmol/min/ml sample)	Total activity	Recovery (%)	Specific activity (pmol/min/mg)	Overall fold purification
PNS	66.7	130	1,868	100	28	1
Peak SG[b]	13.1	239	6,846	366	523	19
Concentrated SG	11.6	246	858	46	74	3
Peak S-400	3.7	70	2,849	153	770	28
Peak Heparin-Sepharose	0.04	1,108	8,854	474	221,355	7,906

[a]Monitored by assay of chymotrypsin-like activity.
[b]Isopycnic sucrose gradient fractions.

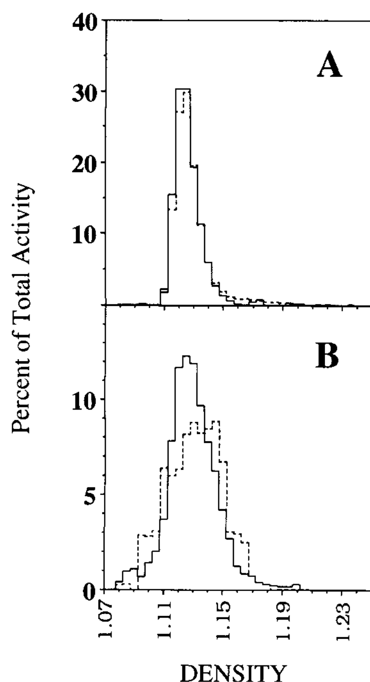

Fig. 4. Isopycnic sucrose density gradient analysis of the rat hepatic and A-NK cell proteasomes. Trypsin- (---) and chymotrypsin-like (—) activities of the rat A-NK cell (**A**) and hepatic (**B**) proteasomes were assayed in 0.1 M Tris-HCl containing 0.1% Triton X-100 (pH 8.0) and 9 μM substrate. Approximately 460 pmol AMC/min of the trypsin-like and 180 pmol AMC/min of the chymotrypsin-like activity was applied (A), and approximately 400 pmol AMC/min of the trypsin-like and 840 pmol AMC/min of the chymotrypsin-like activity was applied (B).

between 77 kDa and 97 kDa and a faint band with a corresponding molecular weight of 14.4 kDa did not have counterparts among hepatic proteasomal subunits (Fig. 6, lane 5); several hepatic proteasomal bands with apparent molecular weights between 25 kDa and 42 kDa were not found among the CRNK-16 proteasome subunits. Four different methods have been employed for the purification of the rat hepatic proteasome. Regardless of method, all of the bands in lane 4 were consistently observed [Skilton et al., 1991].

Morphologic Analysis of CRNK-16-Derived Proteasomes by Electron Microscopy

Given the biochemical, immunochemical, and biophysical differences observed between the proteasomes under investigation, electron microscopic analysis of purified CRNK-16–derived proteasomes was performed. Although the characteristic cylindrical, barrel-shaped morphology of the rat liver proteasome was observed (Fig. 7) [Tanaka et al., 1986b], additional dimeric, trimeric, and tetrameric concatemers by end-on apposition of individual proteasome units were noted.

DISCUSSION

We have previously reported that A-NK cells produce A-NKP 2, a nongranzyme, cytosolic protease that might contribute to cell-mediated cytotoxicity [Goldfarb et al., 1992]. Herein we report the identification of A-NKP 1 and A-NKP 2 as constituents of the multicatalytic proteinase complex of these cells. We have also purified both A-NKP 1 and A-NKP 2 to apparent homogeneity and describe the purification of A-NKP 2 via methods successfully employed for the purification of proteasomes from rat liver [Tanaka et al., 1986a].

Although both hepatic and A-NK cell proteasomal chymotrypsin-like activities displayed similar apparent molecular weights (Fig. 2A and data not shown), and were enhanced in the presence of optimal concentrations of SDS, proteasomal rat A-NK cell chymotrypsin-like activity was inhibited by 100 mM NaCl and the protease inhibitor antipain. In addition, the A-NK cell proteasomal trypsin-like activity was completely inhibited by 0.02% SDS, whereas hepatic proteasomal trypsin-like activity was en-

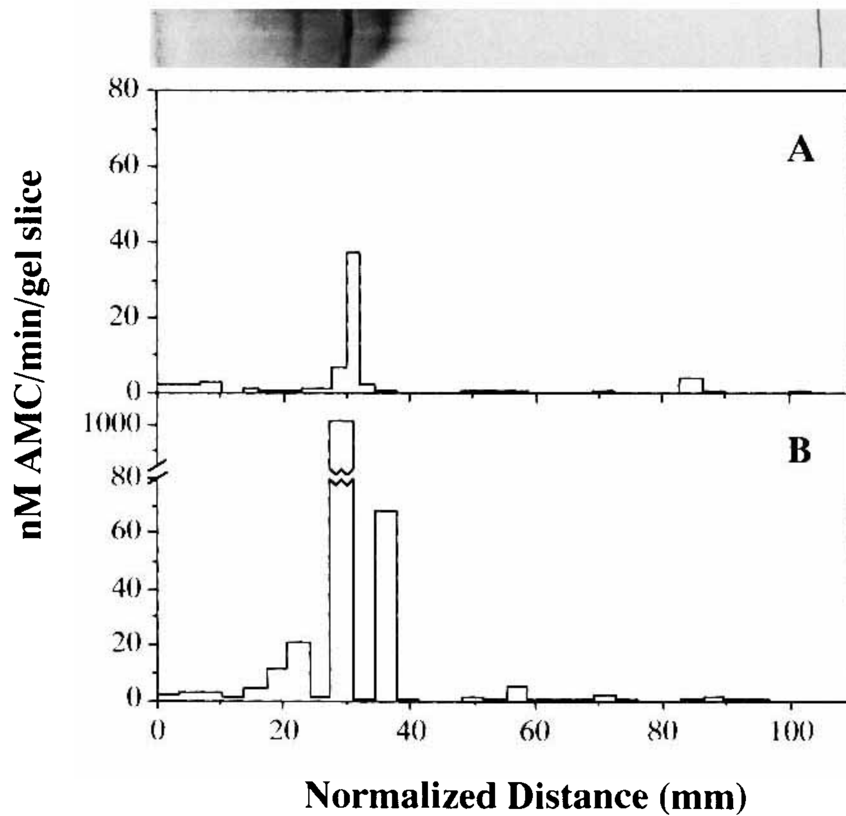

Fig. 5. Gel electrophoresis of purified CRNK-16–derived proteasomal subunit proteins. Approximately 5 μg purified CRNK-16 proteasomes (chymotrypsin-like activity: 110 pmol AMC/min) per gel lane were electrophoresed under nondenaturing conditions in the presence of 0.02% SDS. Contiguous lanes were excised from the gel. One lane was assayed directly following slicing for chymotrypsin-like activity (**A**), the adjacent lane was silver stained, and the next lane was incubated in the presence of Triton X-100 to complex with and extract SDS, and was subsequently assayed for trypsin-like activity (**B**) as described in Materials and Methods.

hanced (Table I). The results of immunoblot analysis also indicated possible differences in subunit relationships within the proteasomes compared, and/or differences in subunit composition (Fig. 6).

We consistently observed total recoveries of chymotrypsin-like activity greater than 100% following gel permeation chromatography. Apparent activation of this activity might be due to putative zymogen/precursor activation and/or separation from reported endogenous inhibitors [Chu-Ping et al., 1992; Murakami and Etlinger, 1986]. Either S-300 or S-400 molecular sieve chromatography could be used for a minimum overall enrichment of at least 28-fold for proteasomes from rat CRNK-16 and A-NK cells, and the results of the heparin-Sepharose® chromatography of the rat CRNK-16 or A-NK cell proteasomes were similar to previous findings [Tanaka et al., 1986a] of approximately 6,000–8,000-fold enrichment. The results of S-400 chromatography of CRNK-16–derived chymotrypsin-like activity, modified (0.02%) SDS-PAGE, as well as the electron microscopic analysis of purified CRNK-16–derived proteasomes indicated the presence of multimeric forms. SDS-PAGE revealed that the preparation was homogeneous since no silver-stained bands were observed in regions of the gel that did not contain proteolytic activity (Fig. 5). The purified proteasomes from both sources displayed both immunoreactivity with monospecific antibodies directed against the rat hepatic proteasome and proteolytic activity (Fig. 6, and data not shown). Therefore, each silver-stained band shown in Figure 5 contained epitopes recognized by these antibodies as well as enzymatic activity associated with proteasomes.

The appearance of two forms of chymotrypsin-like activity, one accompanied by trypsin-like

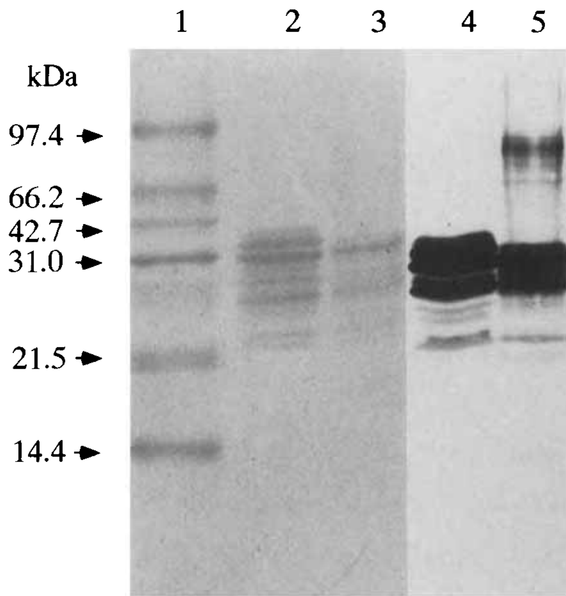

Fig. 6. Immunocrossreactivity between CRNK-16–derived and rat hepatic multicatalytic proteinase complex. **Lanes 1–3:** Ponceau S stained for protein. **Lane 1,** molecular mass standards; **lane 2,** 2 μg purified rat hepatic proteasome; **lane 3,** 1.9 μg purified rat CRNK-16 proteasome; **lane 4,** 2 μg rat hepatic proteasome; **lane 5,** 1.9 μg CRNK-16–derived proteasome.

activity and Z-GGL-AMC cleaving activity and one apparently free of these activities (Fig. 1), suggests that "20S" and "26S" proteasomes (sedimentation values inferred from published reports) in A-NK and CRNK-16 cells differ in their proteolytic activity. The observation that 20S proteasomes of rat liver and rabbit reticulocytes are found within 26S complexes is well documented [Driscoll and Goldberg, 1990; Eytan et al., 1989; Ishiura et al., 1989; Rivett and Sweeney, 1991]. Previous reports have indicated that 1–1.5 MDa 26S particles with "dumbbell" ultrastructure contain 20S proteasome units [Ikai et al., 1991; Peters et al., 1991], and both forms have been identified in separate peaks by density gradient centrifugation [Orino et al., 1991; Peters et al., 1991; Shimbara et al., 1992]. The 26S form of the rabbit reticulocyte proteasome is formed in the presence of nonmetabolizable analogs of ATP [Driscoll and Goldberg, 1990]. Given activation of chymotrypsin-like activity of various proteasomes by SDS [Saitoh et al., 1989; Tanaka et al., 1989] and fatty acids [Orlowski and Michaud, 1989; Yamamoto et al., 1986], the second activity peak in

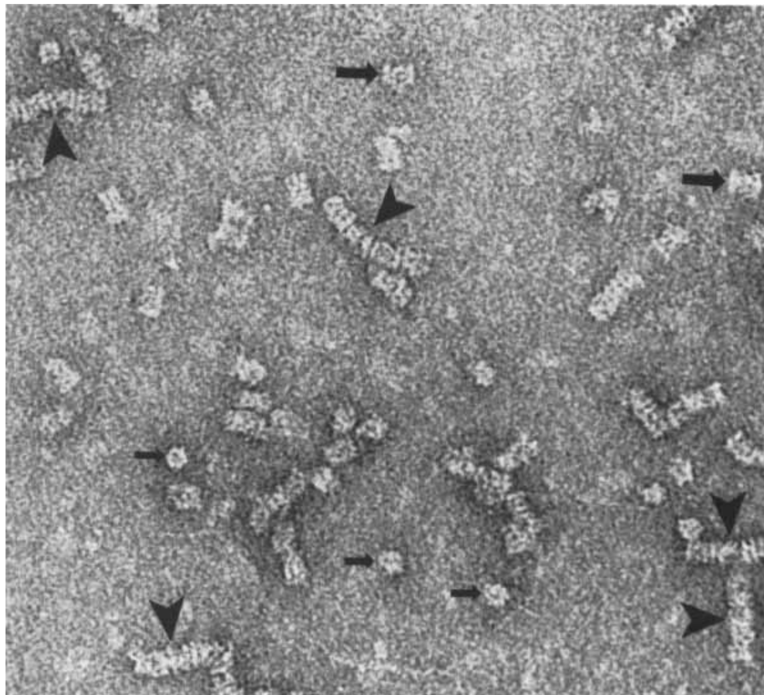

Fig. 7. Electron micrographic analysis of negatively stained CRNK-16 proteasomes. End-on views (small arrows), side view profiles (large arrows), and multimeric (dimeric, trimeric, and tetrameric) forms (chevrons) are shown. Magnification ×646,000.

Figure 1 might represent a membrane-associated form of the proteasome.

The data obtained during four separate purifications of rat A-NK cell and CRNK-16–derived proteasomes were similar to those in Tables II and III. Nevertheless, these data must be interpreted with caution. Rat A-NK cell proteasomal chymotrypsin-like activity was inhibited 89% at 100 mOsM ion concentration (data not shown). Therefore, the specific activity value obtained following the heparin-Sepharose® step might be underestimated. Conversely, given the apparent activation of chymotrypsin-like activity during its purification, the initial activity, and hence the specific activity of the starting material, might actually be higher, thereby causing the final degree of purification to be inflated. Active site cooperativity [Arribas and Castaño, 1990; Ryan et al., 1992] and association with regulatory cofactors/inhibitors [Chu-Ping et al., 1992; Driscoll and Goldberg, 1990; Murakami and Etlinger, 1986] might contribute to variation in the degree of enrichment observed at the molecular sieve step, including differences between the enrichment of trypsin- versus chymotrypsin-like activities (Table II). It is unclear nonetheless how, under the conditions employed, the A-NK cell-derived proteasomal chymotrypsin-like specific activity was approximately 8.7-fold greater than the specific activity value for the CRNK-16–derived enzyme.

Proteasomes are distinct from smaller, monomeric, well characterized serine proteases such as chymotrypsin and trypsin [Orlowski, 1990; Rivett, 1993], and have been found in archaebacteria [Dahlmann et al., 1989; Kloetzel, 1987; Martins de Sa et al., 1986], all eukaryotic cell types, and tissues examined thus far. Many functions for these complexes have been proposed including: ATP/ubiquitin-dependent degradation of proteins [Ciechanover et al., 1991; Driscoll and Finley, 1992], generation/synthesis of enkephalins [Orlowski and Wilk, 1981], degradation of muscle proteins [Dahlmann et al., 1985], roles in cell division and homeostasis [Fujiwara et al., 1990], embryonic development and cell migration [Ahn et al., 1991; Klein et al., 1990], gene regulation [Ciechanover et al., 1991; Grainger and Winkler, 1989; Mellgren, 1990], resistance to virus replication [Horsch et al., 1985, 1989], antigen presentation [Goldberg and Rock, 1992], and interleukin-2 (IL-2)–activated rat natural killer (NK) cell-mediated cytotoxicity [Goldfarb et al., 1992]. While more study is required to resolve the mechanisms by which proteasomes might perform these functions, we remain intrigued by the possibility that proteasomes might play specialized roles in regulating the expression of cytotoxic effector molecules in NK cells.

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