Natural killer (NK) cells and cytotoxic T lymphocytes eliminate virally infected and transformed cells. Target cell killing is mediated by the regulated exocytosis of secretory lysosomes, which deliver perforin and proapoptotic granzymes to the infected or transformed cell. Yet despite the central role that secretory lysosome exocytosis plays in the immune response to viruses and tumors, little is known about the molecular machinery that regulates the docking and fusion of this organelle with the plasma membrane. To identify potential components of this exocytic machinery we used proteomics to define the protein composition of the NK cell secretory lysosome membrane. Secretory lysosomes were isolated from the NK cell line YTS by subcellular fractionation, integral membrane proteins and membrane-associated proteins were enriched using Triton X-114 and separated by SDS-PAGE, and tryptic peptides were identified by LC ESI-MS/MS. In total 221 proteins were identified unambiguously in the secretory lysosome membrane fraction of which 61% were predicted to be either integral membrane proteins or membrane-associated proteins. A significant proportion of the proteins identified play a role in vesicular trafficking, including members of both the Rab GTPase and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and protein families. These proteins include Rab27a and the SNARE vesicle-associated membrane protein-7, both of which were enriched in the secretory lysosome fraction and represent potential components of the machinery that regulates the exocytosis of this organelle in NK cells. *Molecular & Cellular Proteomics* 6:767–780, 2007.
ics to define the protein composition of the NK cell secretory lysosome membrane and hence identify potential components of the exocytic machinery associated with this organelle. To achieve this goal highly enriched secretory lysosomes were obtained by subcellular fractionation of the NK cell line YTS. The integral membrane proteins and membrane-associated proteins were then enriched using Triton X-114 and separated by SDS-PAGE, and the proteins were identified by LC ESI-MS/MS. A total of 221 proteins were unambiguously identified, including members of the Rab GTPase and SNARE protein families. Subsequent validation by immunoblotting with specific antibodies demonstrated that proteins identified by LC ESI-MS/MS were present within the YTS cell secretory lysosome fraction and expressed by primary NK cells.

**EXPERIMENTAL PROCEDURES**

**Materials and Reagents**—Unless otherwise stated all reagents were supplied by Sigma-Aldrich. The following primary antibodies were used: rabbit anti-calnexin (Stressgen), rabbit anti-calreticulin (Stressgen), mouse monoclonal anti-CD63 clone MEM-259 (Serotec), rabbit anti-early endosomal antigen 1 (EAA1; Abcam), rabbit anti-Erp57 (a kind gift from Professor Neil Bulleid, University of Manchester), rabbit ant-GLUT1 (a kind gift from Professor Stephen Baldwin, University of Leeds), mouse monoclonal anti-granzyme B clone ZC5/F5 (BD Biosciences), mouse monoclonal anti-mannose-6-phosphate receptor (MPR) clone 2G11 (Abcam), mouse monoclonal anti-major histocompatibility (MHC) class I heavy chain clone HC10 (11), mouse monoclonal anti-NADH-ubiquinol oxidoreductase clone 20C11 (Molecular Probes), mouse monoclonal anti-Rab8 clone 4 (BD Transduction Laboratories), mouse monoclonal anti-Rab27a clone 4B12 (12) (a kind gift from Professor Miguel Seabra, Imperial College, London, UK), mouse monoclonal anti-RalA (BD Transduction Laboratories), rabbit anti-syntaxin 7 (a kind gift from Dr. Andrew Peden, University of Cambridge), rabbit anti-syntaxin 11 (13) (a kind gift from Dr. Ryts Prekeris, University of Colorado Health Sciences Center), mouse monoclonal anti-transferrin receptor clone H68.4 (Zymed Laboratories Inc.), rabbit anti-transporter associated with antigen processing 1 (TAP1; Calbiochem), sheep anti-TGN46 (a kind gift from Dr. Vas Ponnambalam, University of Leeds), mouse monoclonal anti-vesicle-associated membrane protein-7 (VAMP7) clone CI158.2 (a kind gift from Dr. Thierry Galli, INSERM U536 and UMR144, Paris, France), and rabbit anti-VAMP7 (a kind gift from Professor Paul Luzio, University of Cambridge).

**Cell Culture of YTS Cells and Primary NK Cells**—The human NK cell line YTS was maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 2 mM glutamine, 5 units/ml penicillin, and 50 μg/ml streptomycin. Primary NK cells were isolated from human blood and cultured for 14 days with interleukin-2 as described previously (14).

**Subcellular Fractionation of YTS Cells**—Cells (2 × 10^8) were washed three times in PBS before resuspension into 4 ml of homogenization buffer (10 mM acetic acid, 1 mM EDTA, 190 mM sucrose, 10 mM triethanolamine, pH 7.4) containing 1 mM PMSF and a protease inhibitor mixture (Complete EDTA-free, Roche Applied Science). The cell suspension was homogenized on ice by 10 passes through a ball bearing homogenizer with a 10-μm clearance (Isobiotec). Nuclei, mitochondria, and intact cells were removed by centrifugation (3,000 × g for 10 min at 4 °C), and the resulting postnuclear supernatant (PNS) (3 ml) was layered on top of 21 ml of 27% (v/v) Percoll suspension in homogenization buffer and centrifuged (48,000 × g for 45 min at 4 °C) in a T1250 rotor (Sorvall) to separate subcellular organelles. Twenty-four 1-ml fractions were collected from the gradient. An aliquot of each fraction was added to an equal volume of 2× Laemmli sample buffer and heated at 70 °C for 10 min prior to SDS-PAGE and immunoblotting. Each fraction was assayed for N-acetyl-β-D-glucosaminidase (NAGA) activity and alkaline phosphatase activity to determine the distribution of secretory lysosomes and plasma membrane, respectively, through the gradient using methods described previously (15). Fractions 21 and 22, which contained the highest NAGA activity, were pooled and centrifuged (166,000 × g for 60 min at 4 °C) in a TLS-55 rotor (Beckman) to pellet the membrane fraction, which was stored at –80 °C. An aliquot of this secretory lysosome sample was removed for immunoblotting. In total, secretory lysosomes from 2.9 × 10^10 cells were pooled for proteomics analysis.

**Triton X-114 Phase Partitioning**—Integral membrane proteins and membrane-associated proteins were enriched by Triton X-114 phase partitioning. Solutions of Triton X-114 are homogenous at 0 °C but separate into a detergent-enriched phase and a detergent-depleted phase at temperatures above 20 °C (16). Proteins partition according to their hydrophobicity with integral membrane proteins and membrane-associated proteins partitioning in the lower detergent phase and hydrophilic proteins partitioning in the upper aqueous phase (16, 17). Prior to use Triton X-114 was precondensed, to remove hydrophilic molecules to obtain a more homogeneous preparation, according to the method of Bordier (16). Extraction of integral membrane proteins and membrane-associated proteins was performed according to a modification to the method of Bordier (16). Briefly 1.2 ml of 5% (v/v) Triton X-114 containing Complete EDTA-free protease inhibitor mixture at 0 °C was added to the secretory lysosomal sample (3.3 ml), and the mixture was kept on ice for 30 min before being centrifuged (10,000 × g for 10 min at 4 °C) to remove the insoluble residue. The supernatant was added to 10 ml of 0.2 mM EDTA, 5 mM MgCl2, 200 mM NaCl, and 40 mM Tris-HCl (pH 7.5) and incubated for 5 min at 37 °C before being centrifuged at 4,470 × g for 3 min at room temperature. The upper aqueous phase was discarded, and the lower detergent phase was partitioned a further four times. Proteins in the detergent phase were precipitated by adding 20% (w/v) TCA in acetone, incubating at –20 °C for 4 h, and then centrifuging at 13,000 × g for 20 min at 4 °C. The pellet was washed twice in 90% (v/v) acetone and then resuspended in 95 μl of 2× Laemmli sample buffer (0.02% (w/v) bromphenol blue, 20% (v/v) glycerol, 5% (v/v) mercaptoethanol, 6% (v/v) SDS, and 125 mM Tris-HCl (pH 6.8)) and stored at –80 °C until required.

**SDS-PAGE and In-gel Trypsin Digestion of Secretory Lysosome Membrane Proteins**—The Triton X-114-extracted membrane proteins (95 μl) were loaded into a single well of a 1-mm-thick 4% (v/v) stacking gel above a 7–20% (v/v) gradient polyacrylamide gel. SDS-PAGE was performed in an SE 600 Ruby electrophoresis unit (Amer sham Biosciences) at 20 mA/gel for 2 h followed by 30 mA/gel until the bromphenol blue dye front was 0.5 cm from the bottom of the gel. After electrophoresis the gel was fixed overnight in 50% (v/v) ethanol and 3% (v/v) phosphoric acid before the separated proteins were visualized by Coomassie Brilliant Blue staining (17% (w/v) ammonium sulfate, 34% (v/v) methanol, 3% (v/v) phosphoric acid, and 0.035% (w/v) Coomassie Brilliant Blue (G-250)) for 48 h. The stained gel was destained in deionized water for 16–18 h and then scanned. The lane from the polyacrylamide gel was cut into eight segments (A–H) at points corresponding to molecular weight markers, and then each segment was further cut into 2–4-mm slices. Gel slices were subsequently cut into 1-mm³ pieces with a scalpel before being destained and subjected to in-gel trypsin digestion as described previously (18). Peptides were extracted from gel pieces using vigorous shaking at room temperature using a modification to the method of Wilm et al. (19) as described previously (18). Extracted peptides were dissolved in 8 μl of 2% (v/v) formic acid.
After washing, blots were incubated for 1 h with goat anti-mouse, and separated from other less dense organelles by centrifugation. Fractions were collected, and the secretory lysosomes were pelleted by high speed centrifugation. B, the YTS cell homogenate and PNS were immunoblotted with an antibody specific for mitochondrial NADH-ubiquinol oxidoreductase. No immunoreactivity of mitochondrial NADH-ubiquinol oxidoreductase was observed in the PNS.

RESULTS

Isolation of Secretory Lysosomes from the NK Cell Line YTS—Secretory lysosomes were separated from other organelles on the basis of their size and density by subcellular fractionation (Fig. 1A). To reduce disruption of intracellular organelles, YTS cells were homogenized using a ball bearing homogenizer. The homogenate was subsequently centrifuged at 3,000 \( \times \) g for 10 min to generate a PNS. This speed of centrifugation was used because it was also sufficient to sediment mitochondria as evidenced by immunoblotting with an antibody specific for mitochondrial NADH-ubiquinol oxidi-
doreductase (Fig. 1B). The PNS was then layered on top of a self-forming Percoll gradient, and after centrifugation fractions were collected and assayed for activity of the plasma membrane marker alkaline phosphatase and the lysosomal enzyme NAGA (Fig. 2A). Membrane-associated alkaline phosphatase activity peaked in fractions 8–10, whereas non-membrane-associated soluble alkaline phosphatase activity was present in fractions 1–5. In contrast the peak NAGA activity was much deeper in the gradient in fractions 21 and 22, which is consistent with the high density of secretory lysosomes when compared with that of many other organelles. Therefore fractions 21 and 22 were pooled and retained for proteomics analysis.

To assess the purity of the fractions chosen for proteomics analysis, immunoblots were performed using antibodies specific to proteins localized to a range of different intracellular organelles (Fig. 2B). The presence of secretory lysosomes in fractions 21 and 22 was corroborated by immunoblotting with an antibody specific for CD63, an integral membrane protein associated with secretory lysosomes (22). CD63 was enriched in the fractions with the highest NAGA activity. In contrast, the fractions chosen for proteomics analysis showed no detectable contamination with markers of other organelles, namely calnexin (endoplasmic reticulum), TGN46 (trans-Golgi network), EEA1 (early endosome), MPR (late endosome and trans-Golgi network), GLUT1 (plasma membrane), and CD63 (secretory lysosome marker). Immunoblots are representative of four different experiments.

Protein Separation and LC ESI-MS/MS Identification—Because the primary aim of this study was to identify the exocytic machinery associated with the secretory lysosome membrane, integral membrane proteins and membrane-association proteins were enriched prior to LC ESI-MS/MS by Triton X-114 phase partitioning. This enrichment step was included because peptides from highly abundant luminal proteins may have suppressed the ionization of peptides from less abundant integral membrane proteins and membrane-associated proteins. Triton X-114 solutions are homogenous at 0 °C but separate into a detergent phase and an aqueous phase at 30 °C (16, 23–26). Integral membrane proteins and membrane-associated proteins partition into the detergent phase (23–26). Therefore, the pooled secretory lysosome fractions were solubilized in Triton X-114, and the detergent phase was retained for proteomics analysis. The detergent phase was then separated by SDS-PAGE, and protein bands were visualized with Coomassie Brilliant Blue (Fig. 3). The lane was cut into eight segments (A–H) at points determined by the location of molecular weight.
markers, and then each segment was further cut into 2–4-mm slices. Following in-gel digestion of each gel slice, peptides were separated and identified by LC ESI-MS/MS.

In total, 221 proteins were unambiguously identified from one or more peptide sequences (Table I and the supplemental table). However, there was one example when an unambiguous identification was not possible. In this instance we were unable to discriminate between the closely related proteins Rab11a and Rab11b, which share 90.8% sequence identity. Of the 221 proteins unambiguously identified, 103 were predicted to have at least one hydrophobic membrane-spanning domain, 31 had a predicted lipoprotein motif, and 87 were soluble proteins (Table I). Thus 61% of the proteins identified were likely to be integral membrane proteins or membrane-associated proteins. The remaining proteins represent, at least in part, soluble proteins that were not completely removed by Triton X-114 phase partitioning. These include granzyme B and granzyme H, which are abundant within the NK cell secretory lysosome lumen (27). Alternatively some luminal proteins may have physiochemical properties that cause them to partition into the Triton X-114 detergent phase. One such example is perforin, which although found within the secretory lysosome lumen can insert itself into the membrane of a target cell to form pores (3).

Experimental molecular mass values generally compared well with predicted values based upon cDNA sequences. However, there were some notable exceptions. Thirty-seven proteins were found to have higher than expected experimental molecular mass values. The vast majority of these proteins were either known glycoproteins or had predicted glycosylation sites in their sequences. These include LAMP1 and LAMP2, which based upon their cDNA sequences have predicted molecular mass values of 38.3 and 44.8 kDa, respectively, but due to extensive glycosylation both proteins have experimental molecular mass values of ~120 kDa (28). Forty-five proteins displayed lower than expected molecular mass values; this may be because some of these proteins were in the process of being proteolytically degraded within secretory lysosomes.

Functional Classification of Identified Proteins—Functional classification of proteins was based upon information obtained from published literature and from the Swiss-Prot Protein Knowledgebase (www.expasy.org/sprot/) (Table I and Fig. 4). The major functional class represented was channels and transporters (38 of 221). This is not surprising given the role that the secretory lysosome membrane plays in transporting lysosomal degradation products to the cytosol and in maintaining the acidic environment of this organelle. Other functional classes represented include GTPases (33 of 221) with most of these proteins belonging to the Rab protein family, hydrolases (19 of 221), receptors and signal transduction (19 of 221), immunity (18 of 221) with the majority of these proteins being MHC molecule subunits, metabolism (16 of 221), vesicular and protein trafficking (15 of 223), biosynthesis (9 of 221), cytoskeletal (8 of 221), chaperones (7 of 221), membrane structure and lipid rafts (6 of 221), DNA binding (5 of 221), and redox proteins (3 of 221). Twenty-seven proteins were of unknown/uncharacterized function.

Analysis of the Expression of Proteins Identified by LC ESI-MS/MS—A number of potential components of the NK cell secretory lysosome exocytic machinery were identified by LC ESI-MS/MS. The small GTPases Rab8b, Rab27a, and RaLA have all been shown to participate in regulated secretion in other cell types (6, 7, 29–32), whereas the three SNARE proteins identified by LC ESI-MS/MS, namely syntaxin 7, syntaxin 11, and VAMP7, are either associated with conventional lysosomes or have been implicated in NK cell function (33–37). Nonetheless it was important to validate the data obtained by LC ESI-MS/MS with an alternative method to demonstrate that these proteins were indeed expressed by YTS NK cells and present within the secretory lysosome fraction. To this end immunoblots of YTS cell lysates and secre-
### Table I

Identification of proteins in the lysosomal membrane fraction by LC ESI-MS/MS

SC, sequence coverage; ER, endoplasmic reticulum; Hpp, human protective protein; IMAP, immunity-associated protein; NSF, N-ethylmaleimide-sensitive factor.

| Accession number | Protein identified | Peptides matched; SC (%) | Mascot score | Predicted molecular mass (kDa); location | TMHMM |
|------------------|--------------------|--------------------------|--------------|-----------------------------------------|--------|
| **GTPases**      |                    |                          |              |                                         |        |
| 1 P84077         | ADP-ribosylation factor 1 | 2; 16                  | 194          | 20.7; F                                  | 0α     |
| 2 NP_001654      | ADP-ribosylation factor 6 | 1; 6                    | 114          | 20.2; F                                  | 0α     |
| 3 NP_620150      | ADP-ribosylation factor-like 10B | 4; 26              | 418          | 21.6; F                                  | 0      |
| 4 NP_060654      | ADP-ribosylation factor-like 10C | 4; 33              | 624          | 21.7; F                                  | 0      |
| 5 1AJE           | Cdc42              | 2; 10                   | 195          | 21.7; F                                  | 0      |
| 6 BAB70958       | GTPase, IMAP family member 5 | 1; 3                    | 39           | 39.5; E                                  | 1      |
| 7 NP_112243      | Rab1B              | 4; 36                   | 320           | 22.3; F                                  | 0α     |
| 8 NP_002856      | Rab2               | 7; 44                   | 540           | 23.7; F                                  | 0α     |
| 9 NP_116235      | Rab2B              | 2; 12                   | 509           | 24.4; F                                  | 0α     |
| 10 AAP97171      | Rab4B              | 1; 6                    | 109           | 27.8; F                                  | 0α     |
| 11 NP_004153     | Rab5A              | 3; 27                   | 268           | 23.6; F                                  | 0α     |
| 12 AAH40143      | Rab5B              | 2; 10                   | 236           | 29.0; F                                  | 0α     |
| 13 AA06040       | Rab5C              | 6; 37                   | 413           | 23.5; F                                  | 0α     |
| 14 NP_004628     | Rab7               | 7; 49                   | 579           | 23.8; F–H                                | 0α     |
| 15 AAB19881      | Rab8A              | 2; 11                   | 446           | 23.7; F                                  | 0α     |
| 16 NP_057614     | Rab8B              | 3; 17                   | 471           | 23.7; F                                  | 0α     |
| 17 NP_004242     | Rab9A              | 2; 28                   | 181           | 23.1; F                                  | 0α     |
| 18 NP_057215     | Rab10              | 5; 39                   | 491           | 22.7; F                                  | 0α     |
| 19 CAG38732      | Rab11A or Rab11B   | 2; 12                   | 147           | 21.5; F                                  | 0α     |
| 20 CAG33675      | Rab14              | 4; 26                   | 415           | 23.9; F                                  | 0α     |
| 21 AAH21901      | Rab21              | 3; 19                   | 240           | 24.3; F                                  | 0α     |
| 22 AAC51195      | Rab27A             | 4; 19                   | 316           | 24.8; F                                  | 0α     |
| 23 AAH01157      | Rab33A             | 1; 5                    | 37            | 27.0; G                                  | 0α     |
| 24 CAG46484      | Rab35              | 3; 32                   | 449           | 23.0; F                                  | 0α     |
| 25 AAH16615      | Rab37              | 1; 6                    | 139           | 24.8; F                                  | 0α     |
| 26 AAL12244      | Rab39              | 1; 5                    | 42            | 24.8; H                                  | 0α     |
| 27 NP_002875     | RAP1A              | 4; 32                   | 637           | 21.3; F, G                               | 0α     |
| 28 NP_056461     | RAP1B              | 4; 30                   | 314           | 21.0; G                                  | 0α     |
| 29 AAM12824      | RaA                | 3; 16                   | 177           | 24.0; F                                  | 0α     |
| 30 NP_001656     | Ras homolog gene family, member G | 3; 19              | 173           | 21.3; F                                  | 0α     |
| 31 AAA36544      | Ras-related C3 botulinum toxin substrate 1 | 2; 13             | 212           | 20.8; F                                  | 0α     |
| 32 NP_002863     | Ras-related C3 botulinum toxin substrate 2 | 2; 11             | 140           | 20.5; F–H                                | 0α     |
| 33 AAA36565      | Rh protein         | 1; 10                   | 39            | 21.7; F                                  | 0α     |
| **Vesicular and protein trafficking** | | | | | |
| 34 AAH00804      | Adaptor-related protein complex 3, ε1 subunit | 2; 12             | 128           | 21.7; F                                  | 0      |
| 35 NP_004850     | Clathrin heavy chain 1 | 8; 6                   | 600           | 191.5; B                                 | 0      |
| 36 AAH08081      | ER lumen protein-retaining receptor 2 | 4; 27             | 233           | 24.6; F                                  | 0      |
| 37 AAH13314      | NSF protein        | 1; 4                    | 118           | 36.2; E                                  | 0      |
| 38 AAB62724      | Secretory carrier-associated protein 3 | 2; 10             | 133           | 38.7; C, E, F                            | 4      |
| 39 AAAH16509     | Secretory carrier-associated membrane protein 4 | 1; 5             | 60            | 26.0; F                                  | 4      |
| 40 AAD27834      | Sorting nexin 11   | 1; 5                    | 66            | 29.6; F                                  | 0      |
| 41 AAD48491      | Sorting nexin 12   | 2; 10                   | 82            | 19.0; G                                  | 0      |
| 42 CAI12572      | Synaptic vesicle glycoprotein 2A | 1; 3             | 80            | 82.6; C                                  | 12     |
| 43 NP_006745     | Synaptophysin-like 1 isoform a | 1; 4             | 81            | 28.5; A, D–F                            | 3      |
| 44 NP_003560     | Syntaxin 7         | 1; 5                    | 71            | 29.8; E                                  | 1      |
| 45 AAC24031      | Syntaxin 11        | 1; 4                    | 36            | 32.9; E                                  | 0α     |
| 46 CAD70593      | VAMP7              | 5; 42                   | 393           | 20.1; A, F                               | 1      |
| 47 AAH01825      | Transmembrane trafficking protein | 1; 4          | 65            | 25.1; F                                  | 2      |
| 48 751846A       | Ubiquitin          | 3; 5                    | 210           | 8.5; A–H                                 | 0      |
| **Membrane structure and lipid rafts** | | | | | |
| 49 NP_001771     | CD63 antigen       | 1; 5                    | 91            | 25.6; A–E                                | 4      |
| 50 NP_005794     | Flotillin 1        | 13; 46                  | 951           | 47.3; D–F                                | 0      |
| 51 NP_004466     | Flotillin 2        | 12; 36                  | 872           | 41.7; D, E                                | 0      |
### CHANNELS AND TRANSPORTERS

| Accession number | Protein identified                                                                 | Peptides matched; SC (%) | Mascot score | Predicted molecular mass (kDa); location | TMHMM |
|------------------|-------------------------------------------------------------------------------------|--------------------------|--------------|------------------------------------------|-------|
| 55 AAB96347      | ADP/ATP carrier protein (adenine nucleotide translocator 2)                         | 3; 11                    | 227          | 32.8; E                                  | 3     |
| 56 NP_109599     | Amino acid transporter system A1                                                   | 1; 2                     | 41           | 54.0; A                                  | 10    |
| 57 NP_277053     | Amino acid transport system N2                                                     | 1; 2                     | 46           | 51.4; A, C, D                            | 11    |
| 58 BAA77248      | ATPase                                                                             | 2; 2                     | 133          | 130.9; B                                 | 8     |
| 59 NP_775965     | ATPase, class VI, type 11C isoform a                                              | 1; 1                     | 47           | 128.0; D                                 | 7     |
| 60 NP_001685     | ATPase, H⁺-transporting, lysosomal, V0 subunit c                                  | 1; 20                    | 100          | 15.7; G                                  | 4     |
| 61 AAH08861      | ATPase, H⁺-transporting, lysosomal, V0 subunit d1                                  | 2; 8                     | 159          | 40.3; E                                  | 0     |
| 62 NP_005041     | ATP-binding cassette, subfamily D, member 4 isoform 1                             | 1; 12                    | 44           | 11.0; H                                  | 1     |
| 63 NP_064731     | ATP-binding cassette, subfamily D, member 4 isoform 5                             | 2; 4                     | 81           | 55.7; E                                  | 5     |
| 64 NP_004880     | ATP synthase, H⁺-transporting, mitochondrial F0 complex, subunit f isoform 2a     | 1; 12                    | 44           | 11.0; H                                  | 1     |

### HYDROLASES

| Accession number | Protein identified                                                                 | Peptides matched; SC (%) | Mascot score | Predicted molecular mass (kDa); location | TMHMM |
|------------------|-------------------------------------------------------------------------------------|--------------------------|--------------|------------------------------------------|-------|
| 93 AAC51775      | Carboxypeptidase D                                                                  | 2; 2                     | 176          | 152.9; D                                 | 2     |
| 94 CAA60671      | Cathepsin C                                                                         | 1; 3                     | 116          | 53.0; F                                  | 0     |
| 95 ABB28449      | Cathepsin W                                                                         | 2; 11                    | 114          | 42.7; F                                  | 0     |
| 96 AAH28040      | 2',3'-Cyclic-nucleotide 3'-phosphodiesterase                                       | 1; 3                     | 41           | 47.5; E                                  | 0     |
| 97 CAA43118      | Dipeptidyl-peptidase IV                                                             | 1; 2                     | 96           | 88.2; B                                  | 1     |
| 98 NP_001767     | Ectonucleoside-triphosphate diphosphohydrolase 1                                   | 1; 3                     | 51           | 58.5; F                                  | 2     |
| 99 BAA74853      | Endonuclease domain-containing 1 protein                                            | 1; 2                     | 68           | 57.1; D                                  | 3     |
| 100 A32609       | α-Glucosidase (EC 3.2.1.20), lysosomal                                              | 2; 4                     | 157          | 105.3; C                                 | 1     |
| 101 NP_003869    | γ-Glutamyl hydrolase                                                                | 3; 16                    | 184          | 35.9; E                                  | 0     |
| 102 AAA75490     | Granzyme B                                                                          | 1; 4                     | 67           | 27.6; D                                  | 0     |
| 103 NP_219491    | Granzyme H                                                                          | 1; 3                     | 77           | 27.3; E                                  | 0     |
| 104 BAA74902     | KIAA0879 protein                                                                    | 1; 2                     | 48           | 52.5; C                                  | 2     |
| 105 CAA83495     | Lysosomal acid lipase                                                                | 1; 3                     | 71           | 45.4; D                                  | 0     |
| 106 AAH03160     | Lysosomal acid phosphatase                                                           | 1; 3                     | 77           | 48.3; D                                  | 1     |
| 107 BAC11519     | Minor histocompatibility antigen H13                                                | 1; 2                     | 44           | 41.5; C                                  | 7     |
| Accession number | Protein identified | Peptides matched; SC (%) | Mascot score | Predicted molecular mass (kDa); location | TMHMM |
|------------------|--------------------|--------------------------|-------------|----------------------------------------|--------|
| 108 AAH11729     | Presenilin 1       | 1; 3                     | 44          | 52.9; G                                 | 9      |
| 109 AAH72405     | Serine carboxypeptidase 1 protein | 7; 24             | 427         | 50.8; E–G                              | 0      |
| 110 CAD13133     | Signal peptide peptidase-like 2A | 1; 2          | 90          | 58.1; A–D                              | 9      |
| 111 AAH14863     | Tripeptidyl-peptidase I | 3; 8             | 206         | 61.2; D, G                             | 0      |
| 112 NP_001020329 | CD74 antigen isoform c | 1; 9              | 77          | 18.4; H                                | 1      |
| 113 CAD52872     | Cystatin F         | 1; 6                    | 49          | 16.9; F                                | 0      |
| 114 AAH08611     | HLA-A25α           | 6; 23                   | 444         | 41.1; A–G                              | 1      |
| 115 CAC38066     | HLA-B48a           | 2; 8                    | 271         | 40.3; E, F                             | 1      |
| 116 AAS59645     | HLA-B52α           | 1; 4                    | 170         | 41.3; E–H                              | 1      |
| 117 CAG28534     | HLA-DQB1           | 1; 9                    | 154         | 21.4; E                                | 0      |
| 118 CAA25076     | HLA-DRα            | 2; 18                   | 250         | 26.0; A–C, E                           | 1      |
| 119 AAH08611     | HLA-DRA3           | 1; 9                    | 392         | 30.0; E                                | 1      |
| 120 AAP80750     | HLA-DQA2           | 4; 19                   | 303         | 28.5; A–E                              | 1      |
| 121 AAP93137     | HLA-DQA1           | 1; 5                    | 102         | 28.9; E, F                            | 1      |
| 122 AAP80750     | HLA-DQβ2           | 6; 23                   | 144         | 61.1; C, D, F                          | 2      |
| 123 AAH08611     | HLA-DQA2           | 1; 5                    | 418         | 27.0; E                                | 1      |
| 124 AAP80750     | HLA-DRβ3           | 2; 14                   | 299         | 29.9; E                                | 1      |
| 125 AAP80750     | HLA-DRβ2           | 5; 32                   | 423         | 28.9; E                                | 1      |
| 126 AAP80750     | HLA-DRβ3           | 2; 14                   | 299         | 29.9; E                                | 1      |
| 127 AAP80750     | HLA-DRβ2           | 1; 5                    | 56          | 131.9; B                               | 0      |
| 128 AAP80750     | HLA-DRβ2           | 10; 29                  | 775         | 54.0; D–G                             | 0      |
| 129 AAP80750     | HLA-DRβ3           | 4; 13                   | 264         | 60.1; D                                | 0      |
| 130 AAP80750     | HLA-DRβ3           | 1; 4                    | 50          | 21.3; F                                | 1      |
| 131 AAP80750     | HLA-DRβ3           | 1; 4                    | 67          | 19.7; D, F                             | 1      |
| 132 AAP80750     | HLA-DRβ3           | 2; 10                   | 100         | 53.0; C                                | 2      |
| 133 AAP80750     | HLA-DRβ3           | 2; 5                    | 107         | 35.1; A–D                              | 6      |
| 134 AAP80750     | HLA-DRβ3           | 2; 6                    | 123         | 37.3; E                                | 0      |
| 135 AAP80750     | HLA-DRβ3           | 1; 1                    | 63          | 96.9; D                                | 15     |
| 136 AAP80750     | HLA-DRβ3           | 2; 7                    | 81          | 42.8; H                                | 1      |
| 137 AAP80750     | HLA-DRβ3           | 2; 9                    | 81          | 42.8; H                                | 1      |
| 138 AAP80750     | HLA-DRβ3           | 1; 1                    | 56          | 131.9; B                               | 0      |
| 139 AAP80750     | HLA-DRβ3           | 10; 29                  | 775         | 54.0; D–G                             | 0      |
| 140 AAP80750     | HLA-DRβ3           | 4; 13                   | 264         | 60.1; D                                | 0      |
| 141 AAP80750     | HLA-DRβ3           | 1; 4                    | 50          | 21.3; F                                | 1      |
| 142 AAP80750     | HLA-DRβ3           | 1; 4                    | 67          | 19.7; D, F                             | 1      |
| 143 AAP80750     | HLA-DRβ3           | 2; 10                   | 100         | 53.0; C                                | 2      |
| 144 AAP80750     | HLA-DRβ3           | 2; 5                    | 107         | 35.1; A–D                              | 6      |
| 145 AAP80750     | HLA-DRβ3           | 2; 6                    | 123         | 37.3; E                                | 0      |
| 146 AAP80750     | HLA-DRβ3           | 1; 1                    | 63          | 96.9; D                                | 15     |
| 147 AAP80750     | HLA-DRβ3           | 2; 7                    | 81          | 42.8; H                                | 1      |
| 148 AAP80750     | HLA-DRβ3           | 2; 9                    | 81          | 42.8; H                                | 1      |
| 149 AAP80750     | HLA-DRβ3           | 1; 1                    | 56          | 131.9; B                               | 0      |
| 150 AAP80750     | HLA-DRβ3           | 10; 29                  | 775         | 54.0; D–G                             | 0      |
| 151 AAP80750     | HLA-DRβ3           | 4; 13                   | 264         | 60.1; D                                | 0      |
| 152 AAP80750     | HLA-DRβ3           | 1; 4                    | 50          | 21.3; F                                | 1      |
| 153 AAP80750     | HLA-DRβ3           | 1; 4                    | 67          | 19.7; D, F                             | 1      |
| 154 AAP80750     | HLA-DRβ3           | 2; 10                   | 100         | 53.0; C                                | 2      |
| 155 AAP80750     | HLA-DRβ3           | 2; 5                    | 107         | 35.1; A–D                              | 6      |
| 156 AAP80750     | HLA-DRβ3           | 2; 6                    | 123         | 37.3; E                                | 0      |
| 157 AAP80750     | HLA-DRβ3           | 1; 1                    | 63          | 96.9; D                                | 15     |
| 158 AAP80750     | HLA-DRβ3           | 2; 7                    | 81          | 42.8; H                                | 1      |
| 159 AAP80750     | HLA-DRβ3           | 2; 9                    | 81          | 42.8; H                                | 1      |
| 160 AAP80750     | HLA-DRβ3           | 1; 1                    | 56          | 131.9; B                               | 0      |

The Secretory Lysosome Membrane Proteome
| Accession number | Protein identified                                      | Peptides matched; Mascot score | Predicted molecular mass (kDa); location | TMHMM |
|------------------|--------------------------------------------------------|---------------------------------|-------------------------------------------|-------|
| 161              | NP_000166 Glucose-phosphate isomerase                   | 1; 3 73 63.1; D                 | 0                                         |       |
| 162              | CA25833 Glyceraldehyde-3-phosphate dehydrogenase        | 2; 11 158 36.0; E, G           | 0                                         |       |
| 163              | 1402394A Glycogen phosphorylase                         | 6; 8 441 97.1; B               | 0                                         |       |
| 164              | BAC85389 l-Lactate dehydrogenase A chain                | 2; 8 127 30.0; E, F           | 0                                         |       |
| 165              | AAH47621 Nicastrin                                       | 9; 19 478 76.7; B             | 1                                         |       |
| 166              | NP_620061 Phosphoglycerate kinase 2                     | 1; 4 64 44.9; E               | 0                                         |       |
| 167              | AAH62302 Phosphoglycerate mutase 1                      | 1; 5 84 28.8; F               | 0                                         |       |
| 168              | NP_002653 Phospholipase D₁, phosphatidylincholine-specific | 6; 9 442 124.1; A–D          | 0                                         |       |
| 169              | AAA36594 Proactivator polypeptide                        | 1; 2 59 60.3; H              | 2                                         |       |
| 170              | CAA39849 Pyruvate kinase                                | 2; 3 136 57.8; D, F, G        | 0                                         |       |
| 171              | NP_065956 Retinol dehydrogenase 14                      | 1; 4 35 36.8; E               | 1                                         |       |
| 172              | CAA34756 Elongation factor-1 α 1                        | 4; 12 237 50.1; D–F, H        | 0                                         |       |
| 173              | AAH13918 Elongation factor 1 γ                           | 1; 2 41 41.3; D               | 0                                         |       |
| 174              | NP_001952 Elongation factor 2                            | 5; 7 427 95.3; C, D          | 0                                         |       |
| 175              | CAA41027 Ribosomal protein L7                            | 2; 10 129 29.1; E             | 0                                         |       |
| 176              | CAA55816 Ribosomal protein L11                           | 2; 13 115 20.3; F             | 0                                         |       |
| 177              | NP_000967 Ribosomal protein L12                          | 3; 24 196 18.0; F             | 0                                         |       |
| 178              | AAH71674 RPS16 protein                                  | 1; 7 60 17.3; G               | 0                                         |       |
| 179              | AAA36597 Scar protein                                   | 1; 4 68 27.3; E               | 0                                         |       |
| 180              | XP_376420 Similar to ribosomal protein S25              | 1; 8 89 13.8; G               | 0                                         |       |
| 181              | AAA36022 Chaperonin (HSP60)                             | 1; 4 75 61.0; D               | 0                                         |       |
| 182              | CAC15494 DNAJC5                                         | 1; 9 38 19.6; F               | 1                                         |       |
| 183              | BAA02656 DnaJ protein homolog                           | 1; 3 58 44.8; D               | 0*                                        |       |
| 184              | NP_006588 Heat shock 70-kDa protein 8 isoform 1         | 12; 20 663 70.8; C–H          | 0                                         |       |
| 185              | AAH12807 Heat shock 90-kDa protein 1–β                   | 2; 4 167 83.2; C, F, H        | 0                                         |       |
| 186              | CAG32988 Peptidyl-prolyl cis-trans isomerase A           | 5; 39 323 18.2; G             | 0                                         |       |
| 187              | BAA07652 T-complex protein 1 subunit ζ                  | 1; 2 70 58.4; D               | 0                                         |       |
| 188              | NP_859047 Peroxiredoxin 1                               | 4; 20 206 22.3; F             | 0                                         |       |
| 189              | NP_003320 Thioredoxin                                   | 1; 12 42 12.0; H              | 0                                         |       |
| 190              | NP_006397 Thioredoxin peroxidase                         | 1; 4 118 30.7; F              | 0                                         |       |
| 191              | AAC50766 DNA replication licensing factor MCM6          | 1; 1 47 93.0; D               | 0                                         |       |
| 192              | CAA24951 Histone H2A type 1-B                           | 1; 15 85 14.2; G             | 0                                         |       |
| 193              | CAB02542 Histone H2B                                     | 2; 19 148 13.9; G             | 0                                         |       |
| 194              | CAA24918 Histone H4                                     | 1; 12 84 11.3; H             | 0                                         |       |
| 195              | NP_631946 Nucleosome assembly protein 1-like 1          | 2; 7 83 45.3; D              | 0                                         |       |
| 196              | AAH28081 Chromosome 2 open reading frame 18             | 1; 7 64 40.2; E             | 10                                        |       |
| 197              | 1IVYB Chain B, physiological dimer Hpp precursor       | 1; 3 101 51.9; F             | 0                                         |       |
| 198              | AAA35733 Cyclophilin                                    | 1; 6 147 22.8; F, G           | 1                                         |       |
| 199              | AAN08508 Epididymal protein                              | 6; 19 371 43.1; E             | 0                                         |       |
| 200              | BAC03391 FLJ00303 protein                               | 1; 3 91 40.6; D               | 0                                         |       |
| 201              | AAH39741 FLJ11273 protein                               | 1; 5 57 35.1; E               | 1                                         |       |
| 202              | AAH02759 FLJ20489 protein                               | 1; 13 36 10.5; A             | 2                                         |       |
| 203              | NP_006842 Glioma pathogenesis-related protein           | 1; 5 41 30.9; H               | 1                                         |       |
| 204              | AAC97371 HIWI                                          | 1; 1 36 98.5; D             | 0                                         |       |
| 205              | AAD39919 HSPC041 protein                                | 1; 14 63 12.2; G             | *                                         |       |
| 206              | BAB85084 Hypothetical protein FLJ23859                 | 1; 6 39 16.5; F              | 0                                         |       |
| 207              | BAA13378 KIA0247                                        | 2; 12 95 32.7; F             | 2                                         |       |
| 208              | BAA34512 KIAA0792 protein                               | 2; 2 123 94.2; B             | 11                                        |       |
| 209              | BAB67791 KIAA1898 protein                               | 2; 2 157 112.2; E             | 0                                         |       |
| 210              | AAG88807 Multitransmembrane domain immunoglobulin-like protein | 2; 3 123 95.6; C   | 9                                         |       |
| 211              | AAD27000 Osteopetrosis-associated transmembrane protein 1 | 1; 9 75 14.8; D, F, G       | 1                                         |       |
| 212              | NP_009204 Prohibitin                                    | 1; 6 46 33.3; E              | 0                                         |       |
| 213              | AAH24200 Protein FAM3C                                  | 1; 7 35 24.7; F              | 1                                         |       |
tory lysosome fractions were probed with antibodies that recognize Rab8, Rab27a, RalA, syntaxin 7, syntaxin 11, and VAMP7 (Fig. 5). Immunoblots probed with each antibody revealed the presence of a protein of the corresponding size in both the YTS lysate and secretory lysosome fraction, hence providing independent corroboration of the data obtained by LC ESI-MS/MS.

Immunoblots probed with the Rab8 antibody (which recognizes both Rab8a and Rab8b) revealed the presence of a significant proportion of one or both isoforms in the secretory lysosome fraction, consistent with observations in the closely related CTL cell type where this protein is associated with secretory lysosomes (7). RalA, although present, was not enriched in the secretory lysosome fraction implying that the majority of this protein was localized elsewhere in the cell. Of the three SNAREs identified, both syntaxin 7 and VAMP7 were enriched within the secretory lysosome fraction, consistent with a significant proportion of these proteins being localized to this organelle. In contrast syntaxin 11 was not enriched in the secretory lysosome fraction, suggesting that this organelle is not the principal intracellular location of this protein.

Although the YTS cell line is a well established model for NK cells, it was necessary to confirm that the proteins identified in the YTS cells are also expressed in primary human NK cells. Therefore, primary NK cell lysates were immunoblotted with Rab8, Rab27a, RalA, syntaxin 7, syntaxin 11, and VAMP7 antibodies; in each instance the corresponding protein was detected (Fig. 5). Because rabbit antisera were available to detect the SNARE proteins it was also possible to use immunofluorescence microscopy to test for co-localization of syntaxin 7, syntaxin 11, and VAMP7 with secretory lysosomes in primary NK cells by co-staining with a mouse monoclonal antibody specific for CD63 (Fig. 6). CD63 staining was concentrated into large punctate structures in the cytoplasm of the primary NK cells. The intracellular distribution of both syntaxin 7 and VAMP7 paralleled that of CD63 with a high

| Accession number | Protein identified | Peptides matched; SC (%) | Mascot score | Predicted molecular mass (kDa); location | TMHMM |
|------------------|--------------------|---------------------------|--------------|------------------------------------------|-------|
| 214 AA109067     | Proteolipid protein 2 | 1; 9                      | 45           | 17.0; G                                  | 4     |
| 215 AAI9355      | Ring finger protein 149 | 1; 3                      | 88           | 43.1; D, E                               | 1     |
| 216 CAA23754     | Serum albumin        | 1; 2                      | 69           | 69.3; A–C, F                             | 0     |
| 217 AAH02920     | Tetraspanin-14       | 1; 4                      | 55           | 28.8; F                                  | 4     |
| 218 AAH03106     | TMEM59 protein       | 1; 4                      | 38           | 34.4; F                                  | 1     |
| 219 AAH02616     | Transgelin 2         | 2; 10                     | 100          | 22.6; F                                  | 0     |
| 220 NP_060717    | Transmembrane protein 30A | 1; 2                      | 117          | 40.6; D                                  | 2     |
| 221 AA10569      | Unc-93 homolog B1    | 2; 4                      | 96           | 66.6; D                                  | 12    |
| 222 BAC05068     | Unnamed protein product | 1; 2                      | 43           | 66.7; D                                  | 0     |

* Proteins with a putative lipoprotein motif.

Table I—continued
degree of overlap in large punctate structures. This is consistent with the localization of syntaxin 7 and VAMP7 to the secretory lysosome and corroborates the data obtained from the immunoblots of the YTS cell secretory lysosome fractions. In contrast, although there may have been some overlap with CD63, syntaxin 11 staining was more diffuse with some immunoreactivity observed in small punctate structures (Fig. 6). This implies that the majority of syntaxin 11 is not localized to the secretory lysosome in these cells and is in agreement with the results obtained by immunoblotting.

**DISCUSSION**

Subcellular fractionation and proteomics represent a powerful combination when used to analyze the composition and function of intracellular organelles (10). In this study we applied these techniques to NK cell secretory lysosomes with the specific aim of identifying potential components of the exocytic machinery associated with this organelle. Subcellular fractionation of YTS NK cells was performed using self-forming Percoll gradients. Due to their high density it was possible to use this single step fractionation procedure to separate secretory lysosomes from other organelles. Immunoblotting demonstrated that the secretory lysosome fractions were highly enriched with little or no contamination of other organelles. However, LC ESI-MS/MS did identify a number of proteins not normally associated with lysosomes and lysosome-like organelles. One possible interpretation of this finding is that the secretory lysosome fraction may have been contaminated to a small degree with other organelles; this is something that we cannot exclude. However, it is important to note that the secretory lysosome is a degradative organelle, and as such many proteins not classically associated with the secretory lysosome may have been sorted to this organelle to be degraded. This process could occur either via autophagy, in which cytoplasmic components including organelles are delivered to the secretory lysosome (38), or via the endocytic pathway. Consistent with the latter, ubiquitin was identified throughout the molecular mass range of the SDS-PAGE gel suggesting that it was covalently conjugated to a number of different proteins. This is significant because ubiquitin conjugation acts as a signal for the trafficking to the lysosome for membrane proteins that are down-regulated from the plasma membrane (39).

Because the secretory lysosome is a specialized organelle that functions both as a degradative compartment and as a secretory organelle, it is not surprising that many of the proteins identified are also present in conventional lysosomes. Indeed a significant proportion of the proteins identified in this study are orthologues of proteins identified in the proteomics analysis of conventional lysosomes isolated from rat liver (40). These include LAMP1 and LAMP2, both of which are associated with the membrane of conventional lysosomes. However, a substantial number of the proteins identified in this study were not found in rat liver lysosomes, and these proteins reflect the specialized function of the NK cell secretory lysosome. One important function of NK cells is cytotoxicity; correspondingly we identified perforin, granzyme B, and granzyme H in the NK cell secretory lysosome. Another more recently described function of NK cells is antigen presentation to CD4\(^+\) T cells (41); this is also clearly reflected in the composition of the secretory lysosome. Antigen-presenting cells activate CD4\(^+\) T cells by presenting antigens on MHC class II molecules, and these antigens are loaded onto the MHC class II molecules within endocytic/lysosomal compart- ments (42). Indeed not only were 10 different MHC class II molecule subunits identified in the secretory lysosome fraction, but both invariant chain (also known as CD74) and a subunit of human leukocyte antigen-DM (HLA-DM) were identified. Invariant chain is required to target newly synthesized MHC class II molecules to lysosomes, whereas HLA-DM acts as a chaperone to facilitate the removal of the invariant chain and promote the subsequent peptide loading of MHC class II molecules (42). Consistent with the NK cell secretory lysosome serving as a compartment for antigenic peptide loading of MHC class II molecules, HLA-DM is enriched in YTS cell secretory lysosome fractions (data not shown).

The capacity of the NK cell for regulated secretion is reflected by the identification of a number of small GTPases that are known to be associated with this process in other cell types. Rab27a is a key component of the exocytic machinery in CTLs in which it is required to tether secretory lysosomes to the plasma membrane prior to fusion (6, 7). Given that Rab27a was enriched in the YTS NK cell secretory lysosome fraction,

**Fig. 5. Expression of GTPase and SNARE proteins in YTS cells and primary NK cells.** YTS cell lysate, YTS secretory lysosome fraction, and primary NK cell lysate were immunoblotted with antibodies specific for Rab8, Rab27A, RabA, syntaxin 7, syntaxin 11, and VAMP7. 20 \(\mu\)g of protein was loaded in each lane.
it seems highly likely that Rab27a will perform the same function in NK cells. Another potential component of the NK cell exocytic machinery is Rab8b, which also was identified in the secretory lysosome fraction by LC ESI-MS/MS. In the neuroendocrine cell line AtT20, Rab8b co-localizes with adrenocorticotropic hormone, and overexpression of this GTPase stimulates adrenocorticotropic hormone release (30). By analogy Rab8b may also participate in secretory lysosome exocytosis in NK cells, perhaps acting in concert with Rab27a. Indeed a precedent for this notion is granule exocytosis by PC12 cells in which both Rab27a and Rab3a are required for docking of the granules to the plasma membrane (43). Additionally the GTPase RalA was identified by LC ESI-MS/MS, but unlike Rab27a it was not obviously enriched in the secretory lysosome fraction. Nonetheless RalA represents a candidate component for the NK cell exocytic machinery because it is also required for granule exocytosis in PC12 cells, although it is localized primarily to the plasma membrane in these cells (31, 32). Clearly future studies are required to dissect what role RalA and the other small GTPases associated with the secretory lysosome play in the exocytosis of this organelle in NK cells.

LC ESI-MS/MS identified three SNAREs in the secretory lysosome fraction of the YTS NK cell line, and this finding was corroborated by immunoblotting with antibodies specific for each SNARE. Additionally syntaxin 7 and VAMP7 co-localized to a significant degree with the secretory lysosome membrane protein CD63 in primary NK cells when analyzed by immunofluorescence microscopy. Taken together, these results demonstrate the presence of syntaxin 7 and VAMP7 in the NK cell secretory lysosome. This is consistent with previous observations in which syntaxin 7 and VAMP7 were localized to conventional lysosomes (33–35). VAMP7 is of particular interest because based upon literature precedents it is likely to play a central role in the exocytosis of the secretory lysosome. Conventional lysosomes can also undergo exocytosis in non-specialized cells to seal holes in the plasma membrane (44). In this wound repair response, VAMP7 forms a complex with the plasma membrane Q-SNAREs syntaxin 4 and SNAP23 (33); as such VAMP7 may form a complex with the same plasma membrane Q-SNAREs to facilitate secretory lysosome exocytosis. Results obtained by immunoblotting demonstrate that syntaxin 11 was present but not enriched in secretory lysosomes. In addition, immunofluorescence microscopy revealed that although there was a small degree of co-localization with CD63 in primary NK cells, the majority of syntaxin 11 was located on other intracellular organelles. Indeed previous studies have localized syntaxin 11 to a variety of different intracellular locations but not to lysosomes (13, 45, 46). However, our data demonstrate for the first time that syntaxin 11 is expressed in both YTS cells and primary NK cells. This is an important observation because mutations in syntaxin 11 are
linked to familial hemophagocytic lymphohistiocytosis type 4 (36, 37). Familial hemophagocytic lymphohistiocytosis is an autosomal recessive disorder characterized by defective NK cell cytotoxicity, hence implying a role for syntaxin 11 in target cell killing. Given that SNAREs mediate membrane fusion reactions, syntaxin 11 may either be required for the exocytosis of the secretory lysosome or be involved in the trafficking of cytotoxic proteins to this organelle, although further work will be required to determine the precise role of this SNARE.

In summary we describe for the first time the protein composition of the NK cell secretory lysosome membrane. Although many proteins localized to this organelle are also associated with conventional lysosomes, a significant proportion of the proteins identified reflect both the capacity of this organelle for regulated secretion and the immunological role that it plays. Crucially we have used proteomics as a tool to identify the small GTPases and SNARE proteins that represent potential components of the exocytic machinery of the secretory lysosome.

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