IDENTIFICATION AND MOLECULAR CLONING OF LEUKOPHYSIN

A thesis submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements of the degree of
Doctor of Philosophy

BY

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ABSTRACT

Granule exocytosis is one of the mechanisms by which cytotoxic lymphocytes deliver the lethal hit to their targets. A great deal of research has improved our understanding of the molecular biology of granules. In this study a panel of monoclonal antibodies, originally raised against platelet dense granules, were used to identify and clone a leukocyte granule associated protein. By using this approach we have identified leukophysin as a 28 kDa granule membrane protein of $CD3^+$ granulated lymphocytes and U937 monocytic cells. Evidence of leukophysin granule association included the punctate cytoplasmic staining in immuno-fluorescence studies, the increased expression on the plasma membrane upon induction and granule exocytosis as well as the association with a granule marker in subcellular fractions. To clone the leukophysin gene, the antibodies were used to screen a U937 expression library. A partial length clone was isolated and used to rescreen the same library. This led to the isolation of a cDNA with an open reading frame coding for a 235 amino acid polypeptide with a calculated m.w. of 24.309 kDa, a single potential N-glycosylation site, and two potential transmembrane regions. A polyclonal antibody directed against the 17 carboxy terminal amino acids detected the same 28 kDa leukophysin band and reacted with cytoplasmic granular structures in CD3+ lymphocytes suggesting that this clone codes for leukophysin. The nucleotide and the amino acid sequences of the leukophysin cDNA are almost identical to the carboxy terminus of human RNA helicase A. However, leukophysin cDNA lacks the presence of a 104 bp insert detected in RNA helicase and confirmed by reverse transcriptase-PCR . The absence of this insert is suggested to be responsible for the interruption of the reading frame resulting in the production of leukophysin as a granule associated low m.w. isoform of RNA helicase A.

DEDICATION

To my parents

ACKNOWLEDGMENT

I would like to express my deep gratitude to my supervisor, Dr. Arnold Greenberg, for accepting me in his laboratory and for his continuous advice and support. I would like to thank the members of my Ph.D. advisory committee for their guidance. I would also like to thank members of the Department of Immunology at the University of Manitoba and members of the Manitoba Institute of Cell Biology for their help.

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ABBREVIATIONS

AA	amino acid
Ab	antibody
ARF	ADP-ribosylation factor
ATCC	American type culture collection
ATP	adenosine triphosphate
BIRCH	biological research computer hierarchy
BLT	N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester
BSA	bovine serum albumin
CaM	calcium-calmodulin
CCP	cytotoxic cell protease
CD	cluster of differentiation
CGN	cis Golgi network
COP	non-clathrin coat protein
CTL	cytotoxic T lymphocyte
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTNB	5,5-dithiobis, 2-nitro-benzoic acid
ELISA	enzyme linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescent activated cell sorter
FCS	fetal calf serum
GTP	guanosine triphosphate
HBSS	Hanks balanced salt solution
HF	Hanukah factor
IgG	immunoglobulin G
IL	interleukin

IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
LAK	lymphokine activated killer cells
LFA	lymphocyte function associated molecule
LPS	lipopolysaccharide
MAb	monoclonal antibody
MHC	major histocompatibility complex
MOPS	(3-[N-morpholino] propane-sulfonic acid)
MTOC	microtubule organizing center
M.W.	molecular weight
NK	natural killer lymphocyte
NSF	N-ethylmaleimide-sensitive protein
Nuc	nucleotide
ORF	open reading frame
PBM	peripheral blood mononuclear cells
PDG	platelet dense granule
PMA	phorbol myristic acetate
RNA	ribonucleic acid
RNK	rat natural killer cells
SE	serine esterase
SNAP	soluble NSF attachment protein
SNARE	SNAP receptor
TBS	Tris-buffer saline
TCR	T cell receptor
TGN	trans Golgi network
TIA	T cell intracellular antigen

TSP-1 T cell specific proteinase-1

VAMP vesicle associated membrane protein

REVIEW OF LITERATURE

I. Introduction

Protein secretion is a basic function of eukaryotic cells. Intracellular protein transport involves packaging of proteins into vesicles that bud from one membrane and fuse with another. The bulk flow model suggests that protein secretion is constitutive from the endoplasmic reticulum (ER) through the Golgi to the cell surface. Proteins targeted to other locations such as regulated secretory granules and lysosomes divert from the bulk flow at the trans Golgi network (TGN) (Pryer *et. al.*, 1992). In addition to transport vesicles, two types of secretory vesicles have been identified ; constitutive secretory vesicles and regulated secretory granules. Constitutive secretory vesicles are common to all eukaryotic cells and mediate the continuous secretion of newly synthesized proteins. On the other hand, regulated secretory granules release their contents only in response to appropriate stimulation. Secretory granules occur in cells capable of regulated protein secretion (Burgess and Kelly, 1987).

Many leukocytes share the presence of regulated secretory granules containing various molecules. Release of granule contents upon stimulation mediates various biological functions of leukocytes. For example, neutrophil granules are involved in killing and degradation of microorganisms as well as in the mobilization and regulation of mediators of inflammation (Boxer and Smolen, 1988). Mast cell granules contain potent inflammatory and repair mediators (Ishizaka and Ishizaka, 1984). Cytotoxic lymphocytes such as natural killer (NK) cells (Henkart *et. al.*, 1984), cytotoxic T lymphocyte (CTL) lines (Podack and Konigsberg, 1984), and lymphokine-activated killer (LAK) cells (Henkart *et. al.*, 1986) have been shown to contain cytoplasmic granules implicated in the mechanism of lethal hit delivery (Henkart, 1985; Podack, 1985; Young and Cohn, 1986).

II. Lethal hit delivery by cytotoxic lymphocytes

The mechanism by which cytotoxic lymphocytes deliver the lethal hit to their target cells has been the subject of intense investigation. The possibility that CTL kill their targets by acting primarily on their plasma membranes, producing lesions like those of complement, was suggested in the early 70's (Henney, 1974). Later, complement-like lesions were demonstrated by electron microscopy on the membranes of target cells killed by their specific CTL. The putative monomer causing these lesions was named perforin, for its ability to perforate membranes (Dennert and Podack, 1983). The CTL used in the study contained dense cytoplasmic granules that were often found near the site of contact with target cells. The authors, who found similar granules in NK cells and similar membrane lesions in their targets (Podack and Dennert, 1983), suggested an active participation of the cytoplasmic granules in cytolysis. Morphological studies supported this notion by showing rapid reorientation of the cytoplasmic granules in cytotoxic cells to the area of contact with target cell upon binding (Podack, 1985; Yannelli *et. al.*, 1986). Another line of evidence came from studies of the cytotoxic activities of granules. Isolated granules have been shown to be highly cytolytic for erythrocytes as well as tumor targets (Henkart *et. al.*, 1984; Young and Cohn, 1985). This was followed by isolation of perforin as well as other granule components. These findings led to the proposition of a model that explains lymphocytotoxicity termed the granule exocytosis model (Henkart, 1985; Podack, 1985; Young and Cohn, 1986). According to this model, cytotoxic lymphocyte-target cell binding is followed by polarization of the lymphocyte cytoplasmic granules towards the contact zone. Exocytosis of the granules leads to the secretion of perforin monomers together with other granule contents into the intercellular space. Calcium-dependent polymerization of perforin into the target cell membrane leads to the formation of membrane channels similar to those of the

complement-dependent killing (Young *et. al.*, 1988).

Extensive research done by several investigators supports the importance of the granule exocytosis model in lethal hit delivery (Krahenbuhl and Tschopp, 1990). However, there is evidence challenging the generality of this model. First, whereas most of the earlier studies that led to the isolation of cytolytic granules and perforin used CTL lines or clones that were propagated in IL-2 driven cultures, these cells are different from primary *in vivo* primed CTL. Several investigators have failed to detect the cytotoxic granules and perforin in several primary CTLs (Berke and Rosen, 1987; Berke and Rosen, 1988; Dennert *et. al.*, 1987; Nagler-Anderson *et. al.*, 1988; Helgason *et. al.*, 1992). In spite of a report showing the presence of perforin and granzyme A mRNA transcripts in the peritoneal exudate lymphocytes (Nagler-Anderson *et. al.*, 1989), it seems that this expression is not obligatory for the cytotoxic activity (Berke *et. al.*, 1993). Second, some CTL can mediate cytolysis in the absence of calcium which is required for both granule exocytosis and perforin activity, (Clark *et. al.*, 1988; Ostergaard *et. al.*, 1987; Trenn *et. al.*, 1987). Third, some events in lymphocyte-mediated killing have not been entirely accounted for by the granule exocytosis model. Whereas perforin leads to cell death by necrosis (Tschopp and Nabholz, 1990), target cell death induced by cytotoxic lymphocytes is apoptotic (Cohen *et. al.*, 1992). Taken together, these arguments suggest the presence of other cytotoxic mechanisms.

Inhibition studies have shown that DNA fragmentation of the target cell is a cause rather than a result of cell death in CTL-mediated lysis (Helgason *et. al.*, 1993). Several models were proposed to explain the sequence of events that occur during lymphocyte-mediated killing. To explain the prelytic DNA fragmentation of the target cell, a model was proposed, in which interaction of the T-cell receptor complex and associated molecules, such as adhesion molecules, with target cell membrane antigens will alter the permeability of the target cell membrane.

Persistent elevation of the intracellular calcium levels in the target cell would activate endonucleases, ATPases and proteases leading to DNA fragmentation, bleb formation and target cell dissolution (Berke, 1990). However, direct evidence supporting this model is lacking.

The internal disintegration model, in which target cell DNA fragmentation results from the activation of an enzyme within the target cell was proposed (Russell, 1983). Support for the active participation of the target cell in CTL-mediated killing was derived from studies showing that the occurrence and degree of DNA disintegration is dependent on the nature of the target cell (Ucker, 1987; Howell and Martz, 1987). This led to the suggestion of the induced suicide model (Golstein, 1987) in which an inducer molecule in or on the effector CTL binds to a target cell receptor molecule causing activation of programmed cell death in the target cell. Support for Golstein's model comes from recent studies which suggest the involvement of the target cell surface molecule Fas antigen in the Ca^{++}-independent T cell-mediated cytotoxicity (Rouvier *et. al.*, 1993) as well as in $CD4^+$ T cell-mediated cytotoxicity (Stalder *et. al.*, 1994). Fas antigen is a 45 kDa cell surface protein which belongs to the tumor necrosis factor/nerve growth factor receptor family (Itoh *et. al.*, 1991). The importance of Fas antigen in apoptotic cell death has been shown (Yonehara *et. al.*, 1989). Also, Fas has been suggested to be involved in T cell development in the thymus (Watanabe-Fukunaga *et. al.*, 1992; Ogasawara *et. al.*, 1993) as well as in the peripheral tolerance of mature T cells (Russell *et. al.*, 1993). A 40 kDa cell surface protein was recently identified as a Fas ligand. The protein, which was isolated from a CTL hybridoma cell line, induced apoptotic cytotoxic activity against cells expressing Fas antigen (Suda and Nagata, 1994). The identity of the 40 kDa Fas ligand, its expression by other cytotoxic lymphocytes as well as the molecular events following its binding to Fas are all important issues that remain to be determined.

Thus, two basic mechanisms have been suggested to be used by cytotoxic lymphocytes to deliver the lethal hit (Fig. 1), one based on granule exocytosis and the other on the Fas ligand. Better understanding of these mechanisms will follow the study of the involved components. A great deal of research has focused on the study of the cytotoxic granules of lymphocytes.

III. Molecular structure of lymphocyte cytotoxic granules

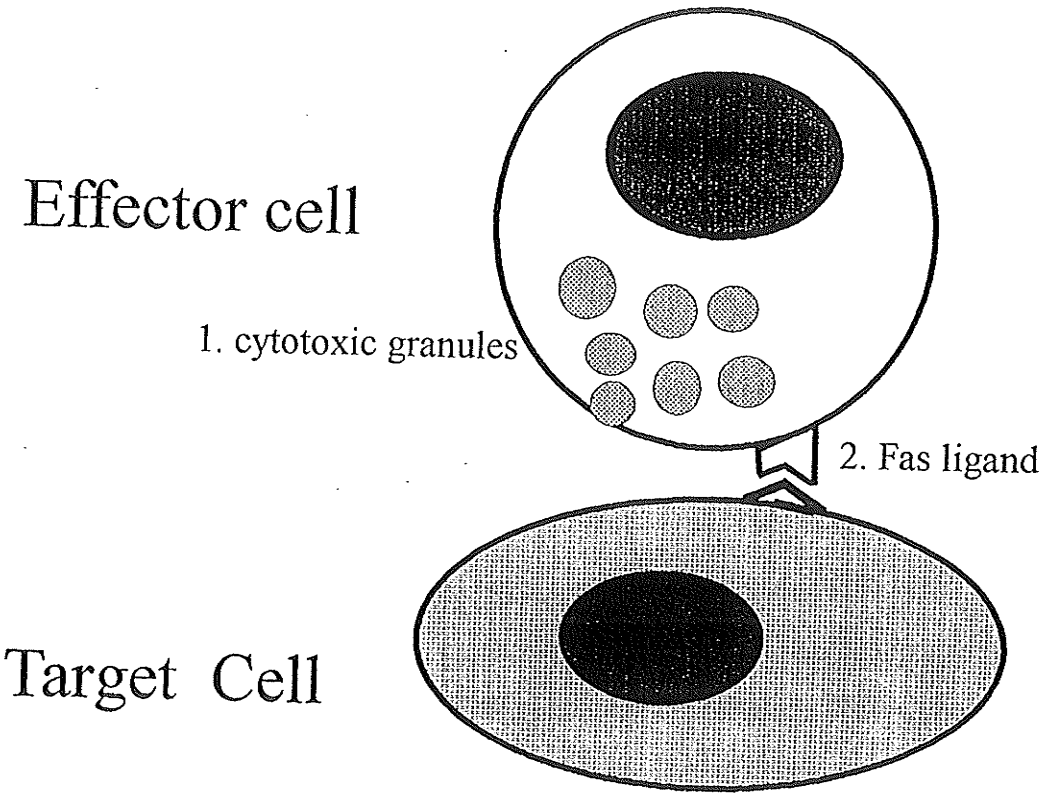
Morphologically, the granules of CTL clones of human and murine origin have been shown by electron microscopy to be membrane-delimited and contain numerous small membrane vesicles as well as a membrane-delimited electron-dense core. The small internal vesicles are thought to be formed through budding from the granule enveloping membrane in a manner similar to the internal vesicles of endosomes and lysosomes. Within the granules, the small vesicles seem to fuse with the limiting membrane of the dense core (Peters *et. al.*, 1989; 1991).

Based on the molecular structure of CTL granules, it has been suggested that their biogenesis combines elements of the biosynthetic and the endocytic pathways (Peters *et. al.*, 1990). The main granule proteins, perforin and granzymes, have been subcellularly localized along the biosynthetic pathway. They have been found in rough ER, Golgi complex, trans-Golgi reticulum and its associated vesicles as well as in all cytotoxic granules. In cytotoxic granules perforin and granzymes have a similar distribution, localized not only in the dense core but also over the region containing the small internal vesicles (Peters *et. al.*, 1991).

T cell surface membrane molecules TCR, CD3, CD8, and class I MHC molecules have been found to be present on the membranes of the granule cores and internal vesicles. These cell surface molecules possibly find their way from the plasma membrane to the granules via endocytosis (Peters *et. al.*, 1989). To test the link between the endocytic pathway and CTL granules, a suspension of bovine

Fig. 1 : Mechanisms of lymphocytotoxicity.

Two basic mechanisms have been suggested to be used by cytotoxic effector cells. In the granule exocytosis model, cytotoxic granules release their contents, including pore forming protein and DNA degrading molecule upon target cell contact. The other mechanism involves the interaction between the 40 kDa Fas ligand molecule on the effector cell and Fas antigen on the target cell.


Effector cell
1. cytotoxic granules
2. Fas ligand
Target Cell

serum albumin (BSA) complexed with colloidal gold was applied to CTL. BSA-gold complexes were internalized and recovered in the cytotoxic granules (Peters *et. al.*, 1989). Moreover, CTL granules share some features with lysosomes. CTL granules are acidic, contain in their lumen cathepsin D and other lysosomal enzymes, and mainly lack the endosomal marker cation-independent mannose 6-phosphate receptor. Also, lysosomal membrane glycoproteins lamp-1, lamp-2 and CD63 (lamp-3) are abundantly present on the granule-delimiting outer membrane. Taken together, these findings suggest that CTL granules represent secretory lysosomes, Fig. 2 (Peters *et. al.*, 1991).

Granules of rat Natural Killer (RNK) cells have also been shown by electron microscopy to consist of a dense core surrounded by a multivesicular compartment (Burkhardt *et. al.*, 1990). However, in contrast to studies of Peters *et. al.* in CTL granules, RNK granules appear more heterogeneous with respect to the relative content of dense core and the multivesicular compartment. Moreover, secretory molecules, such as perforin, granzymes, and chondroitin sulfate, were found only in the dense core region of the granule, whereas pre-lysosomal markers were present mainly in the multivesicular region. These findings suggest that the secretory and pre-lysosomal compartments are segregated within RNK granules (Burkhardt *et. al.*, 1990).

A large number of cytotoxic granule associated proteins have been identified, molecularly cloned and functionally characterized.

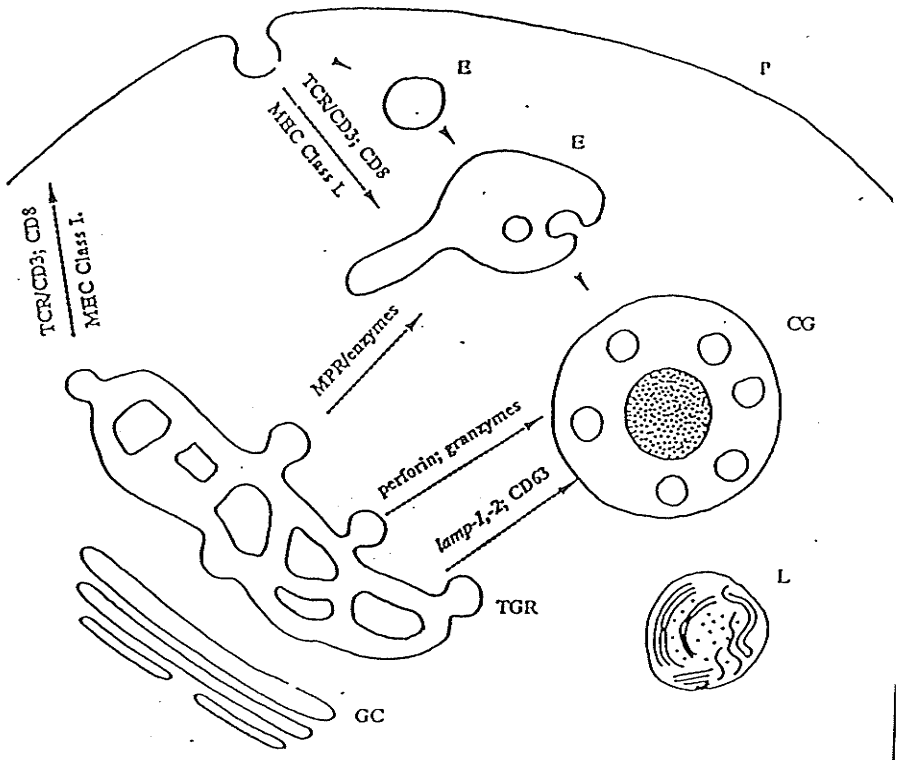
III.1. Intragranular proteins

III.1.1. Pore forming protein

Perforin, also known as cytolysin (Henkart *et. al.*, 1984), pore forming protein (Young and Cohn, 1987), C9-related protein (Liu *et. al.*, 1986), received its name from its ability to perforate membranes (Podack and Dennert, 1983). Perforin is an acidic protein with a molecular weight (m.w.) of 65-70 kDa. It

Fig. 2 : Ultrastructure of cytotoxic granules

The figure shows a model of the molecular structure and biogeneis of cytotoxic granules (Peters *et al.*, 1991). See text for details.


TCR/CD3; CD8
MHC Class I.
TCR/CD3; CD8
MHC Class I.
E
E
P
MPR/enzymes
perforin; granzymes
lamp-1,-2; CD63
CG
L
TGR
GC

consists of 534 amino acids which show high degree of identity among mouse, rat and human (Lowrey *et. al.*, 1989; Ishikawa *et. al.*, 1989; Lichtenheld *et. al.*, 1988). The amino acid sequence of perforin is homologous to the channel-forming terminal complement components, C6, C7, C8α, C8β, and C9, in the region thought to interact with the lipid bilayer (Tschopp and Nabholz, 1990). The structure-function relationship of perforin suggests that the central domain is responsible for polymerization and transmembrane channel formation, whereas the C-terminal domain may be involved in Ca^{++}-dependent membrane binding and insertion (Podack *et. al.*, 1991).

Both murine and human perforins are encoded by three-exon single copy genes located on chromosome 10 and chromosome 17, respectively (Trapani *et. al.*, 1989; Shinkai *et. al.*, 1989; Lichtenheld and Podack, 1989). Two types of murine perforin mRNA, differing in their 5' untranslated region, have been shown to exist and to be translated *in vivo* with similar efficiencies (Garcia-Sanz and Podack, 1993). A region in the 5' end of mouse perforin gene has been shown to contain a sequence capable of enhancing perforin gene expression in a killer cell-specific manner. Within this sequence a motif termed NF-P has been found to be highly homologous to the *Ets* proto-oncoprotein-binding site. The NF-P motif interacts with two protein, NF-P1 and NF-P2. NF-P2 is present exclusively in cytotoxic cells and is induced by agents known to up-regulate perforin message (Koizumi *et. al.*, 1993).

A great deal of work has provided evidence implicating perforin in target cell lysis. First, purified perforin shows lytic activity against target cells. Two steps, membrane binding followed by insertion and polymerization, have been identified in the process of pore formation mediated by perforin. Both steps are calcium and neutral pH dependent. Perforin reversibly binds to the phospholipid headgroups of the membrane in a temperature-independent fashion. This is

followed by a temperature-dependent conformational change of the perforin molecule resulting in unfolding of initially protected hydrophobic domains that insert into and traverse through the membrane. The inserted perforin then polymerizes in the membrane to form large functional membrane channels that lead to target cell lysis by osmotic effects (Tschopp and Nabholz, 1990). Second, the expression of perforin has been correlated with the lytic activity of various cytotoxic lymphocytes (Podack *et. al.*, 1991). Third, selective deposition of perforin at the site of the target cell-cytotoxic lymphocyte interaction has been shown (Ortaldo *et. al.*, 1992). Fourth, perforin antisense oligonucleotide probes led to a reduction of cytotoxic activity proportional to the reduction observed in perforin expression (Acha-Orbea *et. al.*, 1990). Fifth, transfection with perforin cDNA of a nonlytic rat basophilic leukemia cell line rendered it cytotoxic to antigen-coated red blood cells (Shiver and Henkart, 1991). Sixth, $CD8^+$ T cells and NK cells isolated from perforin-deficient mice did not lyse virus-infected or allogeneic fibroblasts or NK target cells *in vitro* (Kagi *et. al.*, 1994).

However, in spite of the above mentioned evidence, perforin-mediated cytotoxicity is not the only lytic mechanism used by cytotoxic lymphocytes. This is suggested by work showing cytotoxic activity in the absence of detectable perforin expression (Helgason *et. al.*, 1992; Berke *et. al.*, 1993).

III.1.2. Cytotoxic cell specific serine esterases (Granzymes)

In search for genes specifically expressed in murine CTL lines that might be related to their cytotoxic function, a family of CTL-specific genes have been isolated. This gene family codes for proteins designated cytotoxic cell proteases (CCP). Members of this family include CCP1, 2, 3, and 4 (Lobe *et. al.*, 1986, Bleackely *et. al.*, 1988a, 1988b). Similar molecules were isolated through conventional protein purification protocols of murine CTL granules. They were named granzymes (granule-associated-enzymes) (Jenne and Tschopp, 1988).

Granzymes B, C, E, and F are identical to CCP1, 2, 3, and 4, respectively (Bleackley *et. al.*, 1988b). Young and his colleagues isolated serine esterases SE-1, and SE-2 which were identical to granzymes A and B, respectively (Young *et. al.*, 1986). Other groups isolated the serine esterases, BLT-esterase (Pasternack and Eisen, 1985), T-cell specific Proteinase TSP-1 (Kramer *et. al.*, 1986), CTL-3 (Brunet *et. al.*, 1988) and Hanukah Factor (Gershenfeld and Weissman, 1986) which were all identical to granzyme A.

Granules of human CTL contain granzymes A and B (Krahenbuhl *et. al.*, 1988; Fruth *et. al.*, 1987; Ferguson *et. al.*, 1988; Gershenfeld *et. al.*, 1988; Poe *et. al.*, 1991) as well as granzyme 3. Granzyme 3 has been isolated from the cytotoxic granules of LAK cells. It shows N-terminal sequence homology with the other granzymes and has trypsin-like activity (Hameed *et. al.*, 1988). Also, another human serine protease gene (h-CCPX) with no apparent murine counterpart has been cloned (Meier *et. al.*, 1990). Recently, a human granzyme, Hu-Met-1, was cloned from NK cells based on its homology to RNK Met-1 (Smyth *et. al.*, 1993).

Granules of the rat Natural Killer cells contain several proteases including RNKP-1, RNKP-2, RNKP-3, and RNKP-4 which are homologous to murine granzymes B, A, F, and C, respectively (Hudig *et. al.*, 1993) as well as RNK Met-1 (Smyth *et. al.*, 1992). A family of three molecules, named fragmentin 1, 2, and 3 was isolated from the granules of RNK cells. Fragmentin 2 is highly homologous to the RNKP-1 and mouse granzyme B. Fragmentin 1 and 3 are homologous to human T cell granzyme A and granzyme 3, respectively (Shi *et. al.*, 1992a, 1992b).

Granzymes are synthesized as inactive precursors with two-residue propeptides preceding the N-terminus of the mature proteases (Tschopp and Nabholz, 1990). Successful production of enzymatically active recombinant granzyme B was obtained after removal of the amino terminal dipeptide. This led

to the suggestion that the dipeptide might regulate the enzymatic activity of granzyme B (Caputo *et. al.*, 1993). Granules of cytotoxic lymphocytes have been shown to contain high levels of dipeptidyl peptidase I which is co-localized with granzyme A and similarly exocytosed upon cell activation (Brown *et. al.*, 1993). Granule- associated dipeptidyl peptidase I might be responsible for the generation of active serine proteases (McGuire *et. al.*, 1993)

The exact biological functions of granzymes are unknown. However, their presence in the cytotoxic granules as well as the correlation of their transcription with cytolytic activity suggest a role in cytolysis (Bleackley *et. al.*, 1988b). A model proposed by Hudig *et. al.*, based on work done in several laboratories, suggests that granzymes might have three roles in target cell lysis. First, upon cytotoxic-target cell contact and before granule exocytosis, granzymes are activated to cleave intragranular proteins, including a putative nucleolysin. Second, after exocytosis they cleave and activate perforin to form pores. Third, they enter through the perforin pores and act on target cell substrates to promote DNA fragmentation (Hudig *et. al.*, 1993).

A role for granzyme A, which exhibits a trypsin-like activity, in aiding the migration of activated lymphocytes into tissues has been suggested based on its proteolytic activity on endothelial-derived extracellular matrix proteins (Simon *et. al.*, 1987). A recent report suggests that granzyme A might be responsible for the degradation of myelin basic protein associated with immune-mediated demeylination (Vanguri *et. al.*, 1993). Suggested roles for granzyme A in lymphocyte cytotoxicity include mediating the rapid detachment of activated lymphocytes from their targets or stimulators (Tschopp and Nabholz, 1987), modulating perforin activity (Munger, 1988), and DNA breakdown of target cells (Munger *et. al.*, 1988; Hayes *et. al.*, 1989). Support of the involvement of granzyme A in target DNA breakdown was obtained by using two approaches.

First, transfection of mouse CTLs with an antisense granzyme A vector led to a reduction of their lytic activity as well as their ability to degrade target DNA (Talento *et. al.*, 1992). Second, target DNA breakdown was observed only after transfection of nonlytic rat basophilic leukemia cells with both perforin and granzyme A genes (Shiver *et. al.*, 1992; Nakajima and Henkart, 1994).

The physiological substrate of granzyme A is still to be defined. However, Pasternak and his colleagues showed that the nuclear fraction of the target cell contained most of the granzyme A binding reactivity. A protein with a m.w. of 100 kDa and two closely migrating proteins with m.w. of 35 and 38 kDa were the predominant reactive moieties. The N-terminal sequence of the 100 kDa protein was identical to nucleolin. Incubation of granzyme A with nucleolin generates a discrete proteolytic cleavage product. This finding suggests that interaction of granzyme A and nucleolin may be important in the process of apoptosis which accompanies CTL-mediated lysis of target cells (Pasternack *et. al.*, 1991).

However, granzyme A appears to be present in non-cytotoxic cells (Brunet *et. al.*, 1987; Garcia-Sanz *et. al.*, 1987; Kramer and Simon, 1987; Gershenfeld *et. al.*, 1988) which might argue against its direct involvement in the lethal hit delivery. Moreover, inhibition of granzyme A activity does not block cytolysis (Masson *et. al.*, 1986; Henkart *et. al.*, 1987).

There is evidence for differential expression of various granzymes in response to different antigenic stimuli. Granzyme B levels were the best in terms of correlation with cytotoxic activity (Prendergast *et. al.*, 1992). Moreover, NK granzyme B, but not perforin or granzyme A, mRNA levels were upregulated in response to target cell binding (Salcedo *et. al.*, 1993). These findings suggest an essential role for granzyme B in lethal hit delivery. Fragmentin 2, the RNK cells homologue of granzyme B, has been shown to induce rapid nuclear apoptotic changes and DNA fragmentation in target cells in the presence of perforin (Shi *et.*

al., 1992a; 1992b). The mechanism by which fragmentin 2 induces apoptosis involves premature activation of the serine-threonine kinase $p34^{cdc2}$, the enzyme which normally controls cell entry into mitosis (Shi *et. al.*, 1994).

Evidence showing the necessity of granzyme B in the induction of rapid DNA fragmentation and apoptosis in allogeneic target cells was recently obtained by using activated CTL from homozygous granzyme B-deficient mice (Heusel *et. al.*, 1994).

Other activities have been suggested for granzyme B. It has been shown that granzyme B has a cytostatic effect on several tumor cell lines (Sayers *et. al.*, 1992). Recently, human granzyme B has been found to degrade aggregan proteoglycan in the matrix synthesized by chondrocytes. This finding suggests that granzyme B secreted by cytotoxic lymphocytes within the joint might be involved in the pathogenesis of rheumatoid arthritis by degrading resident aggregan (Froelich *et. al.*, 1993).

The role of other granzymes and their natural substrates are yet to be determined.

III.1.3. Proteoglycans

Cytotoxic granules of mouse CTL (Tschopp and Masson, 1987), human NK cells (Parmley *et. al.*, 1985) and rat LGL (Stevens *et. al.*, 1987) contain proteoglycans of the chondriotin sulfate A type. Proteoglycans are released into the medium upon granule exocytosis (Schmidt *et. al.*, 1985). The exact role of proteoglycans is not clear. However, based on their strong negative charge, it is suggested that they from ionic complexes with the basic granzymes (Kamada *et. al.*, 1989). By binding to perforin, they may also have a role in preventing proteolytic inactivation of perforin as well as protecting the effector cell from perforin lysis (Tschopp and Nabholz, 1990).

III.1.4. Lysosomal enzymes

A number of lysosomal enzymes have been identified in cytotoxic granules. These include cathepsin D, arylsulfatase, β-glucoronidase, β-hexosamidase, and α-glucosidase (Peters *et. al.*, 1989; Burkhardt *et. al.*, 1990; Tschopp and Nabholz, 1990). Cytotoxic granules have been suggested to be relatives of lysosomes, however they differ from lysosomes in that their lysosomal enzymes are secreted in a regulated fashion (Peters *et. al.*, 1991). Suggested roles for the lysosomal hydrolases in lymphocyte killing include enhancing the efficiency of cytolysis by damaging the target cell and protecting the effector cell from harmful target cell debris (Burkhardt *et. al.*, 1990).

III.1.5. Calreticulin

Dupuis and his colleagues reported the purification of a major 60 kDa granule-associated protein from human LAK cells and murine CTL. This protein has been found to be identical to the Ca^{++}-binding protein calreticulin. In CTL, calreticulin colocalizes with perforin in the secretory granules. Stimulation of the T cell receptor complex led to the release of granule-associated proteins together with calreticulin. The role of CTL-granule calreticulin is unknown. However, based on calreticulin calcium chelating capacity and the requirement of calcium for perforin activity, it has been suggested that calreticulin might prevent granule autolysis by perforin (Dupuis *et. al.*, 1993). Calreticulin has also been suggested to participate in the destruction of target cells based on its ability to control gene expression through interaction with the DNA-binding domain of steroid receptors (Burns *et. al.*, 1994)

III.1.6. Macrophage-activating factor

Cytotoxic granules of RNK cells have been shown to contain a factor capable of stimulating the tumoricidal activity of macrophages. The responsible factor is heat-resistant and has a m.w. of less than 10 kDa. It requires the

simultaneous presence of LPS for maximal activity (Roussel and Greenberg, 1989). The same granules have been also shown to contain a chemoattractant for leukocytes that appears to be distinct from NK-macrophage activating factor (Greenberg *et. al.*, 1986).

III.2. Membrane-associated proteins

III.2.1. Cell surface molecules

The membranes of the dense core and small internal vesicles of CTL granules have been shown to contain TCR/CD3 complex, CD8, and MHC class I molecules facing outwards (Peters *et. al.*, 1989) as well as MHC class II and LFA-1 molecules (Peters *et. al.*, 1991). These molecules possibly reach the granules through endocytosis (Peters *et. al.*, 1989).

The presence of such molecules on the dense core and the internal vesicle membranes of granules, which are released into the effector-target intercellular cleft during exocytosis, prompted the proposal of a new model of lethal hit delivery (Peters *et. al.*, 1990). According to this model, binding of CD3/TCR complex and CD8 to relevant target cell molecules ensures specific and unidirectional delivery of the lethal hit thus avoiding effector and bystander cell killing.

III.2.2. Lysosomal membrane glycoproteins

The granule-delimiting outer membrane of CTL granules contains the lysosomal membrane glycoproteins lamp-1, lamp-2, and CD63 (Peters *et. al.*, 1991). Human lamp-1 and lamp-2 are homologous molecules, with apparent m.w. of 90-120 kDa, consisting of a polypeptide core of ~ 40 kDa which is heavily glycosylated with 16-20 N-glycans. They have a similar overall domain structure which consists of a short cytoplasmic C-terminus tail, a single transmembrane region, and a lumenal N-terminal glycosylated region (Fukuda, 1991).

CD63 (Demetrick *et. al.*, 1992) was originally identified as a marker for the early stages of tumor progression of human melanoma (Hotta *et. al.*, 1988) and as a surface antigen of activated platelets (Nieuwenhuis *et. al.*, 1987; Hildreth *et. al.*, 1991). CD63 is a glycoprotein with a m.w. of 30-60 kDa which contains a core peptide of ~ 25 kDa and three potential N-glycosylation sites. CD63 is different from lamp-1 and -2 in its amino acid sequence and the presence of four potential transmembrane regions (Metzelaar *et. al.*, 1991). However, the three molecules share the presence of a Gly-Tyr motif in the cytoplasmic tail which appears to act as the signal for their trafficking to lysosomes, therefore CD63 has been designated lamp-3 (Fukuda, 1991). The function of lamp glycoproteins is unknown. However, based on their extensive glycosylation, it has been suggested that they might have a role in protecting the plasma membrane from degradation by lysosomal hydrolases (Fukuda, 1991). A similar role in protecting CTL membrane from the released granule protein has been suggested to explain their presence in the membrane of cytotoxic granules (Peters *et. al.*, 1991).

III.2.3. RNA binding proteins

Human CTL granules have been shown to contain a family of RNA binding proteins designated TIA (Tian *et. al.*, 1991). TIA-1 is a 40 kDa protein which contains three RNA-binding domains similar to the poly A-binding proteins as well as a carboxy terminal glutamine-rich domain. Other members of the TIA family include a 15 kDa and the TIA-R isoforms. The 15 kDa isoform is the major granule-associated species and appears to be derived from the carboxy-terminal domain of the 40 kDa TIA-1 by proteolytic processing. TIA-R is a 42 kDa isoform which shares more than 85% amino acid homology with TIA-1 (Kawakami *et. al.*, 1992). Although TIA family members lack potential hydrophobic transmembrane regions, their carboxy termini contain the Gly-Tyr motif which has been implicated in lysosomal targeting.

Members of the TIA family are able to induce DNA fragmentation in permeabilized target cells which led to the suggestion that they represent the potential nucleolysins involved in the induction of apoptosis in CTL targets. Their mechanism of action is hypothesized not to involve the RNA binding domains. The glutamine rich carboxy terminal region is thought to be responsible for this activity possibly through the interaction with and the activation of target cell endonucleases (Tian *et. al.*, 1991; Kawakami *et. al.*, 1992).

IV. Membrane-associated proteins of other granules and vesicles

Recent work done by using different systems has led to identification of a number of vesicle and granule associated proteins. Some of these proteins constitute large families with members present in different types of vesicles and granules. The following sections include discussion of some of the granule membrane-associated molecules suggested to mediate aspects of granule biology.

IV.1. Proteins involved in the biogenesis of secretory granules and vesicles

Vesicle budding requires the deformation of membrane from a planar surface to a curved vesicle. One of the models proposed to explain the mechanism of vesicle formation suggests that vesicle formation is initiated by assembly of a coat made of specific proteins on the budding membrane (Pryer *et. al.*, 1992). Two types of coated vesicles have been described. The first type is clathrin-coated vesicles which includes vesicles that mediate the endocytic uptake from the plasma membrane as well as vesicles diverted from the constitutive pathway at the TGN (Brodsky, 1988; Morris *et. al.*, 1989). The second type of coated vesicles are the vesicles involved in the intra-Golgi transport (Rothman and Orci, 1992). Evidence supporting the importance of coat protein in vesicular budding was derived from the studies done with the fungal metabolite brefeldin A. Brefeldin A has been shown to inhibit non-clathrin-coated vesicle budding from the Golgi compartment

as well as clathrin-coated vesicles from the TGN through inhibition of binding of coat components (Orci *et. al.*, 1993; Robinson and Kreis, 1992; Wong and Brodsky, 1992; Rosa *et. al.*, 1992).

A clathrin-coated vesicle forms by invagination of a clathrin-coated indentation of membrane, a coated pit, which is converted into mature coated vesicle and is then released from its membrane attachment (Pryer *et. al.*, 1992). Native clathrin is a hexamer containing three copies of heavy chains (190 kDa) and three copies of light chains (23-27 kDa). Clathrin heavy chains alone are capable of self assembly in physiological buffers to form the polyhedral lattice characteristic of clathrin-coated pits and vesicles. Clathrin heavy chains serve a structural role in vesicle formation (Pearse and Crowther, 1987; Brodsky *et. al.*, 1991). On the other hand, clathrin light chains are thought to play a regulatory role in the disassembly of the clathrin coat (DeLuca-Flaherty *et. al.*, 1990).

In addition to clathrin heavy and light chains, clathrin coated vesicles have adaptin containing protein complexes with m.w. of 250-300 kDa, called adaptors. Two types of adaptors are recognized, HA1 and HA2. Both HA1 and HA2 are heterotetramers, comprising two ~100 kDa adaptins, one medium chain of ~50 kDa and one small chain of ~20 kDa. The two types of adaptors differ in their content of adaptin and their subcellular localization. Thus, HA1 adaptor contains β' and γ adaptin and is associated with the TGN, whereas HA2 adaptor contains α and β adaptin and is associated with the plasma membrane. Adaptins have been suggested to mediate clathrin binding to membranes and to play a role in the selection of vesicle cargo (Robinson, 1992).

The second type of coated vesicle is found in the intercisternal transport in the Golgi stack. The non-clathrin coat proteins (COPs) of the Golgi intercisternal transport vesicles include α-COP (170 kDa), β-COP (110 kDa), γ-COP (98 kDa), δ-COP (61 kDa) and a small GTP-binding protein called ADP-ribosylation factor

(ARF, 21 kDa). Coatomer (coat protomer) consists of a complex of 650-700 kDa of coat proteins in addition to two subunits of 20 and 36 kDa (Rothman and Orci, 1992).

Two major differences exist between clathrin and non-clathrin coated vesicles. First, in clathrin coated vesicles the coat selects the vesicle content. On the other hand, COP-coated vesicles seem to carry the proteins that diffuse into their walls or lumen during budding, which is consistent with their function as carriers of the anterograde bulk flow in the intercisternal Golgi compartment. Second, the clathrin coat is removed before docking at the target membrane, whereas non-clathrin Golgi-derived coats are removed after contact with the target membrane (Rothman and Orci, 1992).

However, there is a striking similarity of the m.w. of the coatomer subunits of COP-coated vesicles and those of clathrin coats. More particularly, the sequence of β-COP is homologous to that of members of the 100-115 kDa adaptin family, suggesting that elements of the two kinds of vesicle coats might share similar functions (Robinson, 1992; Rothman and Orci, 1992).

IV.2. Proteins involved in the regulation of vesicle formation

The formation of constitutive and regulated secretory vesicles from the TGN as well as Golgi transport vesicles have been shown to require GTP hydrolysis. This led to suggestion of the involvement of G proteins in the regulation of vesicle formation.

IV.2.1. Small *ras*-like G-proteins

Studies suggesting the involvement of small G-proteins in vesicular transport started in yeast by the finding that sec4 (Salminen and Novick, 1987) and YPT1 mutations (Segev *et. al.*, 1988) led to inhibition of vesicular transport. The sequences of sec4 (Kabcenell *et. al.*, 1990) and YPT1 (Gallwitz *et. al.*, 1983) have been found to be similar to the *ras*-like small G- proteins. Mammalian cells

contain several *ras*-like small G-proteins grouped into a family called rab (*ras*-like proteins from rat brain). The rab family consists of at least 30 proteins with a m.w. of 20-30 kDa, with some having several isoforms. Members of the rab family show regions of identity and contain distinct functional domains. These include a nucleotide-binding domain, an effector domain which may be the site where GTP-ase activating proteins bind, and a carboxy-terminal domain which contains sequences that target the proteins to their specific subcellular compartments (Sztul *et. al.*, 1992). Another family of small G-proteins which function in intracellular transport has been identified as ADP ribosylation factor (ARF) (Pfeffer, 1992).

Subcellular organelles involved in exocytic and endocytic processes have a distinct small G-protein ; for example, rab1A is associated with ER and Golgi, rab3A is present on regulated neurosecretory vesicles, rab5 is associated with early endosomes and plasma membrane, and ARF has been located on the Golgi complex (Rothman and Orci, 1992). The rabs and ARF are first synthesized as cytosolic proteins, however, it has been suggested that post-translational modifications, such as carboxy-terminal addition of geranylgeranyl prenyl moiety on cysteine in rab and amino-terminal myristylation of ARF, are required for their functional activity and association with intracellular organelles through specific receptors (Pfeffer, 1992).

It has been suggested that GTP, which is required for vesicle budding, is recruited onto the membrane by binding to a rab or an ARF protein on the membrane or vesicle (Pfeffer, 1992). A regulatory role for ARF in vesicular transport between Golgi cisternae has been suggested by its ability to regulate coating and uncoating of transport vesicles (Rothman and Orci, 1992).

IV.2.2. Trimeric G-proteins

Trimeric G-proteins have been shown to be associated with the membranes of secretory granules (Toutant *et. al.*, 1987), ER (Audigier *et. al.*, 1988), and Golgi

complex (Ercolani *et. al.*, 1990). They also have been shown to regulate protein traffic through the Golgi complex at the level of vesicle formation (Barr *et. al.*, 1991). A trimeric G-protein of the G_i/G_0 class has been found to antagonize the inhibitory effect of brefeldin A on β-COP binding to Golgi membranes (Ktistakis *et. al.*, 1992).

Multiple trimeric G-proteins of the G_i/G_0 and G_s classes have been found to be associated with TGN and to exert opposing regulatory effects on secretory vesicle formation from the TGN in cell free systems. Inhibition of vesicle formation occurred upon activation of G_i/G_0, whereas stimulation of vesicle formation followed either inactivation of G_i/G_0 by pertussis toxin or stimulation of G_s by cholera toxin (Leyte *et. al.*, 1992).

Multiple G-proteins have also been shown to regulate vesicle formation in the endoplasmic reticulum (Schwaninger *et. al.*, 1992) as well as in the clathrin-coated endocytosis (Carter *et. al.*, 1993).

IV.3. Proteins involved in vesicle movement

Microtubules are found in the cytoplasm of almost all eukaryotic cells. They radiate with their minus end from the microtubule organizing center (MTOC) to their plus end at the plasma membrane. This arrangement provides tracks to support the intra-cellular movement of vesicles (Kelly, 1990). Two major types of motor proteins have been identified to mediates microtubule-based movement. Kinesin is an anterograde translocator which mediate vesicle movement towards the plus end of the microtubules (Hoyt, 1994), whereas cytoplasmic dynein is a retrograde translocator (Schroer, 1994).

Microtubule-based movement of synaptic vesicles to the nerve terminal has been shown to occur during fast axonal transport (Sheetz *et. al.*, 1989). In polarized epithelial cells, microtubules play an important role in the development and maintenance of polarity through their distinct distribution as well as their role

in facilitating the transport of vesicles to certain destinations (Mays *et. al.*, 1994).

Kinesin-dependent motility of CTL cytotoxic granules has been shown occur on microtubules *in vitro*. Intact granule membranes were required for microtubule binding as well as movement (Burkhardt *et. al.*, 1993). The granule membrane components that interact with kinesin are still to be defined.

IV.4. Proteins involved in vesicle docking and fusion

To deliver a vesicle to a target membrane, there must be a docking mechanism that determines the specificity of binding as well as a fusion machine to fuse the two lipid bilayers (Warren, 1993). Components of a fusion machine which seem to operate at many steps on the secretory and endocytic pathways have been identified by Rothman and his colleagues (Rothman and Orci, 1992). Recently, they used the components of the fusion machine to isolate proteins thought to be involved in the docking process (Sollner *et. al.*, 1993a,b).

The N-ethylmaleimide-sensitive fusion protein (NSF) is a water-soluble tetramer of 76 kDa subunits which has been purified on the basis of its ability to restore intercisternal Golgi transport in a cell-free system. The requirement of NSF for transport vesicle fusion has been suggested by the accumulation of vesicles at the acceptor membrane in its absence (Block *et. al.*, 1988). The carboxy-terminal part of NSF consists of two direct repeats of homologous ATP domains, each of which possesses an ATP-binding site. Mutating either ATP sites eliminates the fusion activity of NSF and reduces the ATPase activity, suggesting that ATP hydrolysis by NSF is required for fusion (Tagaya *et. al.*, 1993).

Additional proteins are required to allow NSF binding to Golgi membranes. These proteins are called soluble NSF attachment proteins or SNAPs. Three SNAPs have been identified and molecularly cloned ; α-, β-, and γ- SNAPs with m.w. of 35, 36, 39 kDa, respectively. α- and β- SNAPs are structurally very similar and differ significantly from γ-SNAP (Rothman and Orci, 1992). β-SNAP

is a brain specific isoform of α-SNAP (Whiteheart *et. al.*, 1993). SNAPs neither bind NSF in solution to form stable complexes nor stimulate its ATPase activity, however, SNAPs do bind to membrane receptors present in the Golgi membranes. When bound to membranes, SNAPs are able to bind NSF (Rothman and Orci, 1992).

It has been shown that NSF/SNAP link proteins from the donor vesicle to proteins on the target membrane. These proteins have been termed v- and t-SNAREs for vesicle- and target- SNAP receptors, respectively. Thus, a transport vesicle chooses its target for fusion when v-SNARE of the vesicle pairs with its cognate t-SNARE at the target membrane (Fig 3). In synaptic vesicles, v-SNARE has been identified to be synaptobrevin (VAMP), whereas t-SNARE has been shown to be syntaxins and SNAP-25 (Sollner *et. al.*, 1993a).

The existence of numerous SNARE-related proteins, each apparently specific for a single kind of vesicle or target membrane, indicate that NSF and SNAPs may be universal components of a vesicle fusion apparatus common to both constitutive and regulated fusion, in which the SNAREs may help to ensure vesicle-to-target specificity (Sollner *et. al.*, 1993a).

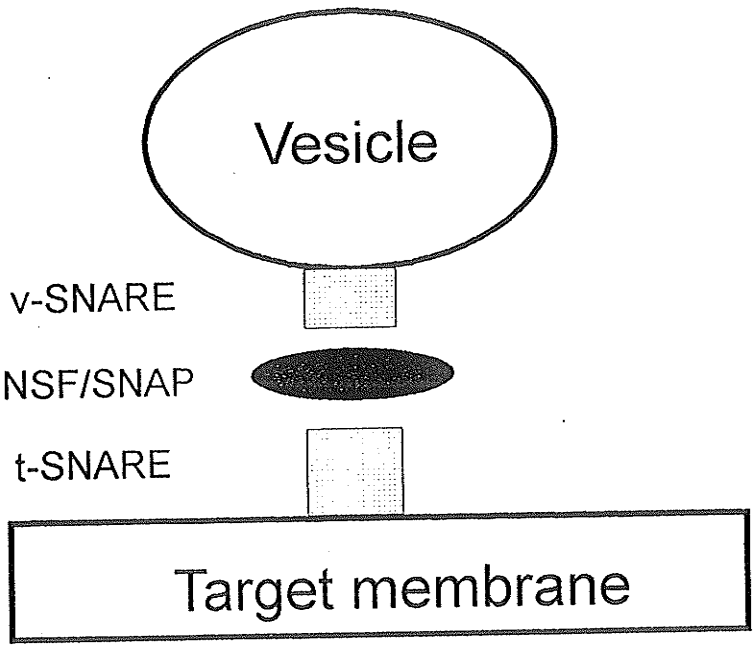
Beside their potential role in the regulation of vesicle formation, rab proteins have also been suggested to play a role in vesicle docking and fusion. A specific rab protein is thought to be recruited on the transport vesicle in its GTP-bound form where it can interact with rabphilin. Subsequent hydrolysis of GTP by the rab protein is thought to trigger fusion of the vesicle with the acceptor membrane. The GDP-bound rab protein is released into the cytosol and returned to the donor membrane (Fig. 3) (Zerial and Stenmark, 1993).

V. Sorting of vesicle and granule proteins

The TGN is a distinct compartment at the exit site of the Golgi complex where segregation of membrane and lumenal proteins into different transport

Fig. 3 : Molecules involved in vesicle docking and fusion

N-ethylmaleimide-sensitive fusion protein (NSF) complex with soluble NSF attachment proteins (SNAP) to link proteins from the donor vesicle to protein on the target membrane. These proteins are termed v- and t-SNAREs for vesicle- and target-SNAP receptors, respectively.


Vesicle
v-SNARE
NSF/SNAP
t-SNARE
Target membrane

vesicles destined for endosomes, lysosomes, secretory granules and the plasma membrane occurs (Pryer *et. al.*, 1992). Mechanisms for sorting both membrane and soluble proteins into various destinations have been proposed.

V.1. Sorting of soluble proteins

The sorting of soluble hydrolytic enzymes into lysosomes is the best studied example. Lysosomal enzymes are identified through a conformation-dependent domain and modified by the addition of mannose 6-phosphate residue in the CGN. Modified enzymes bind to one of two related mannose 6-phosphate receptors in the TGN. A sorting signal has been suggested to be present in the cytoplasmic domain of the mannose 6-phosphate receptor. Receptor-protein complexes are sorted into clathrin-coated vesicles that bud from the TGN. The binding of clathrin to the mannose 6-phosphate receptor is probably mediated by adaptin. The vesicles are then transported to an acidic, prelysosomal compartment where the protein-receptor complex is uncoupled. The receptor is recycled back to the TGN whereas the enzymes are transported further to lysosomes (Pryer *et. al.*, 1992). It has been suggested that granzymes A and B use the mannose-6-phosphate receptor mechanism for their targeting to the lymphocyte cytotoxic granules (Burkhardt *et. al.*, 1989; Griffiths and Issaz, 1993).

Two non-mutually exclusive mechanisms have been proposed to explain the diversion of soluble proteins at the TGN to the regulated instead of the constitutive secretory granules. In the active sorting mechanism, secretory proteins destined for the regulated secretory granules bind to sorting receptor(s) in the TGN. However, neither the identity of the sorting receptor nor the location or structure of a consensus sorting domain on the secretory proteins has been elucidated. On the other hand, the passive sorting mechanism suggests that selective aggregation of regulated secretory proteins leads to their segregation from the constitutive pathway. The *in vitro* aggregation of several regulated secretory

proteins in the presence of calcium and low pH has provided support for this mechanism (Chidgey, 1993; Pryer *et. al.*, 1992).

It has been shown that the formation of regulated secretory granules is preceded by budding of immature secretory granules from the TGN. The current evidence suggests that active sorting of soluble regulated proteins takes place in the immature granules. Granule maturation which occurs by fusion of immature granules is associated with passive sorting of regulated proteins (Arvan and Castle, 1992; Bauerfeind and Huttner, 1993).

V.2. Sorting of membrane proteins

Some evidence suggests that vesicle membrane proteins bear sorting signals in their amino acid sequence. The major lysosomal membrane glycoproteins (lamp-1, lamp-2, and CD-63) share the presence of glycine-tyrosine motif at the same relative position in their C-terminal cytoplasmic tail. An essential role for the tyrosine residue in lysosomal targeting has been suggested (Fukuda, 1991). Similarly, C-terminal cytoplasmic tyrosine has been implicated in sorting cell surface receptors to clathrin-coated vesicle during receptor-mediated endocytosis (Pryer *et. al.*, 1992).

A targeting signal to regulated granules has been shown to be present in the C-terminal cytoplasmic domain of P-selectin (Disdier *et. al.*, 1992) as well as in the membrane associated peptidylglycine α-amidating monooxygenase (Milgram *et. al.*, 1992)). However, not all granule membrane proteins share this property. Synaptic vesicles are by far the most extensively studied among regulated granules. The major classes of membrane proteins have been identified and molecularly cloned. No obvious common motifs that might explain selective targeting to synaptic vesicles have been identified (Jahn and Sudhof, 1993).

VI. Synaptic vesicles as a model of granule membrane structure

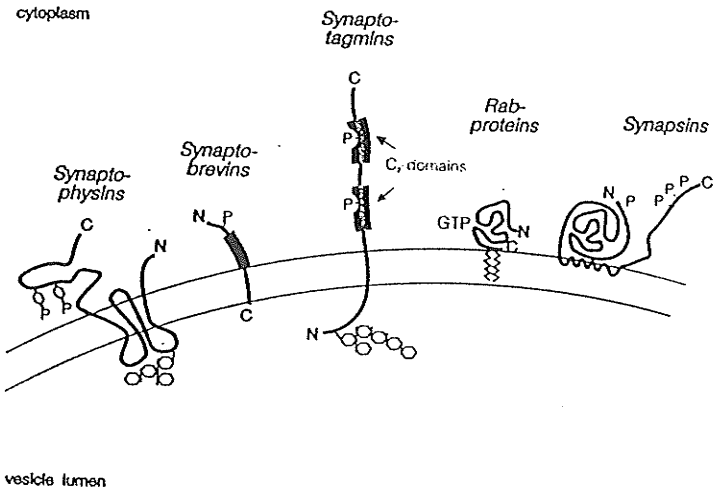
Synaptic vesicles are membranous organelles of the nervous system which store various neurotransmitters. The regulated calcium-dependent exocytosis of synaptic vesicles occurs rapidly upon stimulation of the presynaptic cell. The abundance, uniformity as well as small size made the synaptic vesicle a model for the study of organelle membrane structure. Recently, a great deal of information regarding the protein structure of synaptic vesicle membranes has been gathered. Some of the identified proteins have been shown to be members of large protein families with relatives present in other granules and vesicles. This work has improved our understanding of some of the basic processes of the regulated exocytotic pathway, such as vesicle interaction with the cytoskeleton as well as docking and fusion (Sudhof and Jahn, 1991; Jahn and Sudhof, 1993; Greengard *et. al.*, 1993). Fig. 4 schematically shows the overall structure of the major synaptic vesicles proteins that will be discussed in the following sections.

VI.1. Synapsins

The synapsin family consists of four homologous phosphoproteins which are associated with the cytoplasmic surface of synaptic vesicles. The family includes synapsin Ia, Ib, IIa, and IIb, which are derived by differential splicing from the transcripts of two genes, synapsin I and II. The four proteins, which are differentially distributed in the brain, have identical amino-termini whereas the carboxy-termini consist of sets of differentially spliced domains (Sudhof and Jahn, 1991). Binding of synapsin I to the synaptic vesicle membrane occurs through the hydrophobic amino terminus region, which interacts with vesicle phospholipids and penetrates the hydrophobic core of the membrane, as well as the carboxy terminus region, which binds to the α subunit of CaM kinase II (Benfenati *et. al.*, 1992). Synapsins I and II contain distinct phosphorylation sites which are phosphorylated by several protein kinases.

Fig. 4 : Overall structure of major synaptic vesicle proteins

The overall domain structure of major synaptic proteins is shown. N denotes the amino terminus, C denotes the carboxy terminus, whereas P are the sites of phosphorylation (Jahn and Sudhof, 1993).


cytoplasm
Synapto-
tagmins
C
P
P
C2 domains
Rab-
proteins
Synapsins
Synapto-
brevins
Synapto-
physins
N
P
C
GTP
N
N
P
P
P
P
C
C
N
P
C
N
vesicle lumen

Recent studies suggest a role of synapsin phosphorylation in regulating the availability of synaptic vesicles for exocytosis. Dephosphorylated synapsin I interacts *in vitro* with various cytoskeletal proteins including actin, promoting its polymerization. Actin polymerization is prevented by phosphorylation of synapsin I by CaM kinase II. The current model suggests that, under resting conditions, dephosphorylated synapsin I anchors synaptic vesicles to the cytoskeleton. Neuronal activation is followed by CaM kinase II- dependent phosphorylation of synapsin I resulting in the release of synaptic vesicles from the cytoskeleton (Greengard *et. al.*, 1993).

VI.2. Synaptobrevin

Synaptobrevin or vesicle-associated membrane protein, VAMP, family consists of VAMP 1 and 2 in synaptic vesicles (Archer *et. al.*, 1990), cellulobrevin in other tissues (McMahon *et. al.*, 1993), and homologous proteins in *S. cerevisiae* (Protopopov *et. al.*, 1993). Synaptobrevins are integral membrane proteins with a cytoplasmic N-terminal region, a transmembrane domain and an intravesicular C-terminal region.

The finding that both tetanus and botulinum toxins inhibit neurotransmitter release by acting as specific proteases of synaptobrevin suggest an essential *in vivo* role for synaptobrevin in vesicle exocytosis (Schiavo *et. al.*, 1992). Synaptobrevin has been found to be the v-SNARE of synaptic vesicles which binds to syntaxin (t-SNARE) to ensure specific docking of synaptic vesicle during exocytosis (Sollner *et. al.*, 1993a). The identification of cellulobrevin in all cells and tissues tested as a membrane trafficking protein of the constitutive pathway suggests that both regulated and constitutive pathways use homologous proteins for vesicle traffic control (McMahon *et. al.*, 1993).

VI.3. Synaptotagmin

Synaptotagmin is a 65 kDa integral membrane protein which contains a short amino-terminal intravesicular domain and a long carboxy-terminal cytoplasmic domain. The cytoplasmic domain contains two copies of an internal repeat homologous to the C2 domain in the regulatory Ca^{++} binding region of protein kinase C (Perin *et. al.*, 1990). Synaptotagmin binds Ca^{++} (Brose *et. al.*, 1992), phospholipids, and calmodulin (Perin *et. al.*, 1990). Synaptotagmin also interacts with the presynaptic plasma membrane protein, α laxotroxin receptor (Petrenko *et. al.*, 1991). Several approaches including microinjection of anti-synaptotagmin antibodies, pieces of synaptotagmin fusion protein (Elferink *et. al.*, 1993), and C2 domain peptides (Bommert *et. al.*, 1993) as well as manipulations of the synaptotagmin gene (Littleton *et. al.*, 1993) pointed to an essential role played by this protein in neurotransmitter release.

Synaptotagmin coimmunoprecipitates with the presynaptic plasma membrane protein synatxin (Bennett *et. al.*, 1992), which has been shown to be the t-SNARE (Sollner *et. al.*, 1993a). Recent studies suggest that synaptotagmin acts as a clamp which prevents the fusion of v- and t-SNAREs from proceeding in the absence of the appropriate signal (Sollner *et. al.*, 1993b).

VI.4. Small GTP-binding proteins

Rab proteins, a subfamily of the *ras* superfamily of small GTP-binding proteins, are thought to be important in controlling specific steps in intracellular membrane traffic (Pfeffer, 1992). Synaptic vesicles contain at least two rab proteins, rab3A (Fisher v. Mollard *et. al.*, 1990) and rab3B (Matusi *et. al.*, 1988). Rab 3A is present in two forms, a soluble form and a synaptic vesicle membrane associated form ; dissociation from synaptic vesicle membrane occurs after exocytosis (Fischer v. Mollard *et. al.*, 1991).

Rabphilin-3A, a protein that interacts with the GTP-bound rab3A, has been shown to be highly homologous to synaptotagmin (Shirataki *et. al.*, 1993). It is suggested that rab3A-rabphilin interaction might have a regulatory role in proof-reading the correct interaction between v- and t-SNAREs (Fig. 3) (Zerial and Stenmark, 1993).

VI.5. Synaptophysins

Synaptic vesicle membrane contains at least two synaptophysins, I and II. Synaptophysin I is a 38 kDa major integral membrane protein of synaptic vesicles (Jahn *et. al.*, 1985 & Wiedenmann and Franke, 1985). Based on sequence data, a model has been proposed in which synaptophysin has four transmembrane regions with both the amino and carboxyl-termini facing the cytoplasm. The cytoplasmic carboxyl terminus region of synaptophysin I contains ten copies of an imperfect tyrosine-rich repeat with a consensus sequence of YG(P/Q)QG (Sudhof *et. al.*, 1987).

Native synaptophysin I is a homomultimer homologous to gap junction channel proteins (Thomas *et. al.*, 1988) and is found in the membrane complexed with a second, unidentified low molecular weight protein (Johnston and Sudhof, 1990). Synaptophysin I appears to be phosphorylated on serine residues by Ca^{++}-calmodulin-dependent protein kinase II (Rubenstein *et. al.*, 1993) and on tyrosine residues by the proto-oncogene product $pp60^{c\text{-}src}$ (Barnekow *et. al.*, 1990).

The exact function of synaptophysin I is unknown. However, the requirement of synaptophysin I in neurotransmitter release has been illustrated in inhibition experiments by using specific antibodies as well as antisense oligonucleotides (Alder *et. al.*, 1992a ; 1992b). A role in the formation of the putative fusion pore during synaptic vesicle exocytosis has been proposed (Sudhof and Jahn, 1991). Alternatively, transfection studies suggested that synaptophysin I may be involved in the biogenesis and stability of synaptic vesicles (Leube *et. al.*,

1989; Johnston *et. al.*, 1989).

Synaptophysin II, also known as synaptoporin, is a 37 kDa protein which shows high homology to synaptophysin I except for the C-terminus region (Knaus *et. al.*, 1990; Fykse *et. al.*, 1993). Synaptophysin II is similar to synaptophysin I in its presence as a mutlimeric complex associated with a low molecular weight protein as well as its targeting to the receptor-mediated endocytosis pathway after transfection into fibroblasts. However, unlike synaptophysin I which is homogeneously expressed in most nerve terminals, the expression of synaptophysin II is more restricted. Moreover, the two proteins were not complexed with each other in the synaptic vesicles found to contain both, suggesting that they have similar but nonidentical functions (Fykse *et. al.*, 1993).

VII. Identification of granulophysin by using MAbs

In an attempt to identify granule membrane proteins of human platelets, Gerrard and colleagues generated a panel of MAbs against human platelet dense granules (PDG). The following MAbs stained permeabilized platelets in punctate pattern in immunofluorescence studies : D503 (IgG1), D525 (IgG1), D545 (IgG1), D519 (IgG2a), D531 (IgG3). These MAbs together with other MAbs such as D495, which did not react in immunofluorescence studies, strongly reacted only with the soluble fraction of the lubrol extract of the PDG preparation in ELISA. In Western blot studies, however, only D545, D503, and D495 recognized a 40 kDa glycoprotein that was enriched in the membrane fraction of PDG. This glycoprotein was affinity purified by using D519 column and named granulophysin (Gerrard *et. al.*, 1991). Studies with anti-granulophysin MAb showed that granulophysin was deficient in patients with Hermansky-Pudlak syndrome, a platelet storage pool deficiency disorder (Gerrard *et. al.*, 1991 ; Shalev *et. al.*, 1992b). The epitopes recognized by anti-PDG have been found to be widely distributed and frequently associated with granules. These include the

granules of skin melanocytes, neurons, endocrine gland cells, exocrine glands and surface lining cells. Western blot studies have shown the presence of bands with similar size to that of granulophysin in the lung, adrenal gland, liver, brain, prostate, and pituitary. Atypical bands were present in the pancreas head (47 kDa) and skeletal muscle (34 kDa) (Hatskelzon *et. al.*, 1993).

Anti-granulophysin MAbs have been shown to cross react with synaptophysin, the 38 kDa major integral membrane glycoprotein of synaptic vesicles. Moreover, anti-synaptophysin Abs, including one that is directed towards the repetitive motif in the carboxy terminus end, react with platelet granulophsyin. These results suggest the presence of common epitope(s) in the two granule membrane proteins (Gerrard *et. al.*, 1991; Shalev *et. al.*, 1992a).

Epitope mapping studies by D545, D519, D503, D531, D521 and D495 MAbs against granulophysin have suggested that these MAbs recognize distinct but largely overlapping epitopes within a compact region probably constructed by the three-dimensional configuration of the molecule (Shalev *et. al.*, 1992a).

Recent studies, however, have shown granulophysin to be identical to the lysosomal membrane glycoprotein CD63. Western blot studies showed that both anti-CD63 Abs and the anti-granulophysin MAb D545 recognized the same protein band which was deficient in Hermansky-Pudlak syndrome. Immunofluorescent studies showed that both antibodies recognized similar numbers of granules in normal subjects as well as in Hermansky-Pudlak syndrome patients. Moreover, anti-CD63 Abs effectively blocked the protein detected by D545 in immunofluorescent, Western blot, ELISA and FACS analyses. Finally, amino terminal sequencing of the affinity purified granulophysin over the first 37 amino acids showed identity to the amino terminus sequence of CD63 (Nishibori *et. al.*, 1993).

More recently, granulophysin has been shown to be present in the membrane of azurophilic granules of human neutrolphils and to mobilize to the plasma membrane following cell stimulation. Moreover, support of the identity of granulophysin to CD63 was obtained by inhibition studies and the similar pattern of distribution (Cham *et. al.*, 1994).

RATIONALE AND AIM OF THE WORK

Much of our current understanding of the biology of granules has come from the identification, molecular cloning and functional characterization of a large number of granule-associated proteins. The list of proteins associated with lymphocyte cytotoxic granules is growing rapidly. This has dramatically improved our understanding of granule function. However, the majority of the already described proteins are intragranular. Identification of the proteins associated with cytotoxic granule membrane is expected to shed more light on their biology, as has been the case with synaptic vesicles.

We took advantage of the availability of anti-PDG MAbs the identify granulophsyin in platelets to study the presence of granulophysin/synaptophysin homologue(s) in cytotoxic lymphocyte and leukocyte granules and to clone the putative protein(s).

MATERIALS AND METHODS

1. Antibodies

1.1. Anti-platelet dense granule monoclonal antibodies (anti-PDG MAbs)

The production and characterization of the anti-PDG MAbs have been described previously (Gerrard *et. al.*, 1991). Briefly, PDG were isolated then used either intact or after detergent solubilization to immunize BALB/c mice. Following fusion of the splenocytes from the immunized animals with the mouse myeloma cell line PX63AG8653, supernatants from fused cells were screened for MAb production by ELISA using PDG. MAbs were purified by ammonium sulfate precipitation followed by gel filtration chromatography through a G-200 column. D545, an IgG1 MAb, was used in indirect immunofluorescent and Western blot studies. D519 (IgG2a), D503 (IgG1), D531 (IgG3) as well as D545 (IgG1) stained the platelets in a punctate pattern. These four MAbs were used in addition to D495 (IgG1) to immunoscreen the U937 expression library.

1.2. Anti-peptide antibody

A 17 amino acid peptide identical to the carboxy terminal region of the deduced amino acid sequence of clone U.3.20 was synthesized by Dr. David Litchfield at The Manitoba Institute of Cell Biology by using 431A peptide synthesizer (Applied Biosystems Inc., Foster City, CA). Two mg of the peptide were conjugated to two mg of Imject (Maleimide activated keyhole limpet hemocyanin, KLH) (Pierce, Rockford, IL) according to the supplier's instructions. Female rabbits were immunized with the KLH-conjugated peptide according the following schedule. Preimmune serum was obtained at day -1. The initial immunization consisted of a 100 μg of antigen in Ribi Adjuvant (Ribi ImmunoChem. Research, Inc., Hamilton, MO). This was followed by boost immunization using 100 μg antigen in the Ribi Adjuvant at monthly intervals. Bleeding was done 10 days after each boost and the antiserum was tested in

Western blot analysis by using the bacterial fusion protein.

1.3. Anti-RNA helicase A antibody

The rabbit polyclonal antihelicase A antibody (Lee and Hurwitz, 1993) was kindly provided by Dr. J. Hurwitz of the Memorial Sloan-Kettering Cancer Institute in New York .

2. Cells

2.1. $CD3^{\pm}$ granulated lymphocytes

IL-2 stimulated $CD3^+$ granulated lymphocytes were generated by a culture method developed in our laboratory (Roussel *et. al.*, 1990). Briefly, human peripheral blood mononuclear cells (PBM) were obtained from the buffy coat of normal donors (Canadian Red Cross, Winnipeg, MB). After Ficoll Paque isolation (Pharmacia Fine Chemicals, Piscataway), PBM were cultured at $1x10^6$ cells/ml in RPMI 1640 (Gibco, Grand Island, NY) containing sodium bicarbonate (0.2%), penicillin/streptomycin (50 IU/ml and 50 μg/ml, respectively), 10% v/v heat inactivated fetal calf serum, 2 mM L-glutamine and 10 U/ml of recombinant human IL-2 (AMGEN Biologicals, Thousand Oaks, CA) for 6 days at 37°C in a 5% CO_2 incubator. At day 6, the cells were centrifuged and resuspended at 0.25 x 10^6 cells/ml in fresh medium as above and incubated for another 4-6 days until they reached a concentration close to $1x10^6$ cells/ml. At the end of this culture protocol, almost all (94-97%) the cells were $CD3^+$, $CD16^-$ and $CD56^-$. The cells were a mixture of $CD4^+$ (60%), $CD8^+$ (30%), which showed strong non-MHC restricted killing activity (Roussel *et. al.*, 1990).

2.2. Other cells

U937 is a human monocytic cell line obtained originally from the pleural effusion of a patient with diffuse histiocytic lymphoma (Sundstorm and Nilsson, 1976). They were maintained by culture in RPMI 1640 supplemented with 5 % Fetal Calf Serum (FCS) (Gibco Lab, Grand Island, NY). Normal human

neutrophils (97% neutrophils, 3% eosinophils) were prepared by separation on a Percoll density gradient as described previously (Dooley *et. al.*, 1982). Human skin mast cells were kindly provided by Dr. D. Befus (University of Calgary). Human malignant melanoma cell line BOWES was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Human Jurkat T cell line, fibroblast cell line WI38, RAMOS B cell line (originally obtained from ATCC and provided by Dr. J. Wilkins, University of Manitoba) and Natural Killer cell line YT (provided by Dr. H. Young, NIH, Bethesda, MD) were maintained in RPMI 1640 supplemented with 10 % FCS.

3. Indirect immunofluorescence studies

All cells (1-2 x 10^6) in suspension were fixed for one hour by using 2% paraformaldehyde on ice. The melanoma cell line (BOWES) was allowed to adhere to sterile glass slides in tissue culture dishes. After washing three times with Tris-buffered saline (TBS, 0.01 M Tris - 0.85% NaCl, pH 7.4), cells were then fixed by incubation in a solution of 50 % acetone 50 % methanol at -20°C for 10 minutes. Cells were then washed three times with TBS containing 0.1% bovine serum albumin (BSA, Sigma, St. Louis, MO). The primary antibody (anti-PDG MAb D545 10 μg/ml, anti-leukophysin antipeptide antibody 1 in 100, or the polyclonal antibody against human RNA helicase 1 in 100) diluted in TBS was incubated with cells for varying periods of time (between one hour and overnight). Cells were then washed three times with TBS containing 0.1% BSA. The fluorescein labeled secondary antibody (or the biotinylated secondary antibody followed by Fluorescein Avidin D) was then applied in TBS for one hour. After washing as above, cells were resuspended in glycerol and an aliquot was mounted on a glass slide before examination on the fluorescent microscope.

4. Subcellular fractionation of IL-2 stimulated lymphocytes

The method of obtaining subcellular fractions has been described previously (Roussel and Greenberg, 1989). Briefly, cells were washed in phenol-free Hanks balanced salt solution (HBSS, Gibco) and resuspended at $1x10^8$ cells/ml in the disruption buffer (0.25 M Sucrose, 0.01 M Hepes, 4 mM EGTA, 200 U/ml Heparin, pH 7.4). Cells were lysed by decompression at 0°C after equilibrating at 450 *p.s.i.* nitrogen for 20 minutes by using a nitrogen bomb. After the addition of $MgCl_2$ to 5 mM, the homogenate was digested with DNAse I (from bovine pancreas, type IV, Sigma) at 800 U/ml for 25 minutes at room temperature. Nuclei were then removed by filtration through Nucleopore filters (Nucleopore Corp., Pleasanton, CA) of 5 then 3 μm pore size and the resulting homogenate was cooled to 4°C. Four ml aliquots were layered over 20 ml of 48% Percoll (Pharmacia LKP Biotechnology, Uppsala, Sweden) in disruption buffer without heparin and centrifuged in a 70 Ti rotor at 20,000 *r.p.m.* for 5 minutes with 0 deceleration in a Beckman L8-70M ultracentrifuge (Beckman Instruments, Fullerton, CA). The resulting gradient was fractionated from the bottom by careful insertion of a spinal tap needle from the top and the removal of 1 ml fractions via attached polyethylene tubing.

5. Induction of exocytosis of granulated CD3 ±lymphocytes

Phorbol myristic acetate (PMA) and the calcium ionophore A23187 (Sigma, St. Louis, MO) were used to induce exocytosis. They were dissolved in DMSO and diluted in HBSS containing 10 mM Hepes and 1 mg/ml BSA (HBSS-BSA-Hepes buffer, pH 7.4). Cells ($5x10^5$) were added to V-bottomed microtitre plates (Dynatech Lab, Chantilly, Virgina) in 100 μl of HBSS-BSA-Hepes buffer. The combination of PMA and A23187 or control solutions were added to the wells in aliquots of 50 μl, such that the final volume per well was 200 μl. The plates were mixed on a plate shaker for two minutes and incubated at 37°C for various periods

of time. After incubation, the cells were pelleted by centrifuging the plates at 300 *x g* for 10 minutes. BLT-esterase activity of the supernatant was assayed as described below.

6. Flow cytometry

Cell surface staining for the anti-PDG MAb 545 reactive epitope was done on ice as described above but without fixation or permeabilization. Stained cells were analyzed by using a Coulter EPICS V flowcytometer (Coulter, Hialea, Fl.) with an argon ion laser of 500 mW output. Forward and orthogonal angles light scatter were used to select viable cells. FITC immunofluorescence was observed at 488 nm. Each analysis was performed with a total of 10^4 cells.

7. Measurement of the BLT-esterase activity

BLT-esterase was quantitatively analyzed by a colorimetric microassay based on the assay described previously (Green and Shaw, 1979). BLT (N-α-CBZ-L-lysine thiobenzyl ester, Sigma) was dissolved in water (10mM) and diluted to 1mM in HBSS containing 10mM Hepes (HBSS-Hepes). DTNB (5,5-dithiobis (2-nitro-benzoic acid) (Sigma) was dissolved in dimethyl formamide (10mM) and diluted to 0.5mM in HBSS-Hepes. The assay was carried out in V-bottomed microtitre plates (96 well/plate). Each well received 100 µl of the diluted DTNB solution. Reaction wells received 50 µl of the diluted BLT solution and blank wells received 50 µl of HBSS-Hepes. To both reaction and blank wells, 100 µl of diluted fractions were mixed. After 30 minutes, the BLT-esterase activity was assessed by measuring the absorbance at 414 nm on a Titertek Multiskan MCC/340 plate reader (Labsystems, Finland).

8. Western blot analysis

Electrophoresis was done according to Laemmli (Laemmli, 1970) by using 12 % Polyacrylamide resolving gels (0.375M Tris, pH 8.8) and 4% stacking gels (0.125M Tris, pH 6.8) in a Biorad Mini-Protean II cell (BioRad Lab, Richmond,

CA). Samples were reduced by using either 100 mM dithiothreitol (DTT) or 2-β mercaptoethanol to the sample buffer followed by incubation in a boiling water bath for 15 minutes. After cooling samples to room temperature, some samples were treated with 100 mM iodoacetamide for half an hour in a 37°C water bath. A 20 µl aliquot of each sample was loaded into wells and electrophoresis was performed at a constant voltage of 170 volts for 45-60 minutes. Gels were then equilibrated in the transfer buffer (25mM Tris, 192mM Glycine, 20% v/v methanol, pH 8.3) for half an hour. Proteins were transferred to nitrocellulose membrane at 100 volts for one hour at room temperature (or at 25 volts overnight) using the Biorad Mini Trans-blot electrophoretic transfer cell. Membranes were then blocked for an hour in 10 % non-fat powdered carnation milk in TTBS (100mM Tris, 0.9% NaCl, pH 7.5 with 0.1 v/v Tween 20). This was followed by washing the membrane three times with TTBS over a period of 15 minutes. A 10 ml aliquot of TTBS per membrane containing the primary antibody (D545 MAb 10 µg/ml or anti-leukophysin antipeptide antibody 1 in 1000) added for varying periods of time (one hour to overnight) followed by washing as above. This was followed by incubation with the secondary antibody. In some experiments, the biotinylated goat anti-mouse IgG from the Vectastain ABC kit (Vector Lab. Inc., Burlingame, CA) was used, followed by incubation with the Vectastain ABC Avidin-Alkaline Phosphatase conjugate. Blotting was then completed by incubating the nitrocellulose membrane with alkaline phosphatase substrate Kit II (Vector Lab. Inc., Burlingame, CA) in the substrate buffer (100mM Tris-HCl, pH 9.5) and the reaction was stopped after development by adding distilled water. In other experiments, Peroxidase conjugated goat anti-rabbit IgG (1 in 5000) or goat anti-mouse IgG (1 in 5000) (Sigma ImmunoChemicals, St. Louis, MO) was used followed by blot development by using enhanced chemiluminescence kit (ECL, Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

9. Slot blot analysis

In the slot blot assay, 50 µl aliquots of percoll fractions of $CD3^+$ cell homogenate were absorbed to the nitrocellulose membrane by using a slot blot apparatus (Schleicher and Schuell, Keene, N.H.). The membrane was then reacted with D545 MAb (10 µg/ml in TTBS, followed by peroxidase conjugated goat anti-mouse IgG (1 in 5000) and developed by using the ECL chemiluminescence kit. The densitometer tracing was obtained by using Quick Scan R&D densitometer (Helena Laboratories, Beaumont, Texas).

10. Affinity purification of leukophysin

The Percoll fractions which showed reactivity in the slot blot assay were pooled and then centrifuged at 36,000 *r.p.m.* for 2 hours. The protein ring was carefully isolated from percoll and then solubilized in MOPS buffer (5mM MOPS, 150mM NaCl, 0.02% NaN_3) containing 0.2 % Lubrol, 2mM PMSF, 10 µg/ml Leupeptin. This is followed by centrifugation at 10,000 *xg* for 30 minutes. The supernatant was then applied to an affinity column, made by coupling D519 anti-PDG MAb to Affigel 10 (Gerrard *et. al.*, 1991) (BioRad Lab., Richmond, CA). Columns were sequentially washed with MOPS buffer/0.2 % Lubrol, MOPS/2mM EDTA, MOPS alone, 4M NaCl in 5mM MOPS then MOPS buffer alone. The bound protein was eluted with 80 % ethylene glycol in 1mM MES, pH 5.6. This eluate was dialyzed overnight at 4°C against MOPS/0.2 % lubrol, then concentrated by using Centricon microconcentrators (Amicon Canada Ltd., Oakville, Ontario).

For U937 cells, the postnuclear lysate was used for protein purification. The postnuclear lysate was prepared as follows : cultured cells were harvested and resuspended in the sonication buffer (0.1M KCl, 2mM $MgSO_4.H_2O$, 25mM Hepes, and 10mM dextrose, pH 7.0) supplemented with 2mM PMSF and 10 µg/ml leupeptin. Cells were then disrupted by using a sonicator probe (Brownson Sonic

Power Co., Danbury, CT) for 15 seconds at level 4. The cell homogenate was then centrifuged at 700 *x g* for 15 minutes to remove intact cells, nuclei, and large fragments. Lubrol (0.2 %) was added to the supernatant followed by dialysis against MOPS/0.2 % Lubrol buffer. The postnuclear homogenate was then centrifuged at 10,000 *x g* for 30 minutes. The supernatant, now named the postnuclear lysate, was then applied to the D519 affinity column and the bound protein was eluted as described above.

11. Screening of the U937 and Jurkat cDNA libraries

A Uni-ZAP XR expression library of the U937 cell line was obtained from Stratagene (Stratagene, La Jolla, CA). The library was made with Oligo (dT) primers. The cDNA was unidirectionally inserted into the Uni-ZAP XR vector *Eco*RI and *Xho*I restriction sites in the sense orientation to allow the expression of *lacZ* fusion proteins. Inserts cloned into the Uni-ZAP XR vector can be automatically excised to generate subclones in the pBluescript SK^- phagemid vector. The titer of the library was around 2.5 x 10^{10}/ml and the number of nonrecombinant clones was around 1% at the time of our screening.

Immunoscreening was carried out by using the *pico*Blue Immunoscreening Kit according to the instructions of the supplier (Stratagene, La Jolla, CA). Briefly, the library at a concentration of 5 x 10^4 plaques in 100 µl of SM buffer (100 mM NaCl, 50 mM Tris, 10 mM $MgSO_4$, pH 7.5) was incubated with 600 µl of a solution of $O.D._{600}$ = 0.5 of the bacterial host (XL1-Blue *E. coli*) for 20 min at 37°C. Then 7.5 ml of molten top agarose in NZY medium was added and the mixture was plated immediately into a 150 mm NZY agar plate. Plates were incubated at 42°C until the small plaques became visible (4-6 hour). Dry nitrocellulose filters (Micron Separation Inc., Westboro, MA), previously soaked in 10 mM IPTG (Pharmacia), were applied to the plates which were further incubated for 6-8 hours. Filters were then removed, washed 3 times in TTBS (20

mM Tris-HCl, 150 mM NaCl, 0.05 v/v Tween-20, pH 7.5), and blocked for one hour at room temperature with TTBS with 1 % BSA. The filters were then incubated with the primary antibody (a cocktail of 10 μg/ml of each of the following anti-PDG MAb D545, D503, D495, D519, D531, diluted in TTBS-BSA) for one hour. After washing three times in TTBS, the secondary antibody (alkaline phosphatase conjugated goat antimouse, 10 μg/ml, Stratagene) was applied for one hour at room temperature. After washing as above, Tween-20 was removed through a final wash in TBS. Color was developed in the dark by adding NBT and BCIP, diluted to a final concentration of 0.3 mg/ml and 0.15 mg/ml, respectively, in the color development buffer (100 mM Tris-HCl, 100 mM NaCl, 5mM $MgCl_2$, pH 9.5). Color development was terminated by immersing the filter in the stop solution (20 mM Tris-HCl, 1 mM EDTA, pH 2.9). The filter was air dried and stored protected from light.

Screening with the double stranded cDNA probes of the U937 library and the Jurkat λgt11 library (originally obtained from Clontech Lab. Inc., Palo Alto, CA and kindly provided by Dr. John Wilkins, Department of Medical Microbiology, University of Manitoba) was done as follows. The library was plated as described above except that the bacterial host Y1090 was used for the λgt11 library and the plates were incubated at 37°C for 8 hours. Plates were then refrigerated for two hours at 4°C. Nitrocellulose filters were applied to the plates for two minutes. Filters were then denatured after lifting by submersion in (1.5 M NaCl, 0.5 M NaOH) for two minutes. To make duplicates, the second filter was allowed to transfer for four minutes. Filters were then neutralized by submersion in (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for five minutes, followed by rinsing in (0.2 M Tris-HCl pH 7.5, 2x SCC). The transferred DNA was crosslinked to the filters by baking at 80°C for two hours under vacuum. The filters were then incubated in the prehybridization/ hybridization solution (50% deionized

formamide, 0.5% SDS, 0.8 M NaCl, 20 mM pipes pH 6.5, 100 μg/ml denatured, sonicated salmon sperm DNA) for two hours at 42°C. Labeling of the cDNA probes was done by using ^{32}P-α dCTP (Amersham) and the random primer method of the Oligolabelling kit (Pharmacia). Probes were used at a concentration of 1-5 x 10^6 counts/ml prehybridization/hybridization solution. Hybridization was done by incubating the denatured probe with the membrane at 42°C overnight. Washing was done at room temperature for 20 minutes by using 2X SSC, 0.1 % SDS followed by two washes, twenty minutes each, by using 0.1 X SSC with 0.1% SDS at 60-65°C.

12. Isolation of the positive cDNA clones

The *in vivo* automatic excision allows the excision and the re-circularization of the cloned insert contained in the Uni-ZAP vector to form a pBluescript SK$^-$ phagemid vector. It was done according to the supplier's instructions (Stratagene, La Jolla, CA). Briefly, plaques of interest were isolated and transferred individually to a sterile microfuge tube containing 500 μl of SM buffer and 20 μl of chloroform. 200 μl of this phage stock was added to 200 μl of O.D.$_{600}$ = 1.0 XL1-Blue bacteria and 10 μl of R-408 helper phage (Stratagene, La Jolla, CA). The mixture was incubated at 37°C for 15 minutes in a 50 ml conical tube. 5 ml of 2X YT medium (1.6 % bactotryptone, 1 % yeast extract, 0.5 % sodium chloride) was added and the mixture was incubated for 4-6 hours at 37°C with shaking. The tube was heated at 70°C for 20 minutes and centrifuged at 1000 x *g* for five minutes. 200 μl of the supernatant was added to 200 μl of O.D.$_{600}$ = 1.0 of XL1-Blue cells and the mixture was incubated at 37°C for 15 minutes. 1 - 100 μl of the mixture was then plated on LB/ampicillin plates and incubated overnight at 37°C to allow the colonies containing the pBluescript plasmid with the cloned cDNA insert to grow.

The inserts of the positive clones of the Jurkat λgt11 library was cut by using *Eco*RI, purified through low melting Agarose and GENECLEAN Kit (Bio 101 Inc., La Jolla, CA), then subcloned into the pBluescript plasmid (Stratagene, La Jolla, CA).

13. DNA sequencing

Sequencing of the double stranded DNA was performed according to the dideoxy-mediated chain-termination method of Sagner (Sagner *et. al.*, 1977) by using the Pharmacia ^{T7} (Pharmacia) or the Sequenase (United States Biochemical) sequencing kits and ^{35}S dATP (Amersham). The commercially available SK primer 5' TCTAGAACTAGTGGAT 3' and the M13 primer 3' TGACCGGCAGCAAAATG 5' were used to sequence the 5' end of the upper strand and the 3' end of the lower strand of the cDNA insert, respectively. Other primers specific for internal regions of both strands of the inserts of different clones were synthesized at The Manitoba Institute of Cell Biology by Dr. Mike Mowat using a 380 B DNA synthesizer (Applied Biosystems Inc., Foster City, CA). Internal primers for clone U.3.20 had the following sequence 5'GCCCGATACGACAATGGAAGC3', 5'GCTTCCATTGTCGTATCGGGC3', 5'GCGAGGAGGTGGTAGGGGGGC3', 5'GCCCCCCTACCACCTCCTCGC3', 5'GCCAGGAGGGAGATAACAACA3', 5'TGTTGTTATCTCCCTCCTGGC3'.

Electrophoresis of the sequencing reaction was done by using 8 % acrylamide, 36 % urea, 1X TBE (12.1 g Tris, 0.74 g EDTA, 5.34 g boric acid/1 liter of H_2O, pH 8.3) gel and the DNA sequencing apparatus (Biorad Lab., Richmond, CA).

14. Southern analysis

Plasmid DNA mini-preparations was done by using either the alkaline-lysis method (Sambrook *et. al.*, 1989) or the Miniprep Kit Plus (Pharmaica). U937

genomic DNA preparation was done by using Genomic DNA Isolation A.S.A.P. kit (Boehringer Mannheim Canada, Laval, Quebec) according to the supplier's instructions. DNA was then electrophorsed in 1% agarose gel and then transferred into Nytran membrane (Schleicher & Schuell, Keene, NH) by the capillary elution transfer method (Sambrook *et. al.*, 1989). Labeling of the cDNA probes, hybridization and membrane washing were done as described above.

15. Northern analysis

Total RNA was prepared by using a modification of the guanidinium thiocyanate method (Xie and Rothblum, 1991). Briefly, cells were washed in phosphate-buffered saline (PBS) before lysis. Lysis buffer consisted of water saturated phenol, solution B (4 M guanidinuim thiocyanate and 25 mM sodium citrate, pH 7.0), and 2 M sodium acetate (pH 4.0) in a ratio of 1:1:0.1, and was supplemented with 720 µl of 2 β-mercaptoethanol per 100 ml of solution B. 10^7 cell were lysed in 1.6 ml of lysis buffer. 0.16 ml of chloroform /isoamyl alcohol (24:1) was added and the tubes were vortexed and kept on ice for 15-30 min and the phases were separated by centrifuging at 12000x *g* for 20 minutes at 4 ^{o}C. The upper aqueous phase was transferred to a new tube containing an equal amount of isopropanol. The samples were mixed and kept at -20 ^{o}C for at least one hour. RNA was collected by centrifugation (12000X *g*, 20 min). Pelleted RNA was washed twice with 70 % ethanol, dried and resuspended in DEPC-treated H_2O.

PolyA$^+$ mRNA was isolated by using either FastTrack mRNA isolation kit (Invitrogen corporation, San Diego, CA) or the QuickPrep mRNA Purification Kit (Pharmacia) according to the supplier's instructions.

Electrophoresis of RNA was done by using 1% agarose-formaldehyde gel (Sambrook *et. al.*, 1989). Transfer to Nytran membrane, hybridization and membrane washing was carried out as described above.

16. Reverse transcriptase and polymerase chain reaction (RT-PCR) amplification of U937 mRNA

First-strand cDNA was synthesized from U937 mRNA by using a modification of the protocol provided by AmpliFINDER RACE Kit (Clontech Laboratories Inc., Palo Alto, CA). Briefly, 3 μg of poly A^+ RNA from U937 cell (prepared by the QuickPrep mRNA purification kit, Pharmacia) was used. cDNA was produced by using a sense primer 5' ATGACCTGGGAAGCCAAAGT 3' (position 211- 230 of clone U.3.20) and AMV reverse transcriptase according to the supplier's instructions. After RNA hydrolysis and purification of the cDNA, the cDNA was used as a template for PCR amplification. Forward 5' CTCCCTCCTGGCCTTTG 3' (333-349), and reverse 3' ACCTGTCCCTT CTCCTC 5' (1037-1053) primers of clone U.3.20 were used in a PCR to amplify the synthesized cDNA. A protocol which involves 35 cycles of a three-step cycle (95 °C, 30 seconds for denaturation, 50 °C, 45 seconds for annealing and 72 °C, and two minutes for extension) was performed by using PTC-100 DNA thermal cycler (MJ Research Inc., Watertown, MA). The PCR products were separated by gel electrophoresis through low melting Agarose and identified by transillumination under UV light. This was followed by cutting the DNA bands of interest and isolating the DNA by using GENECLEAN Kit (Bio 101 Inc., La Jolla, CA) according to the supplier's instructions. DNA bands were then subcloned into the pCR vector by using the TA cloning system (Invitrogen Corp., San Diego, CA) and subsequently sequenced by using the Pharmacia ^{T7} Sequencing kit (Pharmacia).

17. Fusion protein production

Primers with the following sequence (forward 5' GGATCCATGAAG TACCCA TCTCCCTTC 3' with a *Bam*HI site and reverse 3' TTCTCCTCCACCGCCGAT AATTCTTAAGTA 5' with an *Eco*RI site) which

flank the longest ORF of clone U.3.20 were synthesized. The two primers were used in a PCR to clone the reading frame by using the insert of clone U.3.20 as a template as described above. The PCR product was purified as described above and subcloned into the *Bam*H1 *and Eco*R1 cloning sites of the pGEX-2T prokaryotic glutathione S-transferase gene fusion vector (Pharmacia). Recombinant vectors were used to transform HB 101 *E. coli* as previously described (Sambrook *et. al.*, 1989). Production of the fusion protein was done by diluting the overnight culture of the transformed bacteria in fresh LB medium (1 in 10) and allowing the bacteria to grow for one hour at 37°C before adding isopropyl B-D-thiogalactoside (IPTG) (Pharmacia) to 0.1 mM. After allowing the bacteria to grow for another 3-7 hours, the cells were then pelleted and resuspended in PBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3). The bacterial lysate was obtained by sonication followed by the addition of triton X-100 to 1 % and incubation for 30 minutes.

18. Computer analysis

Database searches of the nucleotide and protein sequences were done by using the FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et. al.*, 1990) programs. Searches were done through electronic mail by using the computer server at the National Center of Biotechnology Information (Bethesda, MD). Databases used for search included Genbank, EMBL, PIR, SWISS-PROT libraries. Analysis of the DNA and protein sequences was done by using three different packages. These included the Genetics Computer Group Sequence Analysis Software Package (GCG) version 7 at the Canada Institute for Scientific and Technical Information in a VAX/VMS environment, Biological Research Computer Hierarchy (BIRCH) package, compiled and maintained at the University of Manitoba by Dr. Brian Fristensky, in a SUN/Unix environment, and the NIH Molecular Biology PC-tools Distribution Package in an DOS environment.

The Oligo program was used to design the synthetic oligonucleotide primers used for DNA sequencing and PCR.

RESULTS

1. Anti-platelet dense granule MAb reacts with cytoplasmic granules of leukocytes

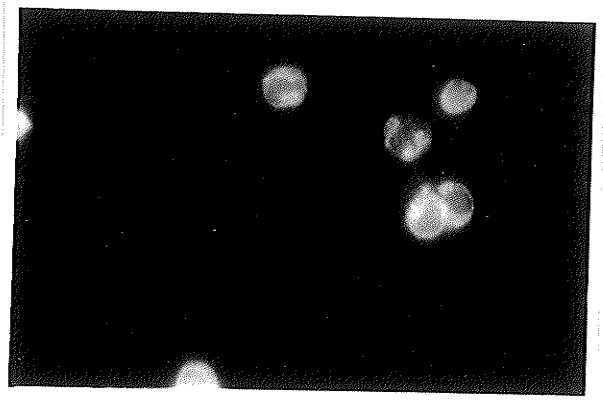
Anti-platelet dense granule (anti-PDG) MAbs have been shown to identify granulophysin as a 40 kDa integral membrane glycoprotein of human PDG (Gerrard et. al., 1991). In order to test the presence and distribution of granulophysin or homologous proteins in leukocytes, we used anti-PDG MAb D545 to examine fixed and permeabilized leukocytes by indirect immunofluorescent staining. $CD3^+$ granulated lymphocytes, generated by culturing PBM cells in recombinant IL-2 (Roussel *et. al.,* 1990), the monocytic cell line U937, human neutrophils and human skin mast cells, panel A-D, respectively, were examined. As shown in Fig. 5, MAb D545 stained intracellular structures in these cells with a punctate staining pattern, suggesting staining of cytoplasmic granules.

2. Association of the D545 reactive epitope with the lymphocyte cytotoxic granules

In order to determine whether the epitope recognized by anti-PDG MAb D545 is associated with lymphocyte cytotoxic granules, we examined the effect of induction of exocytosis on the pattern of antibody staining in $CD3^+$ granulated lymphocytes. These cells display strong non-MHC-restricted killing activity (Roussel *et. al.,* 1990). Studies on the exocytosis of cytotoxic T-cells have shown that the combination of phorbol ester PMA and the calcium ionophore A23187 triggers granule exocytosis. The release of BLT-esterase activity has been taken as a marker of degranulation (Sitkovsky, 1988). Incubation of granulated lymphocytes with a combination of PMA (1 ng/ml) and the calcium ionophore A23187 (5 μg/ml) for one hour resulted in granule exocytosis as determined by the release of BLT-esterase activity (0.21 absorbance at 414 nm compared to 0.06 for

Fig. 5 : **Immunofluorescent staining of granulated leukocytes using D545 MAb.**

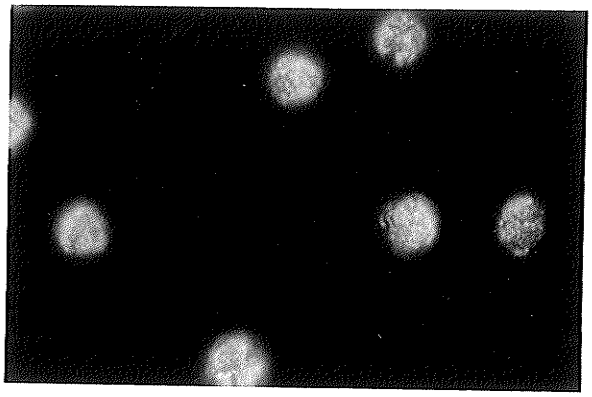
Cells were fixed with 2% paraformaldehyde then permeabilized by using 0.1% Triton X-100 before staining with the anti-PDG MAb D545 (10 μg/ml). The punctate pattern suggest staining of the cytoplasmic granules of human IL-2 stimulated CD3+ cells (A), monocytic cell line U937 (B), human neutrophils (C), and human skin mast cells (D) (x 1000).



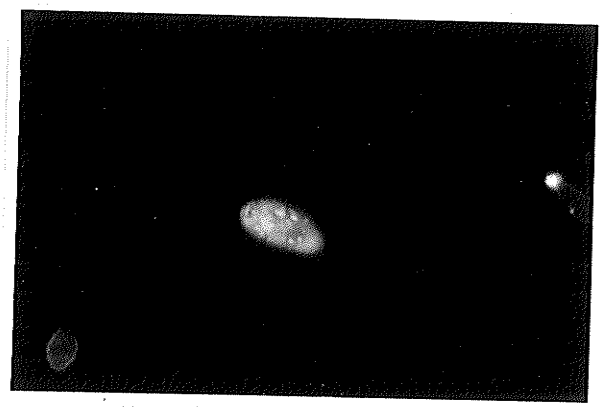
A



B



C



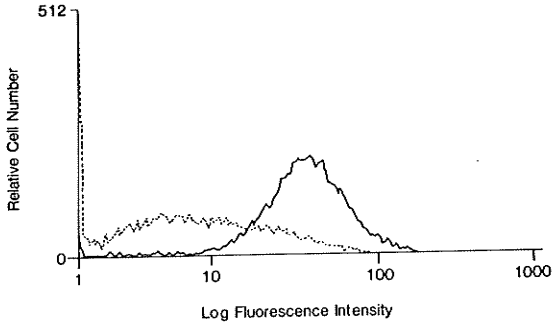
D

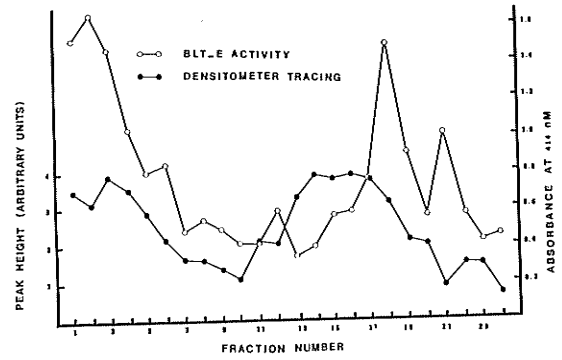
Fig. 6 : **Increased plasma membrane association of the D545 reactive epitope in CD3$^{\pm}$lymphocytes after induction of granule exocytosis.**

The figure shows the result of FACS analysis in which the combination of PMA and A23187 resulted in increased plasma membrane association of the D545 reactive eiptope. IL-2-stimulated CD3^{+} lymphocytes were incubated with the combination of PMA (1 n /ml) and the calcium ionophore A23187 (5 μg/ml) (solid line histogram) or with medium (HBSS with 10 mM HEPES) (dashed line histogram) for 1 hour at 37°C followed by staining with D545 MAb and FACS analysis.

Fig. 7 : **Distribution of BLT-esterase activity and the D545 reactive epitope in subcellular fractions of granulated CD3$^{\pm}$ cells.**

After nitrogen cavitation, the postnuclear homogenate of CD3^{+} cells was layered over Percoll and subjected to ultracentrifugation. The resulting gradient was divided into 24 fractions, and then tested for BLT-esterase activity as well as reactivity with D545 by slot blot analysis and densitometer scanning. Fractions 1 to 6 showed increased BLT-esterase activity as well as high content of the reactive epitope. A second peak of D545 reactivity (fractions 13-18) preceded the second peak of BLT-esterase activity.


512
Relative Cell Number
0
1
10
100
1000
Log Fluorescence Intensity


BLT-E ACTIVITY
DENSITOMETER TRACING
PEAK HEIGHT (ARBITRARY UNITS)
ABSORBANCE AT 414 nM
FRACTION NUMBER

the control medium, data not shown). Fig. 6 shows that cells stimulated in this manner acquire increased levels of the D545 reactive epitope on their plasma membranes, where it is normally present in low density.

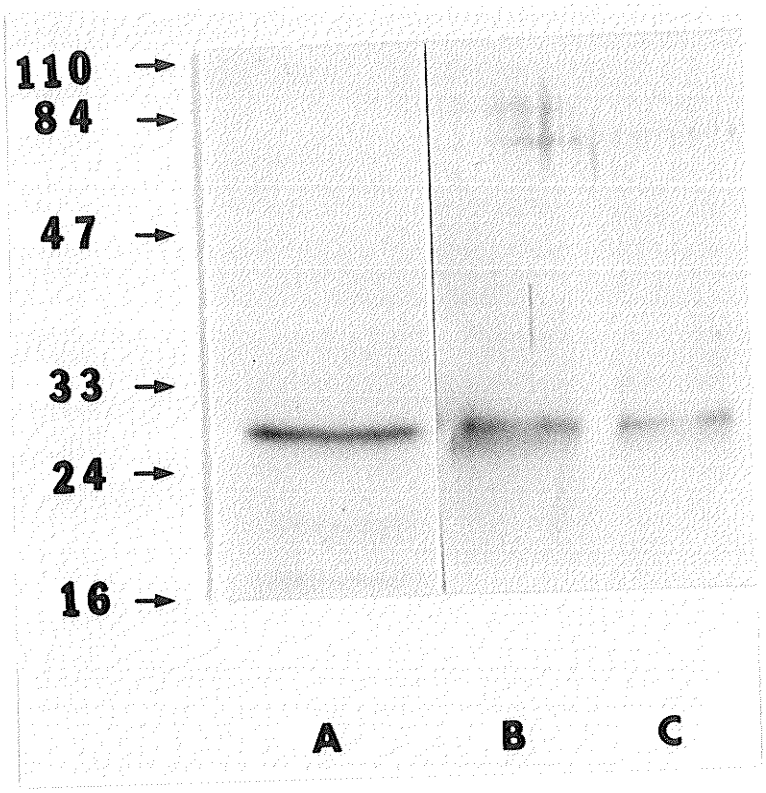
To more directly assess the association of the reactive epitope recognized by D545 with cytoplasmic granules, subcellular fractionation of $CD3^+$ lymphocytes was done. Cells were subjected to nitrogen cavitation followed by density centrifugation of the postnuclear lysate in a 48% Percoll gradient. The gradient was then fractionated from the bottom into 24 fractions, 1 ml each. Individual fractions were tested for the presence of the granule marker, BLT-esterase, as well as the reactivity with D545 MAb by slot blot analysis. As shown in Fig. 7, high reactivity with D545 coincided with the first peak of BLT-esterase activity that corresponded to the dense granules (fraction 1 to 6). Also, a second peak of D545 reactivity was present in lighter fractions (fraction 13 to 18) which preceded and partially overlapped with the second peak of BLT-esterase activity.

3. Affinity purification of leukophysin

In order to determine the relationship between the 40 kDa PDG protein granulophysin identified by D545 and the reactive proteins from the $CD3^+$ lymphocytes and monocytes, proteins from these cells were affinity purified. The Percoll fractions of the $CD3^+$ granulated lymphocytes that reacted with D545 in the slot blot analysis were pooled into two groups, dense fractions (fraction 1 to 6) and light fractions (fraction 13 to 18) (Fig. 7). The reactive proteins from both pools were affinity purified by using the anti-PDG MAb D519 affinity column. This affinity column was used to isolate the 40 kDa granulophysin from PDG (Gerrard *et. al.*, 1991). Western blot studies of the affinity purified protein under reducing conditions showed that D545 MAb mainly recognized a single band with a m.w. of approximately 28 kDa in both the heavy and light fractions (Fig. 8, lane A and B, respectively). The reactive protein from the postnuclear lysate of the

Fig. 8 : **Western blot of the affinity purified proteins from granulated CD3$^{\pm}$ lymphocytes and U937 cells.**

Samples were prepared as described in the materials and methods. Electrophoresis was done in a 12% gel according to Lammeli. After electrotransfer to nitrocellulose membrane, the membrane was stained with the D545 MAb. Lane A, 5 µg of affinity purified protein from dense fractions 1 to 6 of CD3+ lymphocytes ; lane B, 3 µg of the affinity purified protein from light fractions 13 to 18 of CD3^{+} lymphocytes ; lane C the affinity purified protein from the postnuclear lysate of U937 cells (3 µg). D545 identifies a protein in these lanes with identical molecular mass of approximately 28 kDa. The position of the molecular weight markers is shown on the left.


110
84
47
33
24
16
A
B
C

monocytic cell line U937 was also isolated by the D519 affinity column. Lane C in Fig. 8 shows that the monocyte protein has an identical m.w. to the $CD3^+$ lymphocyte cell protein. Some faintly stained higher m.w. bands were also detected in lanes B and C.

The affinity purified proteins from $CD3^+$ lymphocytes and monocytes had a different electrophoretic mobility from that of the affinity purified PDG granulophysin prepared by using the same affinity column and the same elution protocol (Gerrard *et. al.*, 1991). Therefore, leukophysin was proposed as the name for the 28 kDa protein of $CD3^+$ lymphocytes and monocytes (Abdelhaleem *et. al.*, 1991).

4. Isolation of U937 cell cDNA clones by using anti-PDG MAbs

In order to clone the leukophysin gene, a cocktail of five anti-PDG MAbs (D545, D503, D519, D495 and D531) was first used to screen a LAK cell expression library. However, after several rounds of screening, some positive clones which consisted mainly of viral repetitive elements with no obvious open reading frames (ORF) were isolated (data not shown). Because leukophysin from $CD3^+$ cells and U937 cells show similar m.w., we switched to a Uni-ZAP XR expression library of the U937 monocytic cell line. A single positive clone, clone U.1.4, was identified after screening 1.5×10^6 clones. Clone U.1.4 was purified and shown to react with varying intensities with individual anti-PDG MAbs (Fig. 9).

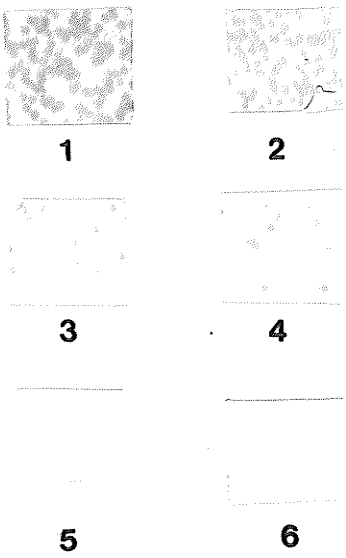
Clone U.1.4 had an 851 bp insert. Sequence analysis showed that the longest ORF of clone U.1.4 coded for a polypeptide with 173 amino acids which began at the 5' end with no start codon and ended in a termination codon. A potential polyadenylation signal and a poly A rich sequence were present at the 3' end, suggesting that this was the 3' end of the gene. In order to obtain a full length cDNA, the library was then rescreened by using either the full size insert or an

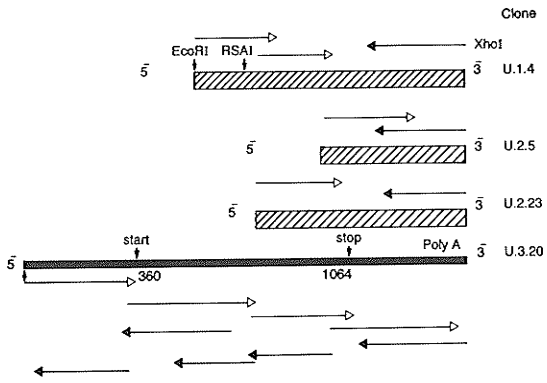
Fig. 9 : **Reaction of individual anti-PDG MAbs with clone U.1.4.**

Isolated phagemid of clone U.1.4 was purified over two rounds of antibody selection (using the anti-PDG MAb cocktail). Purified phagemid was expanded in plate lysate by using XL1-Blue *E. coli.* Dry nitrocellulose filters previously soaked in 10 mM IPTG were applied to the plates which were incubated for 6-8 hours. Filters were then removed, washed three times in TTBS and blocked for one hour at room temperature with TTBS-1% BSA. Individual anti-PDG MAbs (10 μg/ml TTBS-BSA) were applied for one hour. After washing, the secondary antibody (Alkaline Phosphatase conjugated goat anti-mouse) was applied for one hour followed by color development by using NBT and BCIP as described in the materials and methods. The figure shows positive reaction withD519, D545, D531, D503, weak positive reactive detected with D495, the secondary antibody was negative (1,2,3,4,5 and 6 respectively) with purified clone U.1.4

Fig. 10 : **Isolation of cDNA clones from the U937 library.**

Immunoscreening the U937 expression library was done by using a cocktail of anti-PDG MAbs. A single positive clone with 851 bp insert, clone U.1.4, was isolated after screening $1.5x10^6$ clones. The library was then rescreened using either the full size insert or an *Eco*RI / *Rsa*I 164 bp restriction fragment of clone U.1.4. Three clones, U.2.5, U.2.23, and U.3.20 with 434, 561, and 1404 bp inserts, respectively, were isolated. The direction of the arrows indicate the direction of sequencing. Solid arrowheads indicate lower strand sequencing, whereas empty arrowheads indicate upper strand sequencing. Vertical arrows in clone U.1.4 indicate the position of the *Eco*RI and *Rsa*I restriction sites, whereas in clone U.3.20 they indicate the start and stop codons of the longest ORF.


1
2
3
4
5
6


Clone
EcoRI
RSAI
XhoI
5
3
U.1.4
U.2.5
U.2.23
start
stop
Poly A
U.3.20
360
1064

*Eco*RI / *Rsa*I 164 bp restriction fragment of clone U.1.4 (Fig. 10). Three overlapping clones, U.2.5, U.2.23, and U.3.20 with 434, 561, and 1404 bp inserts, respectively, were isolated. Clone U.2.5 and clone U.2.23 were isolated in the screen done by using the full size insert, whereas clone U.3.20 was isolated by using the 164 bp restriction fragment.

The 5' end of the upper strand of clone U.2.5 and clone U.2.23 were sequenced by using the SK primer. The 3' end of the lower strand was sequenced by using the M13 primer. These primers were also used to sequence clone U.3.20. In addition, six internal primers were synthesized and used to complete the sequencing of both strands of clone U.3.20 in both directions.

Fig. 10 shows the sequencing strategy. The arrows indicate the direction of sequencing and the strand being sequenced. Fig. 11 shows the complete nucleotide sequence and the deduced amino acid sequence of the longest open reading frame (ORF) of clone U.3.20. Potential start and stop codon are presented in bold face type, whereas the potential polyadenylation signal is underlined (Abdelhaleem *et. al.*, 1993).

Fig. 12 shows the result of *frames* computer program (GCG7 computer package) which shows the six reading frames of clone U.3.20. The longest ORF in the 5' to 3' direction is present in frame 3 (from 360 to 1064). In order to test whether this ORF is present in a coding region, *Testcode* program (GCG7 computer package) was used. *Testcode* helps identify coding regions of genes with a 95% level of confidence when the specific knowledge of codon preference for the DNA being examined is unknown (Frickett, 1982). As shown in Fig. 13 most the ORF (360-1064) is predicted to be present in a coding region.

Kozak's scanning model for translation (Kozak, 1989) states that the site of translation initiation is the first ATG codon present in a favorable context. The GCCGCC (A/G) CCATGG has emerged as the consensus sequence for initiation

Fig. 11 : **Nucleotide sequence and the deduced amino acid sequence of clone U.3.20.**

Nucleotide sequence and the predicted amino acid sequence of clone U.3.20. Potential start (360-362) and termination (1065-1067) codons are shown in bold face character. Potential polyadenylation signal is underlined.

```
                15                  30                  45                  60
agcattgctgctgctacctgctttccagagcctttcatcaatgaaggaaagcggctgggc

                75                  90                  105                 120
tatatccatcgaaattttgctggaaacagattttctgatcacgtagccctttttatcagta

                135                 150                 165                 180
ttccaagcctgggatgatgctagaatgggtggagaagaagcagagatacgtttttgtgag

                195                 210                 225                 240
cacaaaagacttaatatggctacactaagaatgacctgggaagccaaagttcagctcaaa

                255                 270                 285                 300
gagattttgattaattctgggtttccagaagattgtttgttgacacaagtgtttactaac

                315                 330                 345                 360
actggaccagataataatttggatgttgttatctccctcctggcctttgtagccaagaca
                                                                    M

                375                 390                 405                 420
tgaagtacccatctcccttctttgtatttggtgaaaagattcgaactcgagccatctctg
  K   Y   P   S   P   F   F   V   F   G   E   K   I   R   T   R   A   I   S   A

                435                 450                 465                 480
ctaaaggcatgactttagtcacccccctgcagttgcttctctttgcctccaagaaagtcc
  K   G   M   T   L   V   T   P   L   Q   L   L   L   F   A   S   K   K   V   Q

                495                 510                 525                 540
aatctgatgggcagattgtgcttgtagatgactggattaaactgcaaatatctcatgaag
  S   D   G   Q   I   V   L   V   D   D   W   I   K   L   Q   I   S   H   E   A

                555                 570                 585                 600
ctgctgcctgtatcactggtctccgggcagccatggaggctttggttgttgaagtaacca
  A   A   C   I   T   G   L   R   A   A   M   E   A   L   V   V   E   V   T   K

                615                 630                 645                 660
aacaacctgctatcatcagccagttggaccccgtaaatgaacgtatgctgaacatgatcc
  Q   P   A   I   I   S   Q   L   D   P   V   N   E   R   M   L   N   M   I   R

                675                 690                 705                 720
gtcagatctctagaccctcagctgctggtatcaaccttatgattggcagtacacggtatg
  Q   I   S   R   P   S   A   A   G   I   N   L   M   I   G   S   T   R   Y   G

                735                 750                 765                 780
gagatggtccacgtcctcccaagatggcccgatacgacaatggaagcggatatagaaggg
  D   G   P   R   P   P   K   M   A   R   Y   D   N   G   S   G   Y   R   R   G

                795                 810                 825                 840
gaggttctagttacagtggtggaggctatggcggtggctatagcagtggaggctatggta
  G   S   S   Y   S   G   G   G   Y   G   G   G   Y   S   S   G   G   Y   G   S
```

```
          855              870              885              900
gcggaggctatggtggcagcgccaactcctttcgggcaggatatggtgcaggtgttggtg
  G  G  Y  G  G  S  A  N  S  F  R  A  G  Y  G  A  G  V  G  G

          915              930              945              960
gaggctatagaggagtttcccgaggtggctttagaggcaactctggaggagactacagag
  G  Y  R  G  V  S  R  G  G  F  R  G  N  S  G  G  D  Y  R  G

          975              990             1005     .       1020
ggcctagtggaggctacagaggatctgggggattccagcgaggaggtggtagggggcct
  P  S  G  G  Y  R  G  S  G  G  F  Q  R  G  G  G  R  G  A  Y

         1035             1050             1065             1080
atggaactggctactttggacagggaagaggaggtggcggctattaaaacttggttatgt
  G  T  G  Y  F  G  Q  G  R  G  G  G  G  Y
         1095             1110             1125             1140
cagttcctgtgtgtagacagtaaggaaaaaaaggcatgctatgtgttacgtgtttttcc

         1155             1170             1185             1200
agtatgtttatttgccaccaaaaagtaaatgcattttcacccattctgtggttcattgta

         1215             1230             1245             1260
gtttaaggaaaccaagcatatagatgcattagtgattttgtttatattatgtaaaatata

         1275             1290             1305             1320
acgatctcttaaaaataccacagtttgtatttttttctttaaggagtaaagatttgccttt

         1335             1350             1365             1380
aaataacttggtattttcctggctttcgtttaatacaatagaaaataaagtattacaccg

         1395
aaaaaaaaaaaaaaaaaaaaaaa
```

Fig. 12 : **The open reading frames of clone U.3.20.**

The figure shows the output of *frames* program (GCG 7 package). The open reading frames for the six translation frames of clone U.3.20 are shown. Potential start codons are indicated by line lines extending above the reading frame box and stop codons are indicated by lines extending below the box.

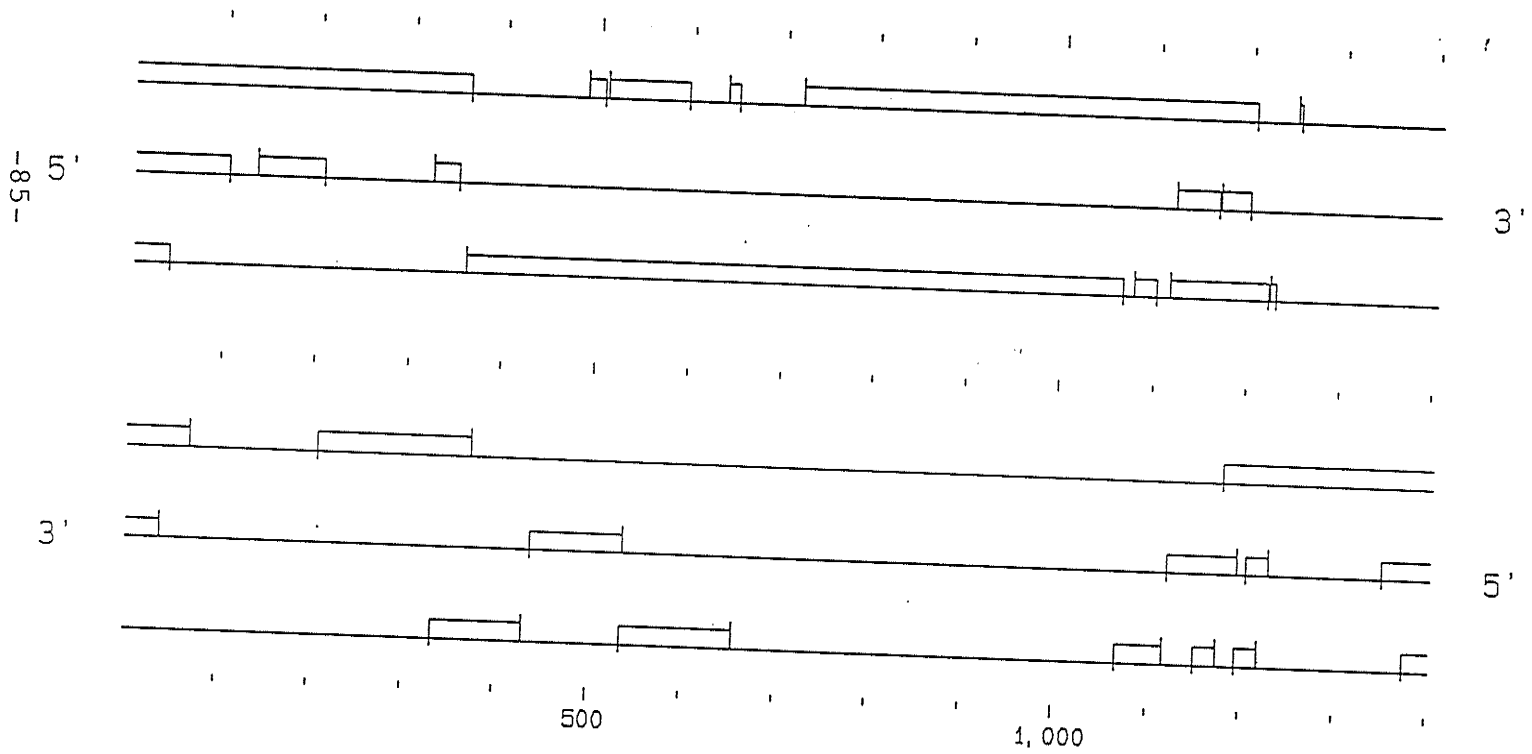
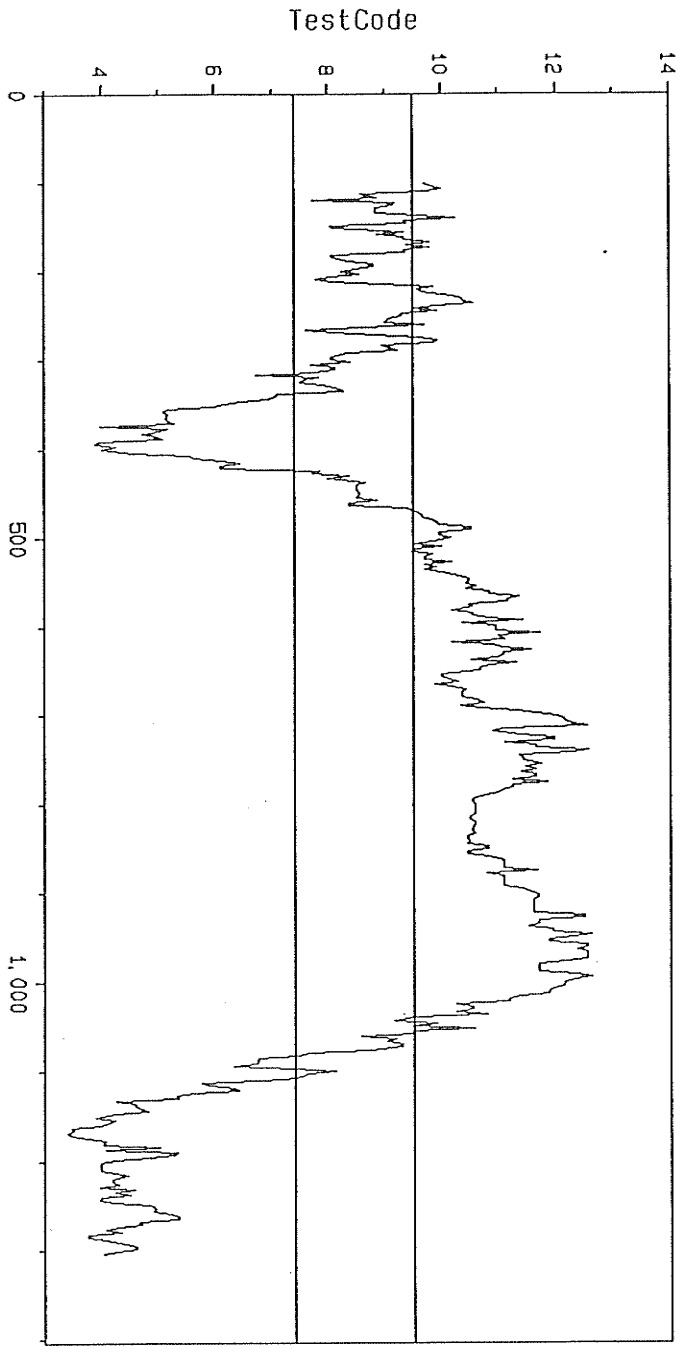

-85-
5'
3'
3'
5'
500
1,000

Fig. 13 : Identification of the protein coding sequence in clone U.3.20.

Testcode program (GCG 7 package) helps to identify protein coding sequences independent of the reading frame by plotting a measure of the non-randomness of the composition at every third base. The plot is divided into three regions, the top region is designed to predict coding regions to a 95 % level of confidence. The bottom region is designed to predict non-coding regions to the same confidence level. In the middle region no significant prediction can be made.


TestCode
4
6
8
10
12
14
0
500
1,000

in higher eukaryotes. A purine in position -3 is the most highly conserved nucleotide in all eukaryotic mRNAs. However, reinitiation is considered to be an exception to the first ATG & favorable context rule. Reinitiation can occur from an ATG in a favorable context downstream, provided that the upstream start codon has a termination codon inframe (Kozak, 1989). The longest ORF of clone U.3.20 is present in frame 3. It starts with an ATG codon (position 360-362) in a favorable context (G at position -3). There is a termination codon upstream in this frame (after position 41). Therefore, this ATG can be potentially used as a reinitiation site.

5. Analysis of the deduced amino acid sequence of clone U.3.20

The longest ORF of clone U.3.20 codes for a polypeptide of 235 amino acids with a calculated molecular weight of 24.309 kDa (*DM5* program of the NIH package). The isoelectric point was determined by to be 11 by using *isoelectric* program (GCG7 computer package) (Fig.14).

By searching the protein sequence motifs in the prosite dictionary (published and updated by Dr. Amos Bairoch, University of Geneva, *motifs* program in the GCG7 package) the following potential motifs were found in polypeptide :

1. a single potential N-glycosylation site, residue 134.
2. 4 protein kinase C phosphorylation sites, serine at positions 20, 37, 117 and 170.
3. 2 casein kinase II (CK-2) phosphorylation sites, serine at positions 87 and 195.
4. a single glycosaminoglycan attachment site, serine at position 146.
5. 21 N-myristylation sites, glycine at positions 67, 142, 147, 148, 151, 152, 157, 162, 166, 176, 178, 181, 185, 189, 193, 204, 215, 216, 219 and 227.

Fig. 14 : Determination of the isoelectric point of deduced amino acid sequence of clone U.3.20.

Isoelectric program (GCG 7 package) calculates the isoelectric point of a protein from its amino acid composition assuming no electrostatic interactions occur that perturb ionization. The plot shows the total positive (+) and negative charges (-) and the net charge (solid curve) as a function of pH. The isoelectric point is indicated on the plot (*) to be 11.00.

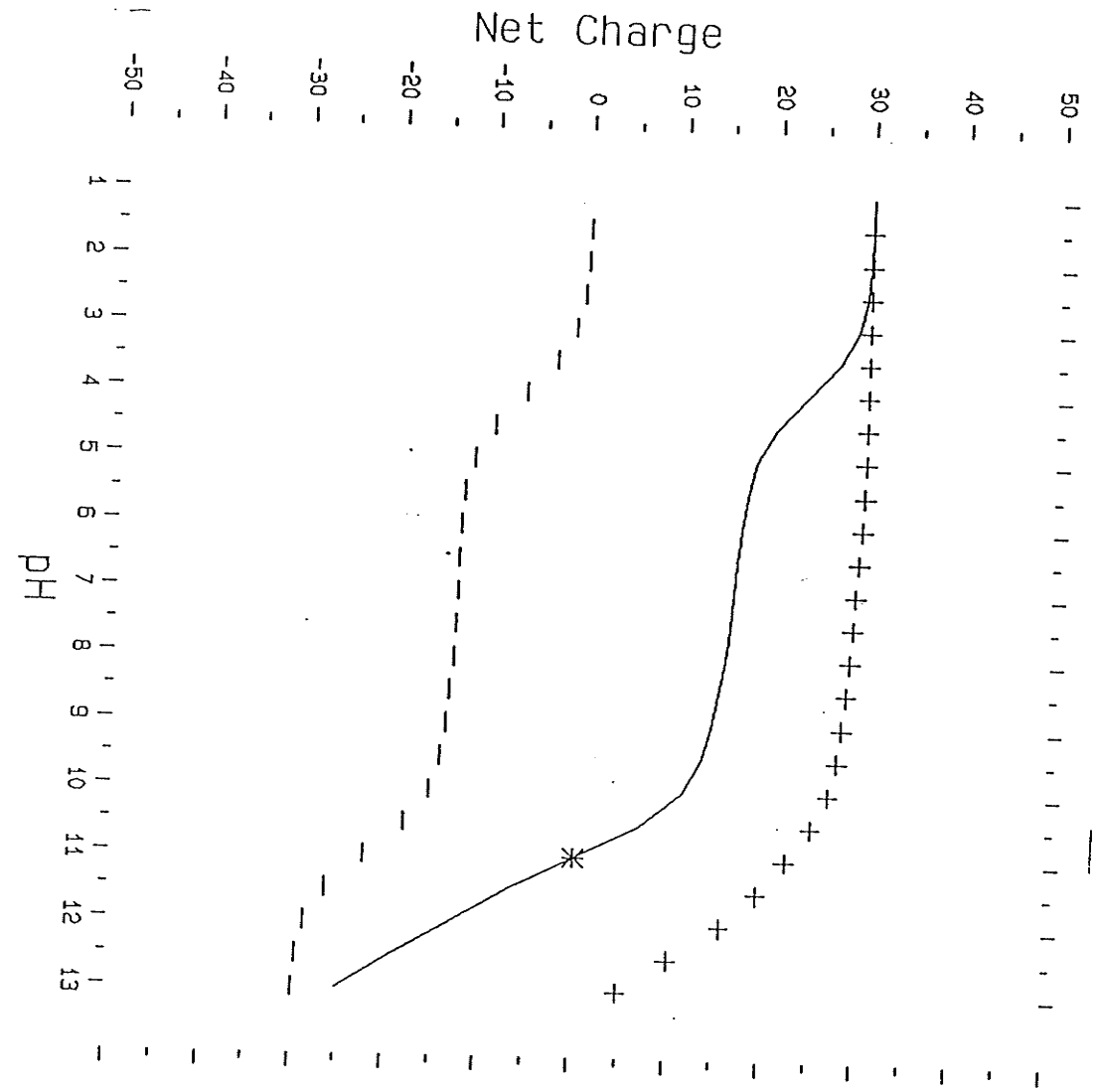

Net Charge
-50
-40
-30
-20
-10
0
10
20
30
40
50
pH
1
2
3
4
5
6
7
8
9
10
11
12
13

Fig. 15 shows prediction of the secondary structure of the polypeptide according to Garnier-Osguthorpe-Robson (Garnier *et. al.*, 1978) by using *PeptideStructure* and *PlotStructure* programs (Jameson and Wolf, 1988) of the GCG 7 computer package. As shown in the figure, the protein can be viewed as consisting of two halves. The amino-terminus half has four alpha helical regions, five beta sheets and few turns. The carboxy-terminus half has no alpha helices, five beta sheets and several turns. Kyte-Doolittle measure of hydropathy (Kyte and Doolittle, 1982) is superimposed on the curve. The amino-terminal half contains two clusters of hydrophobic residues (diamonds). Kyte-Doolittle measure of hydropathy is the most widely used method to calculate the hydrophobic index. Hydrophobic index is the mean value of the hydrophobicity within a window of thirteen amino acids. The hydrophobic index is used to predict potential transmembrane regions (Brandon and Tooze, 1991). Fig. 16 shows a representation of the hydrophobic index within a window of 13 amino acids according to Kyte and Doolittle by using *PepPlot* program of the GCG 7 package. Two potential regions, of sufficient length and hydrophobicity, can be identified in the amino-terminus half of the protein (amino acids 20-39, 57-84).

At the primary amino acid sequence level, the carboxy-terminus region of clone U.3.20 protein is characterized by the presence of a tyrosine-rich imperfect pentapeptide repetitive motif. This motif spans 63 amino acids and has a consensus sequence of YG(G/S)GG. A similar repetitive motif with a consensus sequence of YG(P/Q)QG is present in the carboxy-terminus region of synaptophysin I , the 38 kDa major integral protein of synaptic vesicle (Sudhof *et. al.*, 1987). Synaptophysin also shares the overall secondary structure with leukophysin in that the amino-terminus half has alpha helical and trans-membrane regions whereas the carboxy-terminus half has several turns (data not shown).

Fig. 15 : Prediction of clone U.3.20 protein secondary structure.

The figure shows the prediction of leukophysin secondary structure according to Garnier-Osguthorpe-Robson method (*peptidestructure and plotstructure* programs of the GCG 7 package). Helices are shown with a sine wave, beta sheets are shown with a sharp saw-tooth wave, turns are shown with 180 degree turns and coils are shown with a dull saw-tooth wave. The Kyte-Doolittle measures of hydrophobicity and hydrophilicity are superimposed over the wave as diamonds and ovals, respectively.

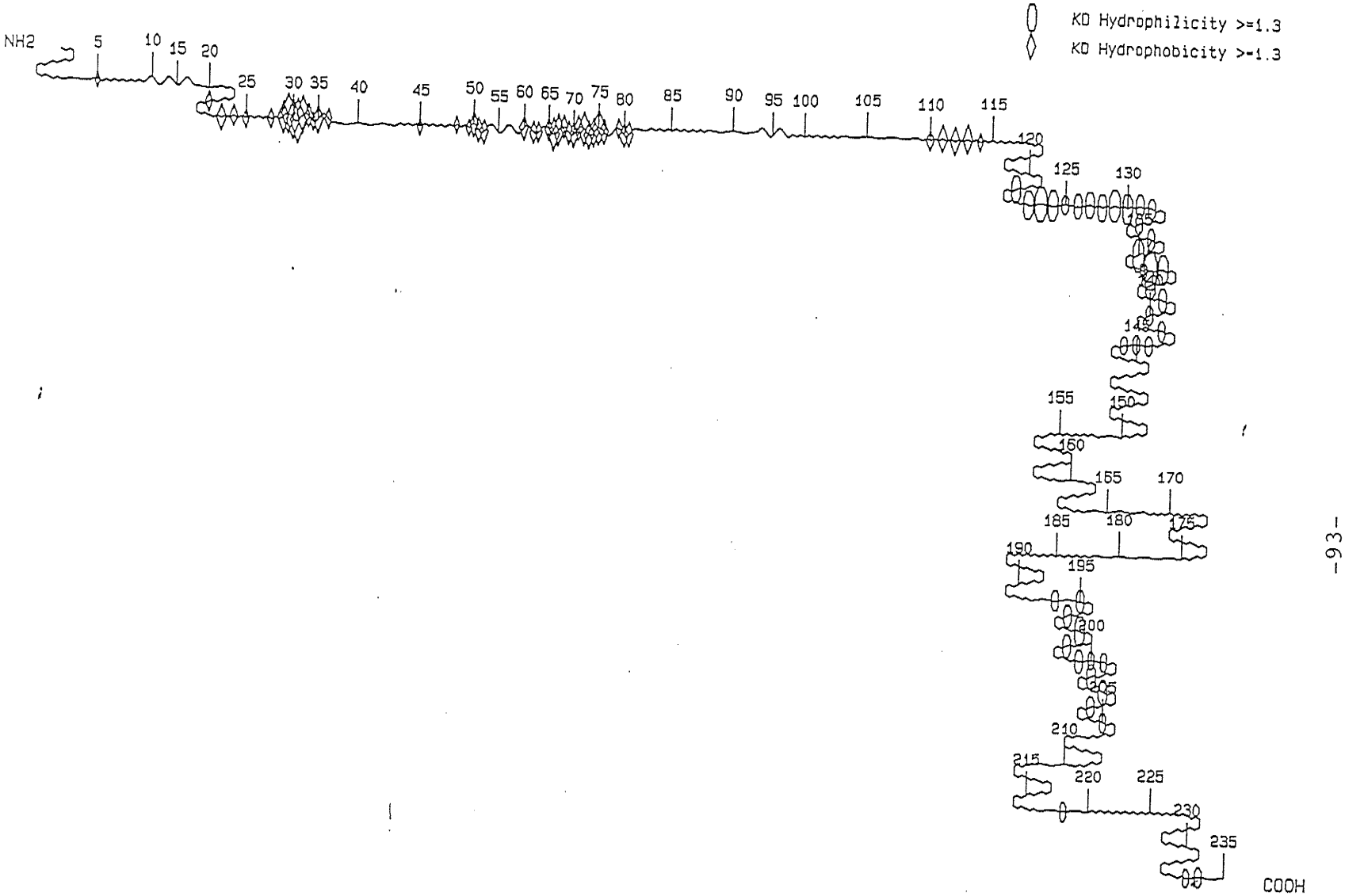
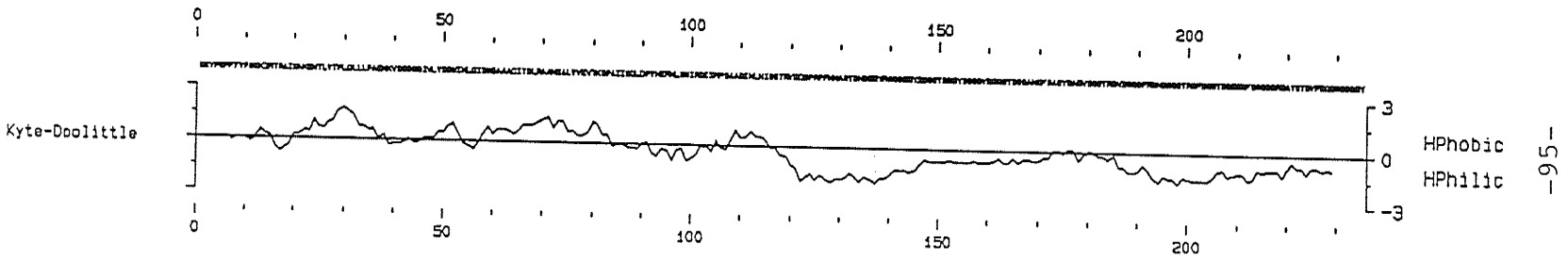

KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130
155
160
165
170
185
180
190
195
210
215
220
225
230
235
COOH

Fig. 16 : Prediction of potential transmembrane regions.

The figure is the output of *pepplot* program (GCG 7 package) which shows the Kyte-Doolittle hydrophobic index over a window of 13 amino acids. Two potential transmembrane regions can be identified in the amino-terminus half (amino acids 20-39 and 57-84).

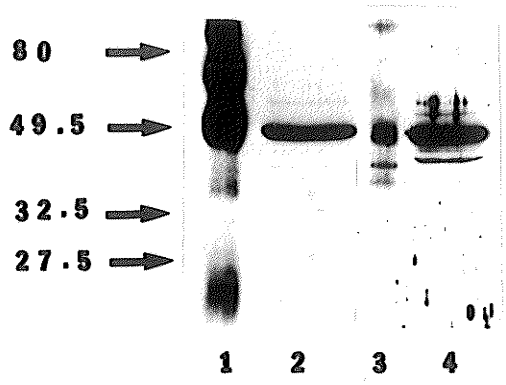

Kyte-Doolittle
0
50
100
150
200
3
0
-3
HPhobic
HPhilic

6. Clone U.3.20 codes for leukophysin

In order to show that the longest ORF of clone U.3.20 codes for leukophysin, we expressed this ORF as a fusion protein in *E. coli*. Primers with the following sequences were synthesized ; forward 5' GGATCCATGAAGTACCCATCTCCCTTC 3' and reverse 3' TTCTCCTCCACCGCCGATAATTCTTAAGTA 5'. The two primers have sequences which flank the ORF. The primers were used in a PCR to clone the ORF as described in the materials and methods. The PCR product was subcloned into pBluescript plasmid and the DNA sequence of the insert was determined. The PCR product was then subcloned into pGEX-2T expression vector which allows the high-level expression of genes as fusion proteins with *Schistosoma japonicum* glutathione S-transferase (GST) (Smith and Johnson, 1988). Western blot analysis of the bacterial lysate of cells transformed with recombinant vector showed a reaction with a band of the expected size (50 kDa, 24 kDa for the cloned gene and 26 kDa for the GST) with both the anti-PDG MAb D545 and the anti-peptide Ab (Fig. 17 lane 4 and 2, respectively). The anti-peptide Ab is rabbit polyclonal antibody generated against the carboxy terminal 17 amino acid sequence (AYGTGYFGQGRGGGGY) of the deduced amino acid sequence of clone U.3.20. Both antibodies also recognize a common band of 50 kDa in the postnuclear lysate of U937 cells (Fig 17, lane 3 and 1). However, the size of this band, 50 kDa, is different from the 28 kDa detected by D545 when the affinity purified fraction of U937 was used as shown in Fig. 8, lane C. Due to unavailability of the affinity purified protein from either U937 cells or $CD3^+$ lymphocytes, the dense granules of $CD3^+$ lymphocytes were isolated from Percoll fractions 1-6 (Fig. 7) by ultracentrifugation. The granules were solubilized in 2 M NaCl with three freeze/thaw cycles followed by ultracentrifugation and isolation of the membrane fraction. Fig. 18 lane A shows that the anti-peptide Ab reacted

Fig. 17 : Western blot of the fusion protein and U937 postnuclear lysate.

Bacterial lysate from HB101 *E. coli* transformed with recombinant pGEX-2T (lane 2 & 4) and the postnuclear lysate from U937 cells (lane 1 & 3) were prepared as described in materials and methods. The anti-peptide Ab (1 in 1000 dilution in TTBS) was used lane 1 and 2, whereas D545 (10 μg/ml in TTBS) was used in lane 3 and 4. Both antibodies detect a 50 kDa band in the bacterial lysate which is the expected size (26 kDa for the GST and 24 kDa for the cloned ORF of clone U.3.20). The position of the molecular weight markers in kDa is shown on the left.


80
49.5
32.5
27.5
1
2
3
4

with a band of approximately 28 kDa from granule membrane fraction. To show that this band is the same as the leukophysin band detected by D545 MAb (Fig. 8), the nitrocellulose membrane was stripped of the anti-peptide Ab and reacted with D545. As shown in Fig. 18 lane B, D545 detects the same band. Moreover, by using the granule membrane fraction of RNK-16 tumor cell line (Fig. 19), several similar bands, with m.w. between 28-30 kDa, were detected by both antibodies. Taken together, these data suggest that clone U.3.20 codes for the 28 kDa protein recognized by the D545 anti-PDG.

7. Immunofluorescent studies using the anti-peptide antibody

In order to study the cellular distribution of leukophysin by using the anti-peptide Ab, indirect immunofluorescence analysis of several cell lines was done. The anti-peptide Ab detected nuclear structures in U937 cells, malignant melanoma cells, and fibroblasts. Comparison with phase contrast microscopy suggested that these structures might represent the nucleoli (Fig. 20, panel A-F). Immunoelectron microscopy staining of U937 cells with the anti-peptide Ab showed similar nuclear pattern of staining (not shown). Some cytoplasmic speckling was detected in fibroblasts. However, in $CD3^+$ granulated cell, the pattern of staining was mainly cytoplasmic and punctate suggesting staining of the cytoplasmic granules.

8. Southern & Northern analyses and chromosome mapping

Fig. 20 shows Southern analysis of the genomic DNA from U937 cell digested with several restriction enzymes and probed with the 164 bp fragment of clone U.1.4. Two bands were detected after either *Hind* III, *Bgl* II or *Pst* I digestion, whereas three bands were detected with *Eco*RI.

In situ hybridization analysis (done in collaboration with Dr. P. McAlpine, Department of Genetics, University of Manitoba and Dr. P. Allderdice, Memorial University of Newfoundland) by using clone U.3.20 showed that this clone map to

Fig. 18 : Western blot of the granule membrane fraction of CD3$^{\pm}$ lymphocytes.

Dense granule were isolated from fractions 1-6 of the Percoll gradient of CD3^{+} granulated lymphocytes. The granules were solubilized in 2 M NaCl through three freeze/thaw cycles. This was followed by ultra-centrifugation of the whole material at 34,000 *r.p.m.* and isolation of the membrane fraction, solubilization in buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF). An aliquot of the granule membrane was added to SDS-reducing sample buffer and run through 12% acrylamide gel. After transfer to nitrocellulose the membrane was reacted with the anti-peptide Ab (1 in 1000 dilution in TTBS) as described in materials and methods. As shown in lane A, a band of approximately 28 kDa is detected by the anti-peptide Ab. The same membrane was stripped of primary and secondary Abs by incubation with the stripping buffer (100 mM 2-β mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50^{o}C for 30 minutes with occasional agitation. After liberal washing in TTBS the membrane was blocked in 10% fat free skimmed milk and incubated with D545 (10 μg/ml in TTBS). Immunodetection was done by using Peroxidase conjugated goat anti-mouse Ab and the ECL detection kit. As shown in lane B the same band is detected by D545. The arrows indicate the position of the molecular weight markers in kDa.

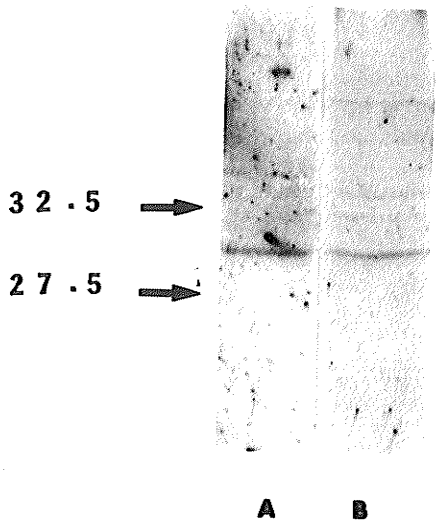

32.5
27.5
A
B

Fig. 19 : Western blot analysis of the granule membrane fraction of rat Natural Killer cells.

Membrane fraction of the RNK-16 granules were prepared by using the same method described for $CD3^+$ lymphocyte granules. Lane A shows the reaction with D545 MAb, whereas lane B shows the reaction with the anti-peptide Ab. Several bands with similar molecular weight (between 28 and 30 kDa) are detected by both antibodies. The arrows indicate the position of the molecular weight markers in kDa.

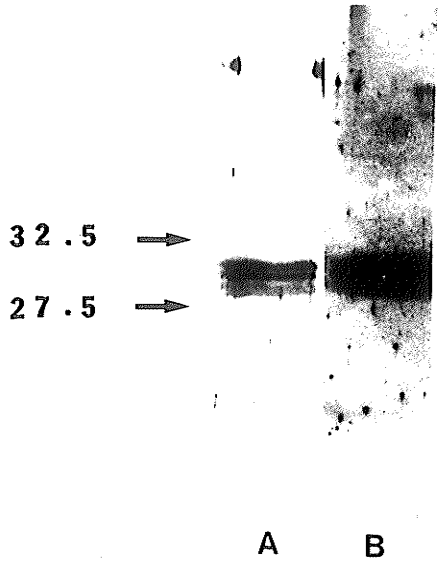
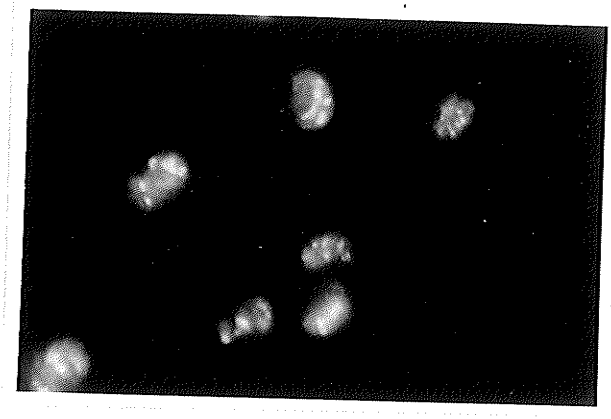
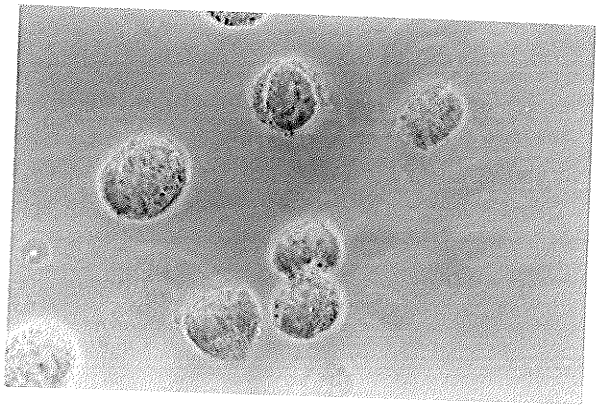

32.5
27.5
A
B

Fig. 20 : Immunofluorescent studies using the anti-peptide Ab.

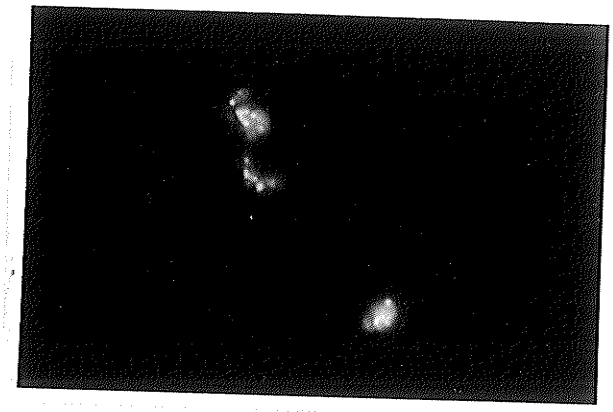
Cells were fixed in 2 % paraformaldehyde and permeabilized in Triton X-100 as described in the materials and methods. The anti-peptide Ab (diluted 1 in 100 in TBS) was incubated with cells overnight. Fluorescein labeled anti-rabbit Ab was added for one hour. Cell were examined by fluorescent as well as phase contrast microscopy. Panel A & B : U937 monocytes show staining of dense nuclear structures. Panel C & D : BOWES malignant melanoma cells show staining of similar nuclear structures. Panel E & F : WI38 fibroblasts show cytoplasmic speckling in addition to the nuclear staining. Panel G & H : $CD3^+$ granulated lymphocytes show staining of cytoplasmic granules. (x1000).



A



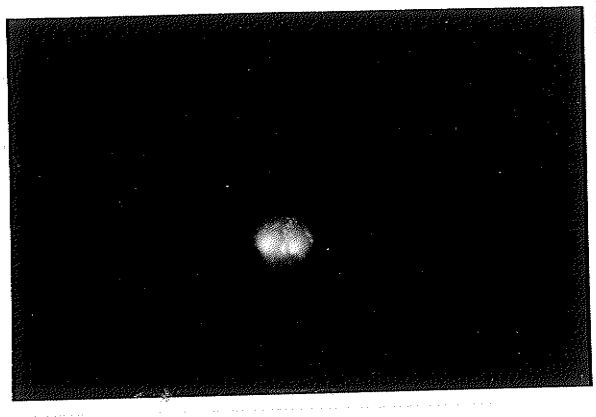
B



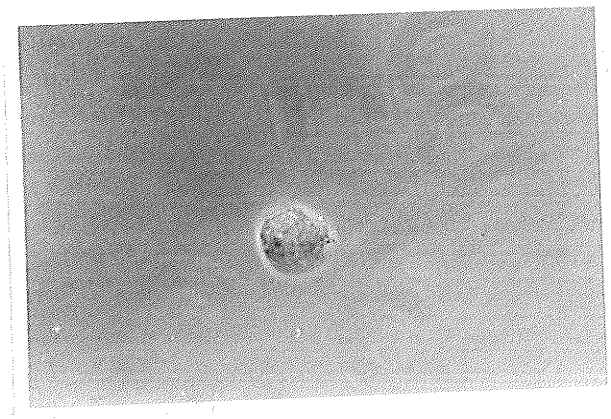
C



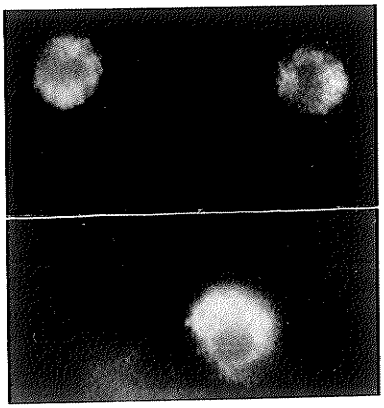
D



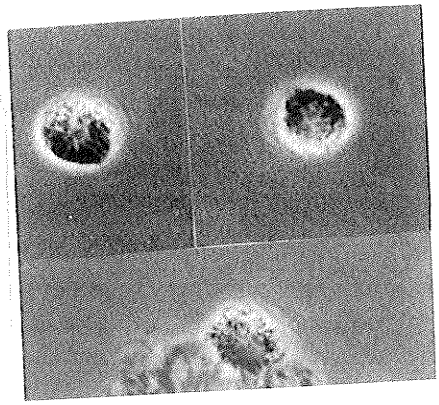
E



F



G



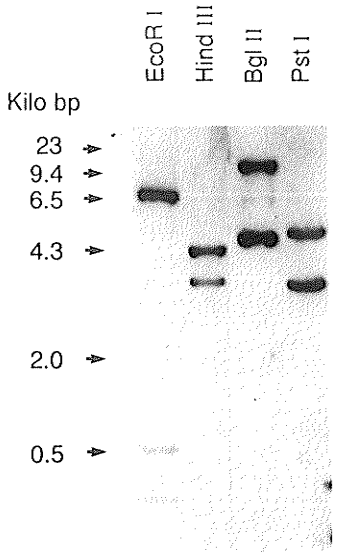
H

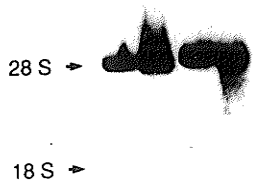
Fig. 21 : Southern Analysis.

Genomic DNA from U937 cells was isolated and subjected to digestion with the shown restriction enzyme. After gel electrophoresis and transfer to the Nytran membrane, the 164 bp probe of clone U.1.4 was used to detect the restriction pattern. The position of the molecular weight markers is shown on the left.

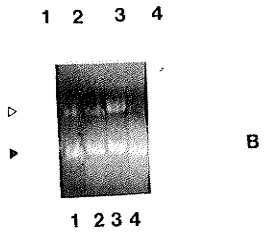
Fig. 22 : Northern analysis.

Total RNA was isolated by using a modification of the guanidinium thiocyanate method. 40 μg from the following cell lines : U937 (monocytes), RAMOS (B-cells), BOWES (malignant melanoma), and YT (Natural Killer) is shown in lanes 1-4. After electrophoresis in 1 % agarose-formaldehyde gel, RNA was transferred to Nytran membrane by using the capillary method. ^{32}P-labeled 164 bp probe of clone U.1.4 was used to detect a message of around 5 kb (Panel A). The position of the 28 S and the 18 S RNA is shown on the left. Panel B shows ethidium bromide staining and UV light transillumination of the RNA. The empty and closed arrowheads points to the position of the 28 S and the 18 S, respectively.


EcoR I
Hind III
Bgl II
Pst I
Kilo bp
23
9.4
6.5
4.3
2.0
0.5


28 S
18 S

A


1 2 3 4
1 2 3 4

B

chromosome 1q25-q31 (unpublished).

Northern analysis showed the presence of a major RNA species of approximately 5 kb in U937 as well as other cell lines Fig. 22. A similar band of approximately 5 kb was also detected in $CD3^+$ granulated lymphocytes (data not shown). We were not able to detect an RNA band of the expected size (1.404 kb) in any of the tested cells even after using poly A+ RNA and exposure for extended periods of time (data not shown).

9. Isolation of a homologous cDNA clone from the Jurkat T cell line

From the above mentioned results, several pieces of evidence suggested to us that the 5' end of the leukophysin gene is still lacking. These included the difference in size between clone U.3.20 and the RNA band detected in Northern analysis (Fig. 22)., the presence of a potential poly A adenylation signal and a poly A rich sequence suggesting the 3' end of the gene (Fig. 11), and the ability of the anti-peptide Ab to recognize proteins with m.w. higher than 28 kDa in U937 postnuclear lysate (Fig. 17).

In an attempt to clone the 5' end of the leukophysin gene, we tried to use primer extension on the U937 library as well as U937 mRNA. However, no further sequence could be isolated (data not shown). We then used the full size insert of U.3.20 clone to screen a λgt11 library from the Jurkat T cell line. This library was chosen because it contained larger inserts compared to the U937 library. A single positive clone with an 804 bp insert, clone J.11, was identified and purified after several rounds of screening. As shown in Fig. 23 the nucleotide sequence of clone J.11 is identical to that of clone U.3.20 at the 5' end. However, the two clones differ in that clone J.11 has a 104 bp insert that is deleted in clone U.3.20. (deletion starts after position 349 in clone U.3.20).

Analysis of clone J.11 showed that it has an ORF extending throughout the its entire length from the 5' end to the 3' end (nucleotide 2 to 802) that codes for

Fig. 23 : Alignment of the nucleotide sequences of clones U.3.20 and J.11.

Nucleotide sequence comparison of clone U.3.20 and clone J.11 was done by using *Nucaln* program (NIH molecular biology package). Identities are depicted by :, whereas gaps are depicted by -.

```
                                10        20        30        40
J.11                    TGCTACCTGCTTTCCAGAGCCTTTCATCAATGAAGGAAAGCGGCTGGGC
                        ::::::::::::::::::::::::::::::::::::::::::::::::::
U320    AGCATTGCTGCTGCTACCTGCTTTCCAGAGCCTTTCATCAATGAAGGAAAGCGGCTGGGC
                10        20        30        40        50        60

      50        60        70        80        90        100
J.11    TATATCCATCGAAATTTTGCTGGAAACAGATTTTCTGATCACGTAGCCCTTTTATCAGTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320    TATATCCATCGAAATTTTGCTGGAAACAGATTTTCTGATCACGTAGCCCTTTTATCAGTA
                70        80        90        100   .   110       120

     110       120       130       140       150       160
J.11    TTCCAAGCCTGGGATGATGCTAGAATGGGTGGAGAAGAAGCAGAGATACGTTTTTGTGAG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320    TTCCAAGCCTGGGATGATGCTAGAATGGGTGGAGAAGAAGCAGAGATACGTTTTTGTGAG
              130       140       150       160       170       180

     170       180       190       200       210       220
J.11    CACAAAAGACTTAATATGGCTACACTAAGAATGACCTGGGAAGCCAAAGTTCAGCTCAAA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320    CACAAAAGACTTAATATGGCTACACTAAGAATGACCTGGGAAGCCAAAGTTCAGCTCAAA
              190       200       210       220       230       240

     230       240       250       260       270       280
J.11    GAGATTTTGATTAATTCTGGGTTTCCAGAAGATTGTTTGTTGACACAAGTGTTTACTAAC
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320    GAGATTTTGATTAATTCTGGGTTTCCAGAAGATTGTTTGTTGACACAAGTGTTTACTAAC
              250       260       270       280       290       300

     290       300       310       320       330       340
J.11    ACTGGACCAGATAATAATTTGGATGTTGTTATCTCCCTCCTGGCCTTTGGTGTGTACCCC
        ::::::::::::::::::::::::::::::::::::::::::::::::::
U320    ACTGGACCAGATAATAATTTGGATGTTGTTATCTCCCTCCTGGCCTTTG----------
              310       320       330       340

     350       360       370       380       390       400
J.11    AATGTATGCTATCATAAGGAAAAGAGGAAGATTCTCACCACTGAAGGGCGTAATGCACTT

U320    ----------------------------------------------------------


     410       420       430       440       450       460
J.11    ATCCACAAATCATCTGTTAATTGTCCTTTTAGTAGCCAAGACATGAAGTACCCATCTCCC
                                          :::::::::::::::::::::::::::
U320    ----------------------------------TAGCCAAGACATGAAGTACCCATCTCCC
                                        350       360       370

     470       480       490       500       510       520
J.11    TTCTTTGTATTTGGTGAAAAGATTCGAACTCGAGCCATCTCTGCTAAAGGCATGACTTTA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320    TTCTTTGTATTTGGTGAAAAGATTCGAACTCGAGCCATCTCTGCTAAAGGCATGACTTTA
        380       390       400       410       420       430
```

```
      530         540         550         560         570         580
J.11  GTCACCCCCCTGCAGTTGCTTCTCTTTGCCTCCAAGAAAGTCCAATCTGATGGGCAGATT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320  GTCACCCCCCTGCAGTTGCTTCTCTTTGCCTCCAAGAAAGTCCAATCTGATGGGCAGATT
      440         450         460         470         480         490

      590         600         610         620         630         640
J.11  GTGCTTGTAGATGACTGGATTAAACTGCAAATATCTCATGAAGCTGCTGCCTGTATCACT
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320  GTGCTTGTAGATGACTGGATTAAACTGCAAATATCTCATGAAGCTGCTGCCTGTATCACT
      500         510         520         530         540         550

      650         660         670         680         690         700
J.11  GGTCTCCGGGCAGCCATGGAGGCTTTGGTTGTTGAAGTAACCAAACAACCTGCTATCATC
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320  GGTCTCCGGGCAGCCATGGAGGCTTTGGTTGTTGAAGTAACCAAACAACCTGCTATCATC
      560         570         580         590         600         610

      710         720         730         740         750         760
J.11  AGCCAGTTGGACCCCGTAAATGAACGTATGCTGAACATGATCCGTCAGATCTCTAGACCC
      ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U320  AGCCAGTTGGACCCCGTAAATGAACGTATGCTGAACATGATCCGTCAGATCTCTAGACCC
      620         630         640         650         660         670

      770         780         790         800
J.11  TCAGCTGCTGGTATCAACCTTATGATTGGCAGTAC
      ::::::::::::::::::::::::::::::::::
U320  TCAGCTGCTGGTATCAACCTTATGATTGGCAGTAC
      680         690         700         710
```

Fig. 24 : Alignment of the deduced amino acid sequences of the longest ORF of clones U.3.20 and J.11.

Prtaln program (NIH molecular biology package) was used to align the deduced amino acid sequences of clone U.3.20 (ORF, 360-1064) and clone J.11 (ORF 2-802). Identities are depicted by :.

```
ATCFPEPFINEGKRLGYIHRNFAGNRFSDHVALLSVFQAWDDARMGGEEAEIRFCEHKRL J.11
         10        20        30        40        50

NMATLRMTWEAKVQLKEILINSGFPEDCLLTQVFTNTGPDNNLDVVISLLAFGVYPNVCY J.11
         70        80        90        100       110

                                    10        20
                                MKYPSPFFVFGEKIRTRAISAKGMTLVTPL U.3.20
                                ::::::::::::::::::::::::::::::
HKEKRKILTTEGRNALIHKSSVNCPFSSQDMKYPSPFFVFGEKIRTRAISAKGMTLVTPL J.11
         130       140       150       160       170

         40        50        60        70        80
QLLLFASKKVQSDGQIVLVDDWIKLQISHEAAACITGLRAAMEALVVEVTKQPAIISQLD U.3.20
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
QLLLFASKKVQSDGQIVLVDDWIKLQISHEAAACITGLRAAMEALVVEVTKQPAIISQLD J.11
         190       200       210       220       230

         100       110       120       130       140
PVNERMLNMIRQISRPSAAGINLMIGSTRYGDGPRPPKMARYDNGSGYRRGGSSYSGGGY U.3.20
:::::::::::::::::::::::::::
PVNERMLNMIRQISRPSAAGINLMIGS                                   J.11
         250       260

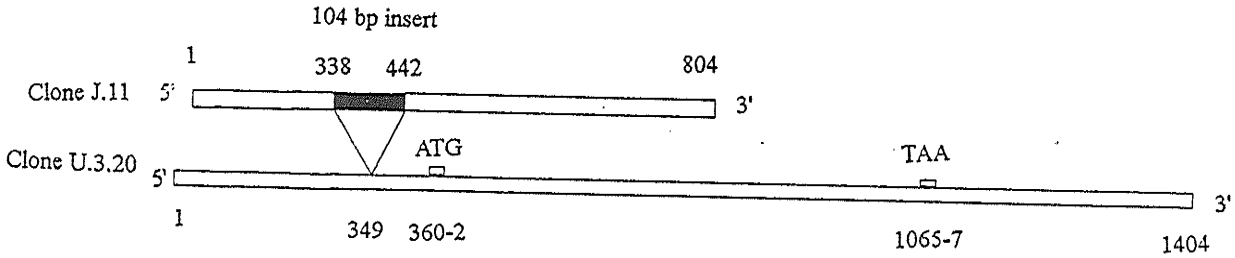
         160       170       180       190       200
GGGYSSGGYGSGGYGGSANSFRAGYGAGVGGGYRGVSRGGFRGNSGGDYRGPSGGYRGSG U.3.20



         220       230
GFQRGGGRGAYGTGYFGQGRGGGGY                                     U.3.20
```

Fig. 25 : The relationship between clones J.11 and U.3.20

The figure shows a simple diagram illustrating the relationship between clone J.11 (804 bp) and clone U.3.20 (1404 bp). The two clones are identical in the overlapping regions (open rectangle). The presence of the 104 bp insert (solid rectangle) in clone J.11 between positions 338 and 442 is shown. The corresponding site of the insert in clone U.3.20 is after position 349. The start and stop codons in U.3.20 are also shown.


104 bp insert
1
338
442
804
Clone J.11
5'
3'
ATG
TAA
Clone U.3.20
5'
3'
1
349
360-2
1065-7
1404

267 amino acids. The 3' end 117 amino acids of clone J.11 are identical to the 5' end 117 amino acids of clone U.3.20 (Fig. 24). This result suggested that deletion of the 104 bp insert from clone U.3.20 results in a frame shift and interruption of the coding region at the 5' end (creation of the stop codon after position 41 in clone U.3.20, which is 5' to 360 ATG of its ORF). The relationship between clone J.11 and clone U.3.20 is illustrated schematically in Fig. 25.

10. Evidence for the presence of an RNA species without the 104 bp insert

In order to test the presence of mRNAs that correspond to both clone U.3.20 and clone J.11, we used reverse transcriptase-PCR to amplify the poly A^+ RNA of U937 cells. A sense primer (5' ATGACCTGGGAAGCCAAAGT 3', position 211-320 of clone U.3.20) was used for first-strand cDNA synthesis. Two other primers were used in the PCR to amplify the cDNA ; the forward primer (5' CTCCCTCCTGGCCTTG 3', position 333-349, i.e. 5' to the deletion site) and the reverse primer (3' ACCTGTCCC TCTCCTC 5', position 1037-1053) of clone U.3.20, respectively. The primers were chosen to allow amplification of the available messages that contain the 104 bp insert. Fig. 26 is a schematic illustration of the primers used.

The PCR product contains two DNA bands detected by ethidium bromide staining which hybridize with the 164 bp probe (Fig. 27 lane 1 and 2, respectively). Both bands were purified (Fig. 28). Sequence analysis of the 5' end showed that the larger fragment (824 bp) contained the 104 bp insert of clone J.11 whereas the smaller fragment (720 bp) lacked the insert and had a sequence identical to that of clone U.3.20.

Fig. 26 : Schematic representation of the primers used in RT-PCR

The figure shows the positions of the primers used in rt-pcr relative to clone U.3.20. The sense primer was used for first strand cDNA synthesis. The forward and reverse primers were used to amplify the cDNA. The positions of the 104 bp insert as well as the start and stop codons of U.3.20 ORF are also shown.

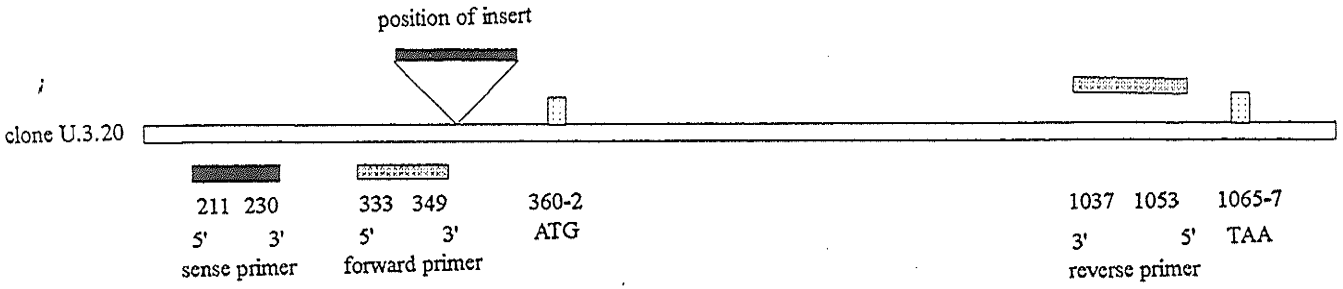
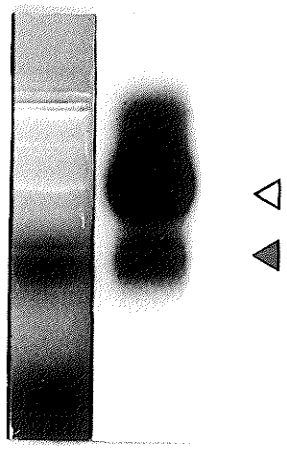

position of insert
clone U.3.20
211 230
5' 3'
sense primer
333 349
5' 3'
forward primer
360-2
ATG
1037 1053
3' 5'
reverse primer
1065-7
TAA

Fig. 27 : Reverse transcriptase-PCR & Southern analysis of U937 mRNA.

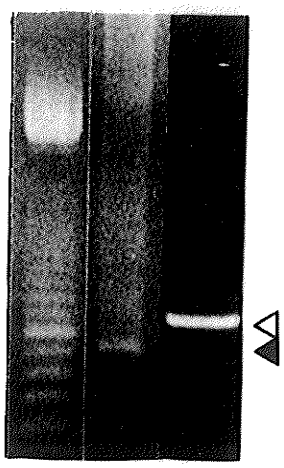
The sense primer 5' ATGACCTGGGAAGCCAAAGT 3' (position 211-230 of clone U.3.20) was used to synthesize first-strand cDNA from poly A^+ RNA of U937 as described in the materials and methods. Forward primer (5' CTCCCTCCTGGCCTTTG 3') and reverse primer (3' ACCTGTCCCTTCTCCTC 5') (position 333-349 and 1037-1053, respectively) were used to amplify the synthesized DNA in PCR. Lane 1 shows ethidium bromide staining of the PCR product after electrophoresis through a 4% agarose gel. Lane 2 shows Southern analysis by using the 164 bp probe of clone U.1.4 Two bands are shown to hybridize with the probe. The empty arrowhead points to the upper band whereas the solid arrowhead points to the lower band.

Fig. 28 : Purification of the RT-PCR products.

The two DNA bands of the PCR product that hybridize with the 164 probe (Fig. 23) were isolated from low melting agarose by cutting the gel band followed by purification through GENECLEAN Kit. Isolated bands were separated through 1% agarose. In the figure, ethidium bromide staining and UV light transillumination shows that the lower band (lane 2, solid arrowhead) is around 100 bp smaller than the upper band (lane 3, empty arrowhead). The positions of the 100 bp DNA ladder is shown in lane 1.



1 2



1 2 3

11. Clone U.3.20 is almost identical to a newly described human RNA helicase

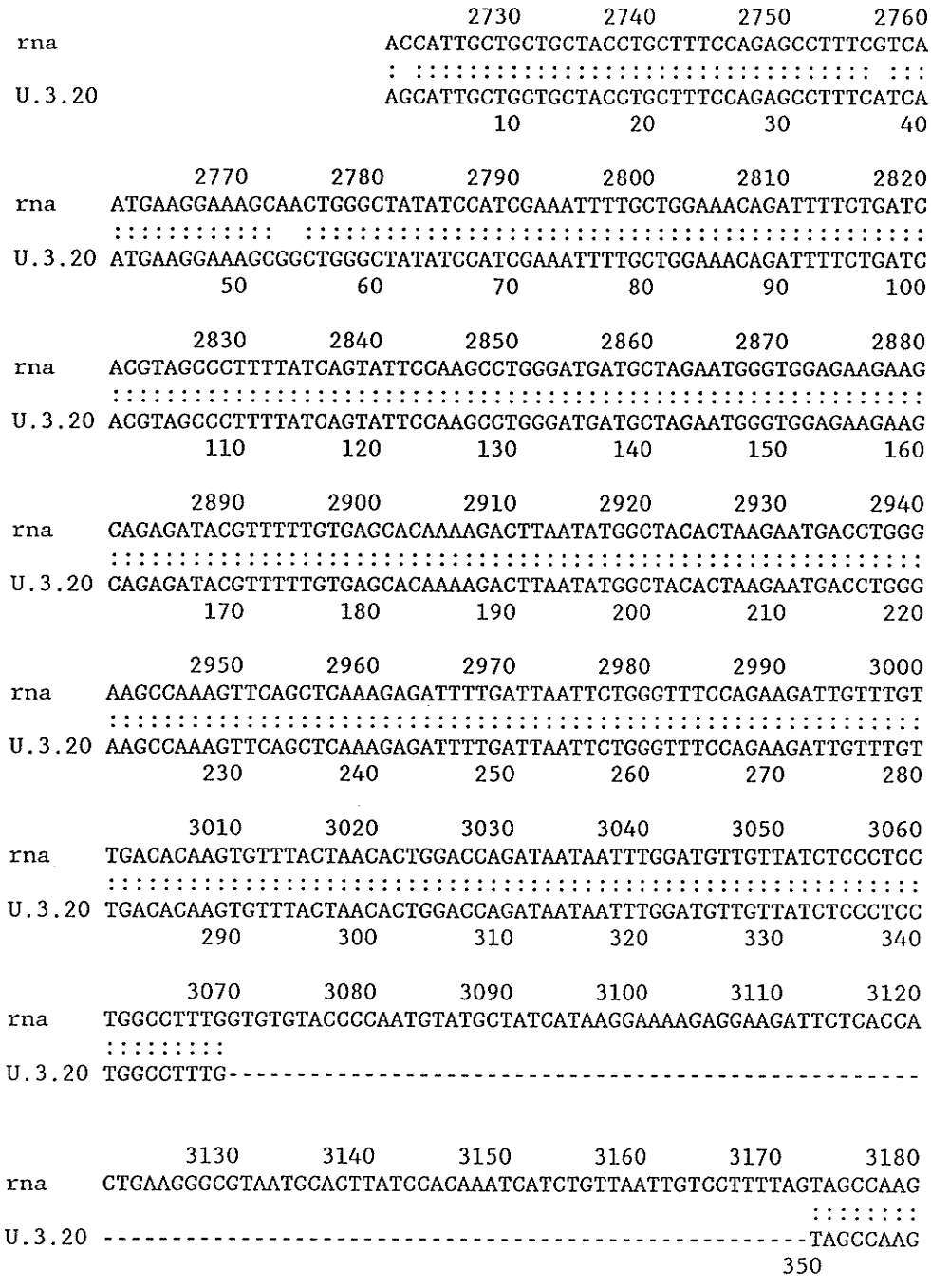
The deduced amino acid sequence of clone J.11 and U.3.20 showed significant homology to the maleless protein of the fruit fly. The degree of homology was determined by the calculating the *Z* value according to the *rdf2* program of the Fasta computer package (Pearson and Lipman, 1988). When compared against the maleless protein, clone J.11 and U.3.20 had Z values of 77.22 and 20.41, respectively (Z values above 6 are considered to represent significant homology) The homologous area was present in carboxy terminus region of the maleless protein which is rich in glycine and tyrosine residues. The maleless protein, which contains conserved sequences that define a family of helicases, is associated with the X chromosome in males and is suggested to be a direct regulator of the dosage compensation of X-linked genes in *Drosophila melanogaster* males (Kuroda *et. al.*, 1991)

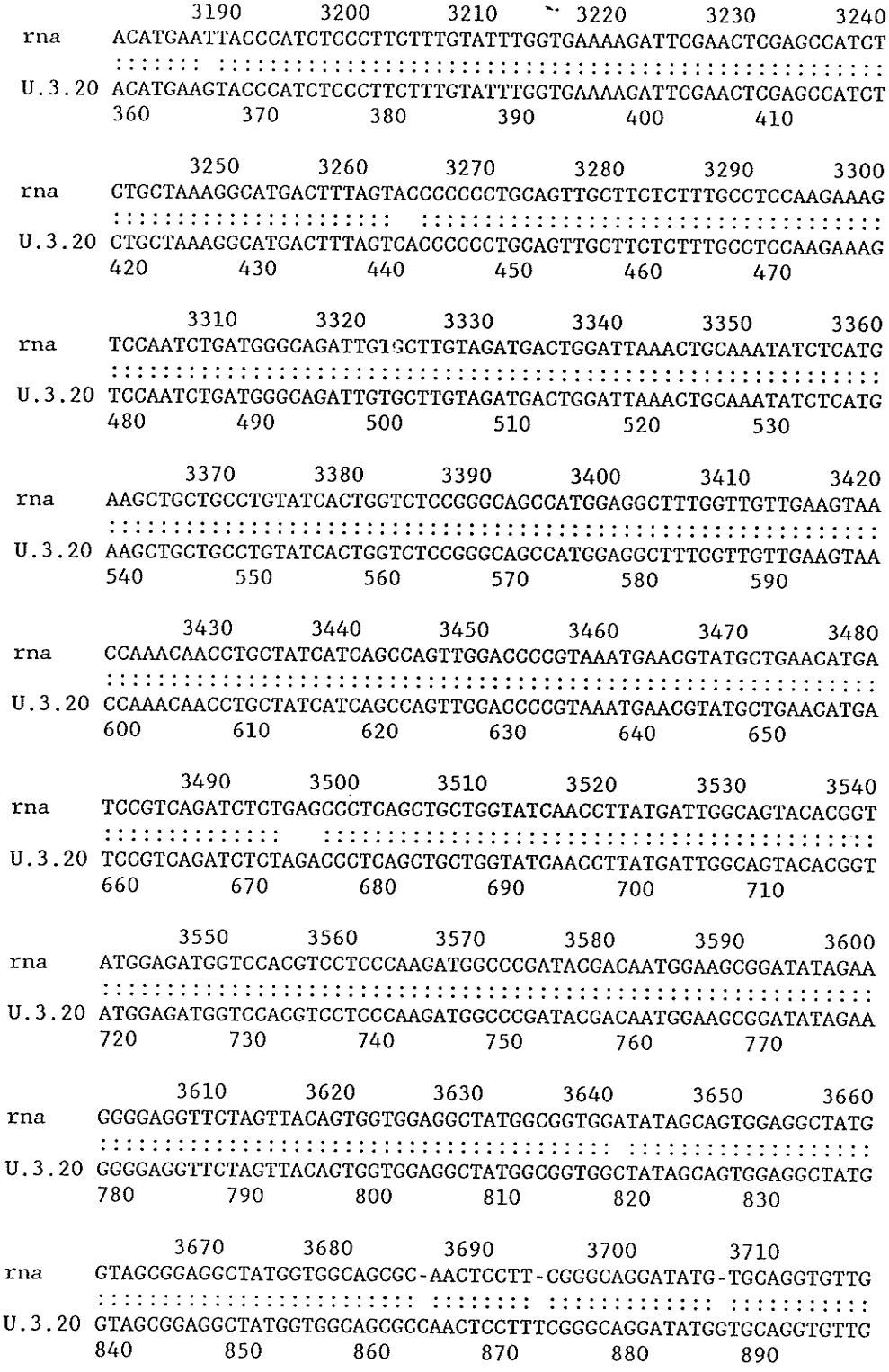
A human cDNA clone coding for a new member of DEAH family of RNA binding proteins with helicase activity, RNA helicase A, was isolated from HeLa cells and shown to be highly homologous to the dorsophila maleless protein (Lee and Hurwitz, 1993). The 4.2 kb cDNA was isolated by using polyclonal antiserum directed against the purified protein. Fig. 29 shows alignment of the nucleotide sequence of clone U.3.20 and 3' end of the nucleotide sequence of human RNA helicase A. As shown in the figure, except for few base mismatches, some deletions, and the presence of the 104 bp insert, the two nucleotide sequences are identical.

RNA helicase A cDNA codes for a protein with 1279 amino acids which shows 49% identity and 85% similarity to the maleless protein of the fruit fly. Fig. 30 shows amino acid sequence alignments of RNA helicase A, the maleless protein of the fruit fly and the deduced amino acid sequence of clone U.3.20.

Fig. 29 : Alignment of nucleotide sequences of clone U.3.20 with the 3' end of the nucleotide sequence of human RNA helicase A.

Align program of the Birch package was used to align the nucleotide sequence of clone U.3.20 with the nucleotide sequence of the homologous region of the cDNA of human RNA helicase A. Identities are depicted as :, whereas gaps are depicted as -.


2730 2740 2750 2760
rna ACCATTGCTGCTGCTACCTGCTTTCCAGAGCCTTTCGTCA
U.3.20 AGCATTGCTGCTGCTACCTGCTTTCCAGAGCCTTTCATCA
10 20 30 40
2770 2780 2790 2800 2810 2820
rna ATGAAGGAAAGCAACTGGGCTATATCCATCGAAATTTTGCTGGAAACAGATTTTCTGATC
U.3.20 ATGAAGGAAAGCGGCTGGGCTATATCCATCGAAATTTTGCTGGAAACAGATTTTCTGATC
50 60 70 80 90 100
2830 2840 2850 2860 2870 2880
rna ACGTAGCCCTTTTATCAGTATTCCAAGCCTGGGATGATGCTAGAATGGGTGGAGAAGAAG
U.3.20 ACGTAGCCCTTTTATCAGTATTCCAAGCCTGGGATGATGCTAGAATGGGTGGAGAAGAAG
110 120 130 140 150 160
2890 2900 2910 2920 2930 2940
rna CAGAGATACGTTTTTGTGAGCACAAAAGACTTAATATGGCTACACTAAGAATGACCTGGG
U.3.20 CAGAGATACGTTTTTGTGAGCACAAAAGACTTAATATGGCTACACTAAGAATGACCTGGG
170 180 190 200 210 220
2950 2960 2970 2980 2990 3000
rna AAGCCAAAGTTCAGCTCAAAGAGATTTTGATTAATTCTGGGTTTCCAGAAGATTGTTTGT
U.3.20 AAGCCAAAGTTCAGCTCAAAGAGATTTTGATTAATTCTGGGTTTCCAGAAGATTGTTTGT
230 240 250 260 270 280
3010 3020 3030 3040 3050 3060
rna TGACACAAGTGTTTACTAACACTGGACCAGATAATAATTTGGATGTTGTTATCTCCCTCC
U.3.20 TGACACAAGTGTTTACTAACACTGGACCAGATAATAATTTGGATGTTGTTATCTCCCTCC
290 300 310 320 330 340
3070 3080 3090 3100 3110 3120
rna TGGCCTTTGGTGTGTACCCCAATGTATGCTATCATAAGGAAAAGAGGAAGATTCTCACCA
U.3.20 TGGCCTTTG----------
3130 3140 3150 3160 3170 3180
rna CTGAAGGGCGTAATGCACTTATCCACAAATCATCTGTTAATTGTCGTTTTAGTAGCCAAG
U.3.20 ----------TAGCCAAG
350


3190 3200 3210 3220 3230 3240
rna ACATGAATTACCCATCTCCCTTCTTTGTATTTGGTGAAAAGATTCGAACTCGAGCCATCT
U.3.20 ACATGAAGTACCCATCTCCCTTCTTTGTATTTGGTGAAAAGATTCGAACTCGAGCCATCT
360 370 380 390 400 410
3250 3260 3270 3280 3290 3300
rna CTGCTAAAGGCATGACTTTAGTACCCCCCCTGCAGTTGCTTCTCTTTGCCTCCAAGAAAG
U.3.20 CTGCTAAAGGCATGACTTTAGTCACCCCCCTGCAGTTGCTTCTCTTTGCCTCCAAGAAAG
420 430 440 450 460 470
3310 3320 3330 3340 3350 3360
rna TCCAATCTGATGGGCAGATTGTGCTTGTAGATGACTGGATTAAACTGCAAATATCTCATG
U.3.20 TCCAATCTGATGGGCAGATTGTGCTTGTAGATGACTGGATTAAACTGCAAATATCTCATG
480 490 500 510 520 530
3370 3380 3390 3400 3410 3420
rna AAGCTGCTGCCTGTATCACTGGTCTCCGGGCAGCCATGGAGGCTTTGGTTGTTGAAGTAA
U.3.20 AAGCTGCTGCCTGTATCACTGGTCTCCGGGCAGCCATGGAGGCTTTGGTTGTTGAAGTAA
540 550 560 570 580 590
3430 3440 3450 3460 3470 3480
rna CCAAACAACCTGCTATCATCAGCCAGTTGGACCCCGTAAATGAACGTATGCTGAACATGA
U.3.20 CCAAACAACCTGCTATCATCAGCCAGTTGGACCCCGTAAATGAACGTATGCTGAACATGA
600 610 620 630 640 650
3490 3500 3510 3520 3530 3540
rna TCCGTCAGATCTCTGAGCCCTCAGCTGCTGGTATCAACCTTATGATTGGCAGTACACGGT
U.3.20 TCCGTCAGATCTCTAGACCCTCAGGTGCTGGTATCAACCTTATGATTGGCAGTACACGGT
660 670 680 690 700 710
3550 3560 3570 3580 3590 3600
rna ATGGAGATGGTCCACGTCCTCCCAAGATGGCCCGATACGACAATGGAAGCGGATATAGAA
U.3.20 ATGGAGATGGTCCACGTCCTCCCAAGATGGCCCGATACGACAATGGAAGCGGATATAGAA
720 730 740 750 760 770
3610 3620 3630 3640 3650 3660
rna GGGGAGGTTCTAGTTACAGTGGTGGAGGCTATGGCGGTGGATATAGCAGTGGAGGCTATG
U.3.20 GGGGAGGTTCTAGTTACAGTGGTGGAGGCTATGGCGGTGGCTATAGCAGTGGAGGCTATG
780 790 800 810 820 830
3670 3680 3690 3700 3710
rna GTAGCGGAGGCTATGGTGGCAGCGC-AACTCCTT-CGGGCAGGATATG-TGCAGGTGTTG
U.3.20 GTAGCGGAGGCTATGGTGGCAGCGCCAACTCCTTTCGGGCAGGATATGGTGCAGGTGTTG
840 850 860 870 880 890

```
        3720      3730       3740       3750       3760       3770
rna     GTGGAGGCTATAGAGGAGTTTCCCGAGGTGGCTTTAGAGGCAACTCTGGAGGAGACTACA
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U.3.20  GTGGAGGCTATAGAGGAGTTTCCCGAGGTGGCTTTAGAGGCAACTCTGGAGGAGACTACA
         900        910        920        930        940        950

        3780       3790       3800       3810       3820       3830
rna     GAGGGCCTAGTGGAGGCTACAGAGGATCTGGGGGATTCCAGCGAGGAGGTGGTAGGGGGG
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U.3.20  GAGGGCCTAGTGGAGGCTACAGAGGATCTGGGGGATTCCAGCGAGGAGGTGGTAGGGGGG
         960        970        980        990       1000       1010

        3840       3850       3860       3870       3880       3890
rna     CCTATGGAACTGGCTACTT-GGACATTGAAGAGGAGGTGGCGGCTAT-AAAACTTGGTTA
        :::::::::::::::::::: ::::: :::::::::::::::::::: ::::::::::::
U.3.20  CCTATGGAACTGGCTACTTTGGACAGGGAAGAGGAGGTGGCGGCTATTAAAACTTGGTTA
        1020       1030       1040       1050       1060       1070

        3900       3910       3920       3930       3940       3950
rna     TGTCAGTTC-TGTGTGTAGACAGTAAGGAAAAAAAAGGCATGCTATGTGTTACGTGTTTTT
        ::::::::: ::::::::::::::::::::::::::::::::::::::::::::::::::
U.3.20  TGTCAGTTCCTGTGTGTAGACAGTAAGGAAAAAAAAGGCATGCTATGTGTTACGTGTTTTT
        1080       1090       1100       1110       1120       1130

        3960       3970       3980       3990       4000       4010
rna     TCCAGTATGTTTATTTGCCACCAAAAAGTAAATGCATTTTCACCCATTCTGTGGTTCATT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U.3.20  TCCAGTATGTTTATTTGCCACCAAAAAGTAAATGCATTTTCACCCATTCTGTGGTTCATT
        1140       1150       1160       1170       1180       1190

        4020       4030       4040       4050       4060       4070
rna     GTAGTTTAAGGAAACCAAGCATATAGATGCATTAGTGATTTTGTTTATTTTATGTAAAAT
        :::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::
U.3.20  GTAGTTTAAGGAAACCAAGCATATAGATGCATTAGTGATTTTGTTTATATTATGTAAAAT
        1200       1210       1220       1230       1240       1250

        4080       4090       4100       4110       4120       4130
rna     ATAACGATCTTCTTTTAAAAATACCACAGTTTGTATTTTT-CTTTAAGGAGTAAAGATTT
        ::::::::::     :::::::::::::::::::::::::: :::::::::::::::::
U.3.20  ATAACGATCTC---TTAAAAATACCACAGTTTGTATTTTTTCTTTAAGGAGTAAAGATTT
        1260           1270       1280       1290       1300       1310

        4140       4150       4160       4170       4180       4190
rna     GCCTTTAAATAACTTGGTATTTTCCTGGCTTTCGTTTAATACAATAGAAAATAAAGTATT
        ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
U.3.20  GCCTTTAAATAACTTGGTATTTTCCTGGCTTTCGTTTAATACAATAGAAAATAAAGTATT
        1320       1330       1340       1350       1360       1370

        4200
rna     ACACGGAATTC------------------
        :::: :::
U.3.20  ACACCGAAAAAAAAAAAAAAAAAAAAAAA
        1380       1390       1400
```

Fig. 30 : Alignment of the amino acid sequences of clone U.3.20, RNA helicase A and maleless protein.

Clustal V multiple sequence alignment program of the Birch package was used to align the amino acid sequence of clone U.3.20 with the entire sequences of RNA helicase A and the maleless protein. Identities, gaps and conserved amino acid substitutions are depicted as (*), (-), and (.), respectively. Note the high homology between RNA helicase A and the maleless protein throughout their entire sequence.

helicase	MGDVKNFLYAWCGKRKMTPTYEIRAVGNKNRQKFMCEVQVEGYNYTGMGNSTNKKDAQSN
MLE	M-DIKSFLYQFCAKSQIEPKFDIRQTGPKNRQRFLCEVRVEPNTYIGVGNSTNKKDAEKN
helicase	AARDFVNYLVRINEIKSEEVPAFGVASPPPLTDTPDTTANAEGDLPTHHGGPLLHILALK
MLE	ACRDFVNYLVRVGKLNTNDVP----ADAGASGGGPRTGLEGAG----MAGGSGQQKRVFD
helicase	AENNSEVGASGYGVPGPTWDRGANLKDYYSRKEEQEVQATLESEEVDLNAGLHGNWTLEN
MLE	GQSGPQDLGEAY--RPLNHDGGDGGNRYSVIDRIQEQRDMNEAEAFDVNAAIHGNWTIEN
helicase	AKARLIQYFQKEKIQGEYKYTQVGPDHNRSFIAEMTIYIKQLGRRIFAREHGSNKKLAAQ
MLE	AKERLNIYKQTNNIRDDYKYTPVGPEHARSFLAELSIYVPALNRTVTARESGSNKKSASK
helicase	SCALSLVRQLYHLGVVEAYTGLTKKKEGETVEPYKVNLSQDLEHQLQNIIQELNLEILPP
MLE	SCALSLVRQLFHLNVIEPFSGTLKKKKDEQLKPYPVKLSPNLINKIDEVIKGLDLPVVNP
helicase	P------EDPSVPVALNIGKLAQFEP-SQRQNQVGVVPWSPPQSNWNPWTSSNIDEGPLA
MLE	RNIKIELDGPPIPLIVNLSRIDSSQQDGEKRQESSVIPWAPPQANWNTWHACNIDEGELA
helicase	FATPEQISMDLKNELMYQLEQDHDLQAILQERELLPVKKFESEILEAISQNSVVIIRGAT
MLE	TTSIDDLSMDYERSLRDRRQNDNEYRQFLEFREKLPIAAMRSEILTAINDNPVVIIRGNT
helicase	GCGKTTQVPQFILDDFIQNDRAAECNIVVTQPRRISAVSVAERVAFERGEEPGKSCGYSV
MLE	GCGKTTQIAQYILDDYICSGQGGYANIYVTQPRRISAISVAERVARERCEQLGDTVGYSV
helicase	RFESVLPRPHASIMFCTVGVLLRKLEAGIRGISHVIVDEIHERDINTSFLLVVLRDVVQA
MLE	RFESVFPRPYGAILFCTVGVLLRKLEAGLRGVSHIIVDEIHERDVNSDFLLVILRDMVDT
helicase	YPEVRIVFMSATIDTSMFCEYFFNCPSLKLW-RTYPVQEYFLEDCIQMTHFVPPPKDKKK
MLE	YPDLHVILMSATIDTTKFSKYFGICPVLEVPGRAFPVQQFFLEDIIQMTDFVPSAESRRK
helicase	-KDKDDDGG------EDDDANCNLICGDEYGPETRLSMSQLNEKETPFELIEALLKYIET
MLE	PKEVEDEEQLLSEDKDEAEINYNKVCEDKYSQKTRNAMAMLSESDVSFELLEALLMHIKS
helicase	LNVPGAVLVFLPGWNLIYTMQKHLEMNPHFG-SHRYQILPLHSQIPREEQRKVFDPVPVG
MLE	KNIPGAILVFLPGWNLIFALMKFLQNTNIFGDTSQYQILPCHSQIPRDEQRKVFEPVPEG
helicase	VTKVILSTNIAETSITINDVVYVIDSCKQKVKLFTAHNNMTNYSTVWASKTNLEQRKGRA
MLE	VTKIILSTNIAETSITIDDIVFVIDICKARMKLFTSHNNLTSYATVWASKTNLEQRKGRA
helicase	GRSTAGFCFHLCSRARFERLETHMTPEMFRTPLHEIALSIKLLRLGGIGQFLAKAIEPPP
MLE	GRVRPGFCFTLCSRARFQALEDNLTPEMFRTPLHEMALTIKLLRLGSIHHFLSKALEPPP

```
helicase        LDAVIEAEHTLRELDALDANDELTPLGRILAKLPIEPRFGKMMIMGCIFYVGDAICTIAA
MLE             VDAVIEAEVLLREMRCLDANDELTPLGRLLARLPIEPRLGKMMVLGAVFGCADLMAIMAS

helicase        -ATCFPEPFVNE--GKQLGYIHRNFAGNRFSDHVALLSVFQAWDDARMGGEEAEIRFCEH
MLE             YSSTFSEVFSLDIGQRRLANHQKALSGTKCSDHVAMIVASQMWRREKQRGEHMEARFCDW

helicase        KRLNMATLRMTWEAKVQLKEILINSGFPEDCLLTQVFTN--TGPDNNLDVVISLLAFGVY
MLE             KGLQMSTMNVIWDAKQQLLDLLQQAGFPEECMISHEVDERIDGDDPVLDVSLALLCLGLY

U.3.20                                                  MKYPSPFFVFGEKIRTRAISAKGMT
helicase        PNVCYHKEKRKILTTEGRNALIHKSSVNCPFSSQDMNYPSPFFVFGEKIRTRAISAKGMT
MLE             PNICVHKEKRKVLTTESKAALLHKTSVNC--SNLAVTFPYPFFVFGEKIRTRAVSCKQLS

U.3.20          LVTPLQLLLFASKKVQ-SDGQIVLVDDWIKLQISHEAAACITGLRAAMEALVVEVTKQPA
helicase        LVPPLQLLLFASKKVQ-SDGQIVLVDDWIKLQISHEAAACITGLRAAMEALVVEVTKQPA
MLE             MVSPLQVILFGSRKIDLAANNIVRVDNWLNFDIEPELAAKIGALKPALEDLITVACDNPS

U.3.20          IISQLDPVNERMLNMIRQISRPSAAGINLMIGST-------RYGDGPRPPKMARYDNG--
helicase        IISQLDPVNERMLNMIRQISEPSAAGINLMIGST-------RYGDGPRPPKMARYDNG--
MLE             DILRLEEPYAQLVKVVKDLCVKSAGDFGLQRESGILPHQSRQFSDGGGPPKRGRFETGRF

U.3.20          --SGY-RRGGSSYSGGGYGGGYSSGGYGS--GGYGGSANSFRAGYGAGVGGGYRGVSRGG
helicase        --SGY-RRGGSSYSGGGYGGGYSSGGYGS--GGYGGSATP-SGRICAGVGGGYRGVSRGG
MLE             TNSSFGRRGNGRTFGGGYGN--NGGGYGNNGGGYGNIGGGY-----GNNAGGY--GNNGG

U.3.20          FRGNSGGDYRGPSGGY-RGSGGFQRGGGRGAYGTGYFGQGRGGG----GY--------
helicase        FRGNSGGDYRGPSGGY-RGSGGFQRGGGRGAYGTGYLDIEEEVAAIKLGYVSSV-CRQ
MLE             Y-GNNGGGYRNNGGGYGNNGGGY--GNNRGGFGDSFESNRGSGGGFRNGDQGGRWGNF
```

Fig. 31 is a schematic representation of the different regions of the three proteins.

Fig. 32 shows the homologous region between clone U.3.20 and the carboxy terminal region of the RNA helicase A. The following is a summary of the differences in the nucleotide sequence in the coding region and the resulting differences in the amino acid sequence :

U.3.20				RNA helicase			
Nuc	position	AA	position	Nuc	position	AA	position
G	365	K	2	T	3188	N	1036
CA	440-1	T	28	AC	3263-4	P	1062
AGA	672-4	R	105	GAG	3495-7	E	1139

(nucleotide substitutions leading to change in amino acid sequence)

C	863	(NSFRAGYG)	-	(TPSGRIC)
T	872	(169-176)	-	(1203-1209)
G	886		-	

(deletion of three nucleotides in the helicase resulting in 7 amino acids compared to 8 for U.3.20 and a frame shift)

T	1037		-	
GG	1043-4		TT	3862-3
T	1065	Creates stop	-	

(an extra base, T at 1037 in U.3.20 leads to frame shift, another extra base T at 1065 in U.3.20 create stop codon resulting in different C- termini, 10 amino acids in U.3.20 and 21 amino acids in the helicase)

12. Immunofluorescence analysis using D545, anti-peptide, and anti-RNA helicase A antibodies

In order to determine the relationship between the epitopes recognized by D545, anti-peptide and anti-RNA helicase A antibodies, immunofluorescence analysis in BOWES cell line was done. This malignant melanoma cell line has the

Fig. 31 : Schematic representation of the alignment the maleless protein, RNA helicase A and leukophysin.

The maleless protein (1293 amino acids) shows 49% identity and 85% similarity with RNA helicase A (1279 amino acids). The region containing the DEAH family conserved motifs is shown as solid rectangles. Leukophysin (235 amino acids) shows 91% identity and 94% similarity with carboxy terminal region of RNA helicase A (starting from amino acid 1035). The region containing the glycine-rich repetitive motif the three protein is shown in dotted rectangels.

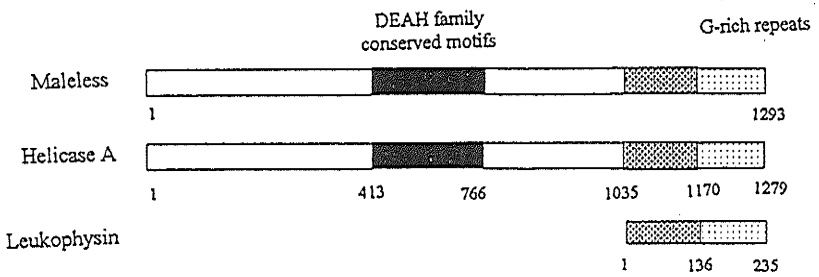

DEAH family
conserved motifs
G-rich repeats
Maleless
1
1293
Helicase A
1
413
766
1035
1170
1279
Leukophysin
1
136
235

Fig. 32 : Alignment of the amino acid sequences of clone U.3.20 and RNA helicase A.

Clustal V multiple sequence alignment program of the Birch package was used to align the amino acid sequence of clone U.3.20 with the homologous region of the amino acid sequence of human RNA helicase A. Identities, gaps, and conserved amino acid substitutions are depicted as (*), (-), and (.) respectively.

```
U.3.20.   MKYPSPFFVF GEKIRTRAIS AKGMTLVTPL QLLLFASKKV QSDGQIVLVD 50

helicase MNYPSPFFVF GEKIRTRAIS AKGMTLVPPL QLLLFASKKV QSDGQIVLVD 1084
         *.******** ********** ******* ** ********** **********

U.3.20.   DWIKLQISHE AAACITGLRA AMEALVVEVT KQPAIISQLD PVNERMLNMI 100

helicase DWIKLQISHE AAACITGLRA AMEALVVEVT KQPAIISQLD PVNERMLNMI 1134
         ********** ********** ********** ********** **********

U.3.20.   RQISRPSAAG INLMIGSTRY GDGPRPPKMA RYDNGSGYRR GGSSYSGGGY 150

helicase RQISEPSAAG INLMIGSTRY GDGPRPPKMA RYDNGSGYRR GGSSYSGGGY 1184
         **** ***** ********** ********** ********** **********

U.3.20.   GGGYSSGGYG SGGYGGSANS FRAGYGAGVG GGYRGVSRGG FRGNSGGDYR 200

helicase GGGYSSGGYG SGGYGGSATP -SGRICAGVG GGYRGVSRGG FRGNSGGDYR 1233
         ********** ******** .   .   **** ********** **********

U.3.20.   GPSGGYRGSG GFQRGGGRGA YGTGYLGQGR GGG----GY- ------     235

helicase GPSGGYRGSG GFQRGGGRGA YGTGYFDIEE EVAAIKLGYV SSVCRQ     1279
         ********** ********** *****..      .    **
```

advantage of having large nuclei with prominent nucleoli as well as numerous cytoplasmic granules. D545 stained the cytoplasmic granules with no detectable staining in the nucleus (panel A, Fig. 33). On the other hand, both the anti-peptide and the anti-helicase Abs stained well-defined nuclear structures (panel, B and C, respectively).

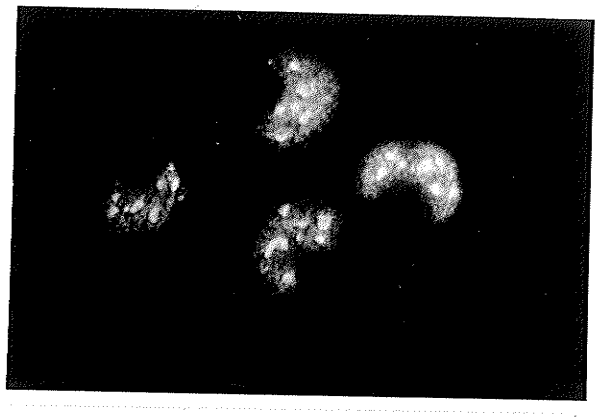
Fig. 33 : Immunofluorescence of the melanoma cell line using different Abs.

Malignant melanoma cell line BOWES was allowed to adhere to sterile glass slides in the tissue culture dish. After washing three times in TBS, cell were fixed by immersion in a solution of 50% acetone-50% methanol at -20°C for 10 minutes. After washing three time in TBS-0.1% BSA, the primary Abs (D545 10 μg/ml, anti-peptide Ab 1 in 100, and anti-helicase Ab 1 in 100) was added and allowed to react with the cells overnight at room temperature. After washing as above, the fluorescenated secondary antibody was added for one hour. Panel A shows the cytoplasmic punctate staining pattern detected by D545. Panel B shows nuclear staining by the anti-peptide Ab, whereas panel C shows the staining pattern observed by using the anti-helicase Ab.

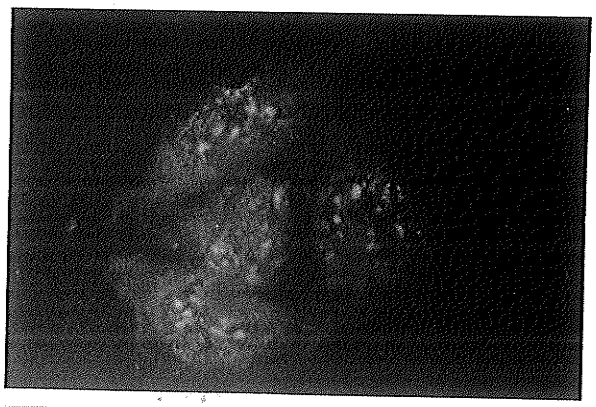
A



B



C



DISCUSSION

In this study we have shown that anti-PDG MAbs identify leukophysin as a 28 kDa granule membrane protein. The antibodies were used to isolate a cDNA which appears to code for leukophysin. This cDNA is almost identical to part of the gene for an RNA helicase.

I. Identification of leukophysin as a granule membrane protein

The initial immunofluorescence results showing that the anti-PDG MAb D545 stains IL-2-stimulated $CD3^+$ cells in a punctate manner suggest an association of the reactive epitope with cytoplasmic granules. During exocytosis, regulated cytoplasmic granules migrate towards the plasma membrane and then fuse with it in such a way that the granule membrane becomes part of the plasma membrane. The granule contents are then released extracellularly (Burgess and Kelly, 1987). Stimulation with PMA and A23187 releases the cytotoxic granule-associated serine esterases. Measuring the BLT-esterase activity of granzyme A has been used as a test to demonstrate granule exocytosis in CTL (Sitkovsky, 1988). The result of increased surface staining with D545 in granulated $CD3^+$ cells following granule exocytosis suggests that granule exocytosis results in exposure of the reactive epitope on the cell surface. This pattern can be seen if the reactive epitope is part of a granule membrane protein which becomes part of the plasma membrane after exocytosis. Similar surface redistribution of granulophysin stained with D545 has been observed after thrombin activation of the platelets (Gerrard et. al., 1991). The low level of D545 staining in unstimulated cells (Fig. 6) might represent plasma membrane association of leukophysin as a result of spontaneous degranulation or the plasma membrane-associated phase of leukophysin during its recycling.

To examine the granule association question more directly, granulated $CD3^+$ cells were subjected to subcellular fractionation. Examination of murine

CTL (Persechini *et. al.*, 1989) and rat NK cells (Henkart *et. al.*, 1984) has demonstrated two populations of lytic granules that differ in density but not in morphology. The results reported here, suggest that subcellular fractions of IL-2-stimulated $CD3^+$ cells also contained two populations of granules as well as two major peaks of D545 reactivity. The first peak of D545 reactivity coincided with the first peak of BLT-esterase activity which probably corresponds to the dense granules. The second peak of D545 reactivity (fractions 13-18 of Fig. 7) preceded and partially overlapped with the second peak of BLT-esterase activity. Murine CTL have been shown to contain a cytoplasmic pool of BLT-esterase activity separate from the granules (Persechini *et. al.*, 1989). Thus, the second major D545 reactivity peak might represent protein associated with granules with lighter density, soluble cytoplasmic protein or cross reactivity with other proteins. However, the finding that the affinity purified proteins from both peaks of $CD3^+$ cells show similar m.w. of approximately 28 kDa would suggest the presence of a single protein carrying the D545 reactive epitope.

Granulophysin has been recently shown to be identical to the lysosomal membrane glycoprotein CD63 (Nishibori *et. al.*, 1993). In light of this result and the finding that CD63 is present in the membrane of CTL granules (Peters *et. al.*, 1991), the possibility that the lymphocyte protein reacting with D545 MAb in immunofluorescence and FACS studies is CD63, rather than leukophysin, can not be excluded at present. This issue can be resolved after repeating the experiments using antibodies specific to leukophysin. However, by using the anti-peptide antibody directed against the carboxy terminal 17 amino acids of leukophysin, a punctate cytoplasmic staining pattern similar to that obtained by D545 was detected in $CD3^+$ lymphocytes (Fig. 20, panel G and H). Moreover, both antibodies detect similar bands in the membrane fraction of both $CD3^+$ lymphocytes (Fig. 18) and RNK cells (Fig. 19).

Leukophysin appears to have two regions of sufficient length and hydrophobicity to qualify as potential transmembrane regions (Fig. 16). These regions also have α-helical secondary structure (Fig. 15) which is the form required to span the lipid bilayer (Brandon and Tooze, 1991). If leukophysin spans the granule membrane twice, both the carboxy and amino termini are expected to face either the cytoplasmic or intragranular sides.

II. The relationship between clone U.3.20, leukophysin, and RNA helicase A

II. 1. Clone U.3.20 and leukophysin

The longest ORF of clone U.3.20 codes for a protein with a calculated m.w. of 24.309 kDa with a single potential N-glycosylation site. If this glycosylation site is used, the m.w. of the protein becomes close to the 28 kDa m.w. of leukophysin observed by SDS-PAGE. The evidence presented here, indicating that clone U.3.20 codes for leukophysin, is provided by antibody studies. Two Abs were used including the anti-granulophysin MAb D545 and the anti-peptide Ab directed against the carboxy terminal 17 amino acids of deduced amino acid sequence of clone U.3.20. On one hand, D545 MAb which was used to identify the 28 kDa leukophysin, has been shown to react with clone U.3.20 fusion protein. On the other hand, the anti-peptide Ab reacts with the same 28 kDa lymphocyte granule membrane protein that reacts with D545 MAb. Moreover, in IF both Abs react with cytoplasmic granules in $CD3^+$ lymphocytes. However, these results provide indirect evidence which rely on the specificity of the antibodies used (see below). This evidence suggests, rather than proves, that clone U.3.20 codes for leukophysin. Direct evidence, however, will await the purification of the 28 kDa leukophysin followed by comparing its amino acid sequence to that of clone U.3.20.

II. 2. Ab specificity

D545 MAb appears to react with at least three proteins, CD63/ granulophysin in platelet dense granules (Nishibori *et. al.*, 1993), synaptophysin of bovine brain (Gerrard et. al., 1991) and the 28 kDa leukophysin (Abdelhaleem *et. al.*, 1991). By using sequence comparison computer programs, we were not able to detect regions of significant homolgoy between the primary amino acid sequences of clone U.3.20, CD63, and synaptophysin (data not shown). This raises the possibility that D545 MAb might react with a conformation-dependent epitope.

In light of the fact that clone U.3.20 is almost identical to the newly described RNA helicase A, it might be argued that D545 MAb cross reacts with RNA helicase A and that clone U.3.20 was isolated based on this cross reactivity. The reactivity of D545 MAb against purified RNA helicase A was not tested. However, RNA helicase A is a nuclear protein (Lee and Hurwitz, 1992) and anti-RNA helicase A antibody stains only the nucleus (Fig. 33). D545 did not stain any nuclear structures in the cells examined so far. This suggests that D545 identifies a unique U.3.20 epitope which is absent in RNA helicase A. The candidate region is the C-terminus end which is different among the two proteins. This might explain why clone U.3.20 rather than RNA helicase A cDNA was isolated by immunoscreening the U937 library despite the marked homology between the two clones.

It is currently unknown whether the anti-peptide Ab cross reacts with RNA helicase A or not. Direct testing of this Ab against purified RNA helicase A protein is required to answer this question. However, of the 17 amino acids of clone U.3.20 against which the anti-peptide Ab is generated, 8 are unique to leukophysin. In the postnuclear lysate of U937 cells (Fig. 17), as well as in other cells (data not shown) the anti-peptide Ab detects several bands suggesting that it might cross react with other proteins.

The anti-RNA helicase polyclonal Ab (Lee and Hurwitz, 1992) was not able to detect either the 28 kDa granule membrane protein in Western blot analysis or any cytoplasmic granular structures in Immunofluorescent analysis in $CD3^+$ lymphocytes (data not shown). The region to which anti-helicase A Ab is directed is unknown. One explanation for these results might be that the anti-helicase Ab is directed against a region in RNA helicase A protein that is not present in the granule associated 28 kDa leukophysin.

In melanoma cells, both the anti-peptide and the anti-helicase A Abs detect similar nuclear structures (Fig. 33). Thus, the two antibodies might be directed against two different regions of the same protein or highly homologous proteins.

Table 1 shows a summary of the specificity and the reactivity of different antibodies used in this study.

II. 3. Clone U.3.20 and RNA helicase A

As shown in the results, the entire length of clone U.3.20 is almost identical to the carboxy terminal region of RNA helicase A cDNA (starting from nucleotide 2722). In Northern analysis, only a single species of mRNA could be detected by either U.3.20 (Fig. 22) or RNA helicase A (Lee and Hurwitz, 1993) cDNAs. Although there is an observed difference in the size of the message (around 5 kb by using U.3.20 and 4.2 kb by using RNA helicase A), it is possible that the two messages are similar in size. The difference might be a result of using different standards. We used the ribosomal RNA to estimate the size, whereas the other group used several RNA markers of known length. Based on the high sequence homology between clone U.3.20 and carboxy terminal region of RNA helicase A, it is predicted that 5' end of the RNA detected by clone U.3.20 might also be highly homologous to the RNA helicase A cDNA. However, cloning and sequencing of the remaining 5' message detected by clone U.3.20 must first be obtained.

Table 1

Summary of the specificity and reactivity of different Ab used in this study

Ab	Immunogen & specificity	Immunofluoresccence	Western blot
D545 MAb	platelet dense granule, unknown epitope.	punctate cytoplasmic staining in platelets, granulated CD3+ lymphocytes, U937, neutrophils, mast cells, and malignant melanoma. increased plasma membrane staining upon degranulation in platelets, granulated CD3+ lymphocytes.	platelet dense granules : 40 kDa granulophysin/ CD63, brain sample : 38 kDa synaptophysin, affinity purified material from U938 cells and granules of CD3+ lymphocytes : 28 kDa leukophysin, not CD63.
Anti-peptide Ab	C-terminal 17 residues of the deduced amino acid sequence of the longest O.R.F. of clone U.3.20.	punctate cytoplasmic staining in granulated CD3+ lymphocytes, nuclear staining in U937, WI38 malignant melanoma cells.	granule membrane of CD3+ cells : same 28 kDa band identified by D545, lysate of U937 cells : several unknown bands.
Anti-RNA helicase Ab	purified RNA helicase A, unknown epitope.	no punctate cytoplasmic staining in CD3+ lymphocytes, nuclear staining in malignant melanoma cells.	no staining in lymphocyte granule membrane.

The differences between clone U.3.20 and RNA helicase A cDNA include few base mismatches and some additions/deletions as well as the presence of a 104 bp insert in the RNA helicase A (Fig. 29). An identical 104 bp insert was also present in J.11 clone isolated from the Jurkat library by using clone U.3.20 (Fig. 23). The 5' and 3' two nucleotides of the 104 bp insert has a sequence of GT and AG, respectively. It has been shown that the 5' GU and the 3' AG are the only universally conserved nucleotides that define the consensus sequence of the intron-exon boundaries (Padgett *et. al.*, 1986). By using RT-PCR, we have shown the presence of two species of RNA, with and without the 104 bp insert, in U937 cells. These observations suggest that clone U.3.20 might be derived from alternative splicing of an RNA helicase A isoform. The resulting RNA without the insert would code for a smaller protein which appears to be granule membrane associated in some cells.

III. Significance of the presence of leukophysin in cytotoxic granules

Given that clone U.3.20 codes for leukophysin, as suggested by the Ab studies, then the identification of an isoform of an RNA helicase in lymphocyte granules is an interesting finding. However, Anderson and colleagues described the presence of a family of RNA binding proteins in CTL granules. Members of this family include several isoforms of TIA-1 (15, 28, 40, and 53 kDa) as well as the 42 kDa TIAR. Also, an alternatively spliced variant of TIAR has been identified through RT-PCR analysis. The 40 kDa TIA-1 and the 42 kDa TIAR contain highly homologous three RNA binding domains and a glutamine-rich carboxyl terminal auxiliary domain. The three RNA binding domains are most homologous to the RNA binding domains of the human poly (A)-binding protein. The 15-kDa TIA-1 isoform, which is the major species in cytotoxic granules, may be derived from the carboxyl terminal auxiliary domain of the 40 kDa TIA-1 by serine-protease mediated cleavage. TIA family has been suggested to be the

cytotoxic granule components responsible for inducing apoptosis in CTL targets. The nucleolytic activity is independent of the RNA binding domains and possibly involves activating endogenous endonuclease in the target cells (Tian *et. al.*, 1991; Kawakami *et. al.*, 1992).

Although TIA was identified as a CTL specific protein, Northern analysis showed the presence of two RNA species of 2.7 and 4 kb in several cell lines. Anderson and colleagues indicated that TIA-1 high m.w. isoforms were expressed in the nuclei of cells that were TIA-1 negative (Tian *et. al.*, 1991; Kawakami *et. al.*, 1992). These results are similar to our findings in that leukophysin might represent a low m.w. isoform associated with lymphocyte granules whereas RNA helicase A is a ubiquitously distributed higher m.w. nuclear isoform.

RNA helicase A has been shown to mediate unwinding of double-stranded RNA in a 3' to 5' direction (Lee and Hurwitz, 1992), however its exact function is unknown. Due to its high level of homology with the maleless proteins of the fruit fly which has been implicated in X chromosome dosage compensation in males (Kuroda *et. al.*, 1991), a similar role for RNA helicase A in selective X chromosome metabolism is under investigation (Lee and Hurwitz, 1993).

The functional significance of leukophysin is currently unknown. It will be important to determine the topography of leukophysin in the granule membrane and the plasma membrane after degranulation. Specific antibodies directed to different regions of the molecule (amino terminal end, carboxy terminal end, and intragranular region) can be used to test that.

Leukophysin does not appear to have any of the RNA binding domains characteristic of RNA binding proteins (Mattaj, 1989). However, Kiledjian and Dreyfuss showed that a 26 amino acid peptide, which has four RGG repeats, derived from the sequence of hnRNP U protein is sufficient for RNA binding. Moreover, they described the presence of repeats with a consensus sequence of

RGG in several RNA binding proteins. They suggested that the presence of RGG repeats can be taken as an indicator of RNA binding activity (Kiledjian and Dreyfuss, 1992). The carboxy terminal end of leukophysin, starting from amino acid 184, contains three motifs with RGG sequence as well as five motifs with RGX, with X being any amino acid (Fig. 34). Whether or not leukophysin is capable of RNA binding will await the availability of either affinity purified or fusion proteins.

The carboxy terminal half of leukophysin is rich in tyrosine, glycine, and serine. These residues constitute imperfect repetitive motifs with a consensus sequence of YG(G/S)GG. Similar repetitive motifs with a consensus sequence of YG(P/Q)QG, suggested to be important in protein-protein interaction, was detected in synaptophysin I (Sudhof *et. al.*, 1987) (Fig. 35). Affinity purified leukophysin or bacterially-produced fusion protein can be used to isolate any protein that might be interacting with it.

Inhibition experiments by using specific antibodies and antisense oligonucleotides have illustrated the importance of synaptophysin I in neurotransmitter release (Alder *et. al.*, 1992a; 1992b). Similar approaches can be used to test the role of leukophysin in granule exocytosis.

Fig. 34 : **The Carboxy terminal end of leukophysin**

Carboxy terminal end of leukophysin starting from amino acid 184 to 235 appears to have three repeats with a consensus sequence of RGG as well as five repeats with a sequence of RGX, with X being any amino acid.

Fig. 35 : **Comparison of the repetitive motifs in leukophysin and synaptophysin**

Nine penta-peptide repetitive motifs with a consensus sequence of YG(G/S)GG in leukophysin are compared with ten motifs of synaptophysin which have the consensus sequence of YG(P/Q)QG.

RGVS

RGGF

RGNSGGDY

RGPSGGY

RGSGGFQ

RGGG

RGAYGTGYFGQG

RGGGGY

245	YGDAG	120	YGDGPRPPKMAR
250	YGQGPGG	132	YDNGSG
257	YGPQDS	138	YRRGGSS
263	YGPQGG	145	YSGGG
269	YQPD	150	YGGG
273	YGQPA	154	YSSGG
278	SGGGG	159	YGSGG
283	YGPQGD	164	YGGSANSFRAG
289	YGQQG	175	YGAGVGGG
294	YGQQG		
Consensus			
Sequence	YG (P/Q) QG		YG(G/S)GG

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