MEMBRANE ASYMMETRY IN APOPTOTIC CELLS, SIGNALS ASSOCIATED WITH PHAGOCYTOSIS AND THEIR IMPACT ON HIV TRANSCRIPTION

A Thesis in

Biochemistry, Microbiology and Molecular Biology

by

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ABSTRACT

Transbilayer membrane phospholipid asymmetry is the defining feature between healthy viable cells and their apoptotic counterparts. Healthy cells maintain all their phosphatidylserine (PS) and a large proportion of their phosphatidylethanolamine (PE) in the inner leaflet of the plasma membrane. Loss of asymmetry and exposure of PS on the cell surface targets apoptotic cells for destruction by macrophages. An aminophospholipid translocase is responsible for maintaining this asymmetry. Since all cells maintain this asymmetric distribution, all cells must have a plasma membrane aminophospholipid translocase; however, the protein responsible for this activity has never been identified. Subfamily IV P-type ATPases comprise amphipathic transporters whose members have yet undetermined function. If a type IV P-type ATPase were the plasma membrane aminophospholipid translocase, it should be expressed in all cells.

Using RT-PCR to measure gene expression, I demonstrate that multiple subfamily IV P-type ATPase members are simultaneously expressed in a single mammalian cell type. Of the ten genes analyzed, 1c, 1d, 2a, 2b, 5c, 6f, 6g and 6h were expressed in J774 cells, NIH 3T3 cells, and PC12 cells, suggesting a more general or housekeeping role for these genes; however, these genes also stand as candidates for the plasma membrane aminophospholipid translocase. 1a was expressed in J774 cells and PC12 cells and not in the NIH 3T3 cells, suggesting that 1a may have a specialized function in these cells.

PS expression on the surface of apoptotic cells is involved in their removal from tissue in order to maintain overall homeostasis. The engagement of phagocytic receptors by ligands such as PS found on the apoptotic cell surface results in the activation of
signaling cascades that facilitate engulfment. Macrophages and CD4⁺ T cells are the primary targets of HIV infection; however, unlike HIV infected macrophages which are invulnerable to the cytopathic effects of HIV, infected CD4⁺ T cells as well as uninfected bystander T cells undergo apoptosis. Consequently, these infected apoptotic cells as well as the HIV virions that are released from them have external PS. I examined how PS associated with virions and apoptotic cells influences HIV replication. Using annexin V and PS vesicles to mask, or compete with virus-associated PS, respectively, I show that PS inhibits HIV infection at a step prior to integration but following the generation of early reverse transcription products such as strong stop DNA. These observations suggest that PS initiated signals participate in HIV infection. Moreover, apoptotic cells inhibited HIV transcription in macrophages that have an established infection. This ability of apoptotic cells to suppress transcription was independent of PS, signifying that signals triggered following the interaction between an undetermined recognition ligand on the apoptotic cell surface and phagocytic receptor on the macrophage surface might affect HIV infection of monocytic cells. Furthermore, Elmo, a key signaling molecule that is associated with phagocytosis inhibited HIV transcription in infected macrophages, demonstrating that signaling events associated with phagocytosis of apoptotic cells participate in the establishment of HIV infection and provirus transcription.
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<thead>
<tr>
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<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>ACAMPS</td>
<td>Apoptotic cell associated molecular pattern</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>AS</td>
<td>Angelman syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AV</td>
<td>Annexin V</td>
</tr>
<tr>
<td>BRIC</td>
<td>Benign recurrent intrahepatic cholestasis</td>
</tr>
<tr>
<td>Ca2⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CDK9</td>
<td>Cyclin dependent kinase 9</td>
</tr>
<tr>
<td>CDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>Ced</td>
<td>Cell death abnormal</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell specific ICAM-1-grabbing nonintegrin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>Env</td>
<td>Envelope</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FIC1</td>
<td>Familial intrahepatic cholestasis 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine triphosphate</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl phosphatidylinositol</td>
</tr>
<tr>
<td>H</td>
<td>Hydrogen</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazine-ethanosulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparin-sulphate proteoglycan</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>K</td>
<td>Potassium</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposis sarcoma-associated herpes virus</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LFA1</td>
<td>Lymphocyte function-associated antigen-1</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL-receptor related protein</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>Mbc</td>
<td>Myoblast city</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte-derived macrophages</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>MFG-E8</td>
<td>Milk fat globule epidermal growth factor 8</td>
</tr>
<tr>
<td>Mg</td>
<td>Magnesium</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor activator of T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PFIC1</td>
<td>Progressive familial intrahepatic cholestasis type 1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-integration complex</td>
</tr>
<tr>
<td>PLAP</td>
<td>Placental alkaline phosphatase</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>Pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PSR</td>
<td>Phosphatidylserine receptor</td>
</tr>
<tr>
<td>P-TEFb</td>
<td>Positive transcription-elongation factor b</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SAPLT</td>
<td>Sperm aminophospholipid transporter</td>
</tr>
<tr>
<td>SH2</td>
<td>Src-homology 2</td>
</tr>
<tr>
<td>SH3</td>
<td>Src-homology 3</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian immunodeficiency virus</td>
</tr>
<tr>
<td>SP-1</td>
<td>Specific beta 1 glycoprotein</td>
</tr>
<tr>
<td>Sph</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SRA</td>
<td>Scavenger receptor A</td>
</tr>
<tr>
<td>SREC</td>
<td>Scavenger receptor from endothelial cells</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

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Chapter 1

LITERATURE REVIEW

The progression of cells from viable to apoptotic is an extensively studied phenomenon. Healthy cells are able to recognizably distinguish themselves from apoptotic cells by maintaining an asymmetric distribution of phospholipids. In spite of unrelenting research, the identification and characterization of the plasma membrane aminophospholipid translocase responsible for maintaining plasma membrane asymmetry has remained elusive, with more conflict than concurrence surrounding this pursuit. By analyzing single cell type expression patterns of the subfamily IV P-type ATPase genes of which the putative aminophospholipid translocase is a member, we hope to offer insight into the unique or redundant nature of this transporter in the various cell types and contribute toward an understanding of what defines healthy versus apoptotic in the cellular milieu.

1.1 Plasma membrane transbilayer phospholipid distribution.

Under normal physiological conditions, the distribution of phospholipids across the plasma membrane bilayer of animal cells is asymmetric. The choline phospholipids, phosphatidylcholine (PC) and sphingomyelin (Sph), are concentrated in the outer leaflet, whereas the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), are restricted and concentrated, respectively, to the inner leaflet of the plasma
Figure 1.1 Plasma membrane phospholipid distribution. Courtesy of Dr. Margaret Halleck
membrane (Williamson and Schlegel, 1994) (Figure 1.1). This asymmetric distribution of phospholipids across the plasma membrane is used to discriminate between healthy cells and their senescent or dying (apoptotic) counterparts. The latter lose their phospholipid asymmetry, exposing PS on the cell surface that serves as a signal for phagocytosis by macrophages (Fadok et al., 1992).

Aminophospholipid translocase activity was first described in the erythrocyte membrane (Auland et al., 1994) and in the membrane of secretory vesicles, specifically bovine chromaffin granules (Zachowski et al., 1989). Chromaffin granules were shown to exhibit an ATP-dependent specific translocation of PS from the lumenal face to the cytosolic face of the vesicle monolayer (Zachowski A et al., 1989). The enzyme’s flippase activity was found to be sensitive to Ca\(^{2+}\); micromolar concentrations of Ca\(^{2+}\) being sufficient for direct inhibition of aminophospholipid transport (Bitbol et al., 1987), N-ethylmaleimide (NEM), vanadate and fluoride (Beleznay et al., 1997), providing evidence for its classification as a P-type ATPase. The enzyme was isolated and purified from chromaffin granules, named ATPaseII, and the gene encoding the enzyme cloned (Tang et al., 1996). Sequence comparisons revealed a protein named DRS2 from *Saccharomyces cerevisiae* (yeast) with 47% identity in amino acid sequence to the mammalian chromaffin granule ATPase II, which when knocked out rendered the drs2 mutants unable to transport PS (Tang et al., 1996).

The rapid exposure of PS on apoptotic cells occurs in part due to the activation of non-specific bi-directional transbilayer lipid movements facilitated by a protein termed the phospholipid scramblase (Comfurius et al., 1996) and concomitant down-regulation
of the activity of the aminophospholipid translocase that normally maintains all the PS and most of the PE in the inner leaflet of the plasma membrane.

Lipid scrambling, resulting in appreciable randomization of lipids over both membrane leaflets, was first observed in blood platelets stimulated with a variety of physiological and non-physiological agonists (Bevers et al., 1982; Bevers et al., 1983). This process, which is reversible and insensitive to the lipid headgroups, is most clearly manifested by the surface exposure of PS and, in addition to platelets, has also been demonstrated in lymphocytes (Fadok et al., 1992; Verhoven et al., 1995), endothelial cells (Bombeli et al., 1997), red blood cells (Chandra et al., 1987), smooth muscle cells (Bennett et al., 1995) and tumorigenic cells (VanDeWater et al., 1985). An increase in intracellular calcium levels is an essential requisite for the onset of the scrambling process (Williamson et al., 1995; Dachary-Prigent et al., 1995). Recently, the successful reconstitution of a protein fraction from platelet and erythrocyte membranes into proteoliposomes with functional Ca\(^{2+}\)-inducible scrambling activity was reported (Comfurius et al., 1996; Basse et al, 1996) and the purified 37-kDa protein was found to be a type II plasma membrane protein. However, subsequent studies revealed this protein not to be the scramblase, and to date its identity remains elusive.

### 1.2 The Aminophospholipid Translocase

The aminophospholipid translocase uses the energy generated by the hydrolysis of ATP to actively translocate PS to the inner leaflet. P-type ATPases are a family of ubiquitously expressed integral membrane proteins. In all living cells, a variety of P-type
ATPases transport cations against their electrochemical gradient at the energetic expense of ATP hydrolysis (Pedersen et al., 1987). P-type ATPases, named for the aspartyl-phosphate intermediate of the enzymes (Lutsenko and Kaplan, 1995), can be classified into five major groups based on predicted topology features and on the substrate transported by the enzyme. Subfamily I P-type ATPases include the heavy metal pumps and bacterial K-pumps, subfamily II P-type ATPases include Ca-, Na,K-, and H,K-pumps, subfamily III include H- and Mg-pumps, subfamily IV are putative amphipath transporters, and subfamily V P-type ATPases consist of pumps that have no assigned function (Axelsen and Palmgren 1998). The different subfamilies of P-type ATPases are in turn divided into various classes and subclasses based on consensus sequences. The aminophospholipid translocase, which is responsible for the confinement of PS to the inner leaflet of the plasma membrane bilayer, is a subfamily IV P-type ATPase (Tang et al., 1996).

The yeast genome contains four genes encoding P-type ATPases closely related to DRS2, namely NEO1 (YIL048W) belonging to class 2, DNF1 (YER166W), DNF2 (YDR093W) belonging to class 3, and DNF3 (YMR162C) belonging to class 4. Neo1p is an essential member of the Drs2 family that localizes to endosomes and Golgi, where it physically interacts with other proteins that are associated with endosomal membranes thus regulating trafficking within the endosomal/Golgi system (Wicky et al., 2004). A recent analysis of strains carrying all possible viable combinations of null alleles for Drs2p, Dnf1p, Dnf2p, and Dnf3p revealed that these genes constitute an essential protein family with overlapping functions in membrane trafficking between the Golgi and endosomal/vacuolar system (Hua et al., 2002). It was also recently revealed that Dnf1p
and Dnf2p play a role in the inward translocation of phospholipids across the yeast plasma membrane. Moreover, it appears that both plasma membrane- and Golgi-associated members of this family contribute to regulation of the transbilayer phospholipid distribution in the plasma membrane and that this regulation is critical for budding of endocytic vesicles (Pomorski et al., 2003). Other genes that are characterized as belonging to subfamily IV P-type ATPase family of proteins have also been identified in Arabidopsis, which has eleven genes (Gomes et al., 2000) belonging to class 1 and 5, Drosophila melanogaster and Caenorhabditis elegans genomes each contain six members (Okamura et al., 2003) belonging to class 1, 2, 5 and 6; and class 1, 2 and 5 respectively. There are several identified mammalian genes belonging to class 1, 2, 5 and 6, which are discussed below (Figure 1.2).

In mammals, there are 14 different genes that are proposed to be amphipath transporters; however, little is known about the nature of the substrate transported by the other members of the subfamily IV P-type ATPases, or their physiological roles. A few biological disorders have been linked or attributed to genes in this subfamily. Cholestasis, or impaired bile flow, is one of the most common and devastating manifestations of hereditary and acquired liver disease (Lidofsky et al., 1997). Mutations in the familial intrahepatic cholestasis 1 (FIC1) gene, found widely expressed in mouse digestive tract tissues (Halleck et al., 1999), cause two forms of cholestasis in humans, benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis type 1 (PFIC1) (Bull et al., 1998). Angelman syndrome (AS) which is associated with neurobehavioral anomalies that include severe mental retardation, ataxia and epilepsy (Nicholls et al., 1998; Jiang et al., 1998) is caused by a partial deletion, or
Figure 1.2 Amphipath transporter subfamily. Courtesy of Dr. Margaret Halleck.
lack of expression of ATP10C (Meguro et al., 2001). Dhar and colleagues have also reported that inheritance of deletions associated with the mouse ATP10C homolog, \textit{pfatp}, resulted in increase body fat (Dhar et al., 2000; Dhar et al., 2002). More recently, it was discovered that over expression of ATP11a confers a drug resistance phenotype to cancer cells (Zhang et al., 2005). Furthermore, sperm aminophospholipid transporter (SAPLT/ATP8b3) was identified by Wang and colleagues as the protein responsible for maintaining PS asymmetry in sperm cells. The protein, which showed about 62% similarity to FIC1, was found expressed exclusively in the acrosomal region of spermatocytes and spermatids. Mice deficient in SAPLT were found to produce sperm cells that underwent premature maturation/activation (involves membrane scrambling), and cells that were unable to interact efficiently with eggs (Wang et al., 2003). Therefore, these amphipath transporters appear to play important roles in a variety of normal and disease processes.

1.3 Apoptosis

There are two common forms of cell death recognized in higher organisms, which are distinct, biochemical and morphological processes (Figure 1.3). Necrosis refers to the process when cells die from severe and sudden injury, such as ischemia, physical or chemical trauma. During necrosis, the plasma membrane is often the major site of damage, losing its ability to maintain membrane integrity and osmotic pressure, the cell swells and ruptures (Cohen, 1991) consequently spilling its contents into the extra cellular space, provoking an inflammatory response.
<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Necrosis</th>
</tr>
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<tbody>
<tr>
<td>Nuclear condensation</td>
<td>Cell swelling</td>
</tr>
<tr>
<td>Cell shrinkage</td>
<td>Cell bursts</td>
</tr>
<tr>
<td>No Inflammation</td>
<td>Inflammation</td>
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</tbody>
</table>
Apoptosis, on the other hand, refers to a more subtle process of cell death. The term apoptosis, used in Greek to describe the “dropping off” or “falling off” of petals from flowers, or leaves from trees, is used to describe a process of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations (Kerr et al., 1979). Apoptosis is implicated in the steady-state kinetics of healthy adult tissues and accounts for focal deletion of cells no longer needed during normal embryonic development and metamorphosis. For example during the maturation of the limb, columns of cells die in the interdigital spaces to form fingers and toes. In parts of the developing nervous system, cells that fail to make the necessary trophic connections die by neuronal apoptosis (Yuan et al., 2000). During the development of the T cell repertoire, cells that do not have a properly rearranged T cell receptor, or those whose receptors exhibit high avidity for self are deleted via negative selection through apoptosis (Cohen, 1991). Apoptosis is also induced in disease states in which there is evidence for cell-mediated immune reactions (Wyllie et al., 1980); for instance, T cell apoptosis has been proposed as a mechanism both in the early steps of HIV infection and in the massive T cell depletion, which leads to immune suppression (Katsikis et al., 1996).

Morphologically, apoptosis is defined by cellular and nuclear shrinkage, chromatin condensation and nuclear fragmentation culminating with the formation of apoptotic bodies (reviewed in Kroemer et al., 2005). Unlike necrosis, which is accompanied by rupture of nuclear, organelle and plasma membranes, electron microscopy shows that the structural changes that occur in apoptosis take place in two discrete stages. The first stage comprises the formation of small roughly spherical or
ovoid cytoplasmic fragments referred to as apoptotic bodies. The formation of apoptotic bodies involves marked condensation of the nucleus and cytoplasm, nuclear fragmentation, and the separation of protuberances that form on the cell surface to produce many membrane-bound cell remnants of varying size (Kerr et al., 1979). Apoptotic bodies are found dispersed in the intracellular tissue spaces, and are either extruded into an adjacent lumen or more commonly phagocyted by resident tissue cells (Wyllie et al., 1980). Thus, the second stage of apoptosis is characterized by the engulfment and degradation of apoptotic bodies by specialized cells.

The physiological consequences of necrosis vs. apoptosis are differentiated primarily on the basis of inflammation. Apoptosis, a controlled process sometimes also referred to as “silent death”, is associated with a lack of inflammation following the removal or engulfment of apoptotic cells while they retain their intact plasma membrane. The mechanism that facilitates this process is described below. Necrosis, on the other hand, is characterized by the generation of an inflammatory response following the loss of plasma membrane integrity, and the release of intracellular contents by these cells.

For apoptotic cells to be recognized by phagocytes, they must distinguish themselves from their normal neighbors. Display of PS on the surface of apoptotic cells is the earliest, most general surface change occurring during apoptosis (Schlegel and Williamson, 2001; Fadok et al., 2001). Annexin V is a 36-kDa member of a large family of Ca^{2+} and phospholipid-binding proteins that specifically binds PS in the presence of physiologic concentrations of Ca^{2+}. Labeling of cells with annexin V has been developed as a tool to study membrane asymmetry, or more directly, PS display on the surface of cells. The universal involvement of PS exposure in cell removal was illustrated by the
injection of biotinylated annexin V intercardially into mouse, and chick embryos, and into the haemolymph of Drosophila pupae. These studies showed that binding of annexin V was mostly restricted to apoptotic cells of every lineage and type in the mammalian, avian and insect tissues where apoptosis was occurring (van den Eijnde et al., 1998). Evidence that PS is required for recognition of apoptotic cells was derived from the observation that liposomes containing PS inhibited macrophage phagocytosis of apoptotic cells in a dose-dependent manner (Fadok et al., 1992). This PS requirement was later confirmed by studies that showed that masking the PS on apoptotic cells with annexin V, or competing away this PS using erythrocytes with PS on their surface, effectively inhibited the recognition and phagocytosis of apoptotic cells by macrophages (Krahling et al., 1999).

Phagocytosis, a rapid, seemingly non-inflammatory and histologically undetectable course of action, is the process that defines the methodical uptake or engulfment of apoptotic cells by macrophages and other related professional scavengers. In the case of apoptotic cells generated in the epithelia, phagocytosis is performed by neighboring epithelial cells; when apoptosis occurs in the tissue, phagocytosis is performed by professional phagocytes (macrophages and dendritic cells), which traffic constitutively from the blood to the tissues.

### 1.4 Macrophages

Macrophages are a major component of the mononuclear phagocyte system that consists of closely related cells of bone marrow origin, including blood monocytes, and
tissue macrophages (Fujiwara et al., 2005). Macrophages are derived from common myeloid progenitor cells, and have three major functions; antigen presentation to CD4+ T cells in an MHC dependent manner, clearance of dead and dying cells, and immunomodulation through production of various cytokines and growth factors. The function of activated macrophages is determined by the activating stimuli to which they are exposed; macrophages can potentially kill and degrade intracellular microorganisms by secreting high levels of inflammatory cytokines and effector molecules such as nitric oxide (NO); however, activated macrophages have also been linked with the secretion of polyamines that promote cell growth, collagen formation and tissue repair (Mosser, 2003).

Macrophages are found in all tissues, serving as sentinels in wait for invading pathogens, and cells undergoing apoptosis, which they eliminate by binding to them via specific innate immune receptors referred to as pathogen recognition receptors (PRR). PRRs recognize pathogen-associated molecular patterns (PAMP) and apoptotic cell-associated molecular patterns (ACAMPS), microbial or apoptotic cell-associated structures that have immunostimulatory activity (Medzhitov et al., 1997). A classic example is CD14, a glycosylphosphatidylinositol (GPI)-anchored protein found on the phagocyte that has been implicated in the clearance of gram-negative bacteria through interaction with bacterial lipopolysaccharide (LPS) (Miller et al., 2005). Additionally, CD14 has also been implicated in the clearance of apoptotic cells through its interaction with externalized PS on apoptotic cells (Schlegel et al., 1999; Devitt et al., 1998).

One distinctive characteristic of viable macrophages is their expression of low levels of external PS, a feature that is unique to these cells, bearing in mind that PS
display is most commonly associated with the early stages of apoptosis. The staining of macrophages with fluoresceinated annexin V suggests that phospholipid asymmetry is not maintained in these cells (Schlegel and Williamson, 1988). Significantly, macrophages pretreated with annexin V are unable to engulf apoptotic cells (Marguet et al., 1999; Callahan et al., 2000), suggesting that PS expression is required both on the apoptotic, and on the phagocytic cell surface for efficient phagocytosis to occur.

1.5 Macrophage recognition of apoptotic cells

The importance of apoptotic cell recognition and clearance by phagocytes to tissue homeostasis is corroborated by the vast redundancy of ligands and receptors involved (Moreira et al., 2004) (Figure 1.4). Some of the receptors that have been shown to recognize apoptotic cells, include, CD14 (Schlegel et al., 1999; Devitt et al., 1998), the integrin αvβ3 (Savill et al., 1990), CD36 (Savill et al., 1992), scavenger receptor A (SRA) (Platt and Gordon, 1998), and the PS receptor (PSR) (Fadok et al., 2000). Presumably, the receptors involved can act in concert to improve uptake efficiency (Pradhan et al., 1997) by enhancing tethering. Additionally, these receptors can also interact with different ligands, triggering distinct intracellular signaling pathways that initiate uptake.

Perhaps the most well characterized apoptotic cell recognition system involves the interaction of PS displayed on the apoptotic cell surface with PS receptors found on the macrophage surface. It was initially hypothesized that phagocytosis was mediated by
bivalent PS-binding annexins that served as bridging molecules between the apoptotic target and macrophage. However, annexin I and annexin II, other members of the annexin family of Ca\textsuperscript{2+}-dependent phospholipid-binding proteins, were found bound on the macrophage surface independent of PS, and constitute part of the repertoire used by macrophages for the recognition of PS on, and engulfment of apoptotic cells (Fan et al., 2004).

Other studies have identified numerous serum proteins, referred to as bridging molecules, that illustrate the importance of PS in mediating the interaction between apoptotic cells and phagocytes. Milk fat globule epidermal growth factor 8 (MFG-E8) secreted by thioglycollate-elicited peritoneal macrophages, binds to PS on apoptotic cells and tethers them to phagocytes via the vitronectin integrin receptor (Hanayama et al., 2002). Mer, a member of the Tyro3 receptor tyrosine kinase family, has been shown to play a role in the recognition of cells exposing PS on their surface by phagocytic cells (Scott et al., 2001; Nakano et al., 1997). These studies showed that the uptake of PS liposomes and of apoptotic cells by macrophages was specifically enhanced approximately two-fold in the presence of the PS-binding Mer ligand Gas 6 (Ishimoto et al., 2000). Other serum bridging proteins that are known to stimulate apoptotic cell uptake include coagulation factors such as vitamin-K dependent Protein S (Anderson et al., 2003) and the matrix component mindin (He et al., 2004).

The engagement of phagocytic receptors triggers essential signaling pathways, which induce cytoskeletal changes in the macrophage that culminate in the engulfment of apoptotic cells. Studies in the nematode, \textit{Caenorhabditis elegans}, identified six genes, cell death abnormal (ced) 1, ced 2, ced 5, ced 6, ced 7, and ced 10, that are important for
Figure 1.4 Molecules implicated in interactions mediating recognition of apoptotic cells. Gregory et al., 2004 Immunology 113: 1-4
the engulfment of cell corpses (Ellis et al., 1991). Mutations in any of these genes block
the engulfment of many cell corpses and cause the phenotype of persisting cell corpses.
All six genes have been cloned and their mammalian homologs identified. Genetic
analysis suggests that these genes fall into two redundant pathways. In the first pathway,
ced-1, which shares homology to mammalian scavenger receptor from endothelial cells
(SREC) (Zhou et al., 2001) and low-density lipoprotein (LDL) receptor-related protein
(LRP) (Ogden et al., 2001), functions as a phagocytic receptor that recognizes cell
corpses. Ced-7 encodes an ATP binding cassette (ABC) transporter similar to
mammalian ABCA1 (Wu and Horvitz, 1998) and is required for clustering of ced-1
around neighboring cell corpses (Zhou et al., 2001). Ced-6, an intracellular adaptor
protein similar to mammalian GULP (Zhou et al., 2001; Liu et al., 1998), contains a
phosphotyrosine binding site, a potential proline-rich motif that mediates protein-protein
interactions, and a leucine zipper region for homodimerization. It is thought that ced-6
may act downstream of ced-1 and ced-7 during phagocytosis of cell corpses.

In the pathway illustrated in Figure 1.5, ced-2, ced-5, ced-10 and ced-12 are
conserved components of a Rac GTPase signaling pathway. Ced-2 is similar to the
mammalian adaptor protein CrkII, which has one N-terminal Src-homology (SH) 2
domain followed by two SH3 domains (Reddien and Horvitz, 2000) that bind
phosphotyrosines and proline-rich sequences respectively (Koch et al., 1991). CrkII is
implicated in transmembrane receptor-mediated signaling pathways that regulate cell
shape and motility (Klemke et al., 1998). Ced-5 is similar to human Dock180, a CrkII-
interacting protein, and to Drosophila melanogaster myoblast city (Mbc) (Wu and
Horvitz, 1998). Dock180 contains an N-terminal SH3 domain, a large central region with
Figure 1.5 Rac1 activation mediated through the CrkII/Dock180/Elmo pathway leading to engulfment of phagocytic cells.
guanine nucleotide exchange factor (GEF) activity termed DOCKER, and a C-terminal proline-rich region that likely binds the first SH3 region of CrkII (Reddien and Horvitz, 2004). Dock180 can localize with CrkII to focal adhesions and affect cell spreading (Kiyokawa et al., 1998). Mbc controls myoblast fusion and dorsal closure in Drosophila, controlling events that, similar to engulfment, involve changes in cell shape (Erickson et al., 1997). Ced-10 is a homolog of mammalian Rac GTPase (Reddien and Horvitz, 2000). Rac GTPases are members of a Ras superfamily subgroup that includes Rho, Rac and Cdc42 that controls cell morphology by regulating the organization and dynamics of the actin cytoskeleton (Hall and Nobes, 2000; Van Aelst and D’Souza-Schorey, 1997).

Modification of the actin cytoskeleton is important for the uptake of apoptotic cells (Chimini and Chavrier, 2000). The mammalian homolog of ced-12 is ELMO, which can physically interact with Dock 180 possibly as part of a CrkII-Dock180-Elmo ternary complex (Gumienny et al., 2001; Wu et al., 2001). Additionally, Elmo contains two functionally indispensable domains; a proline-rich candidate SH3 binding domain, and a putative pleckstrin homology (PH) domain (Zhou et al., 2001), a region of about 100 amino acids, found in many proteins involved in signal transduction or cytoskeletal reorganization that is believed to target proteins to membranes (reviewed in Shaw, 1996). The functional co-operation between CrkII, Dock180 and Elmo synergistically enhances GTP-loading on Rac (thus acting as a GEF), in turn promoting actin reorganization associated with cell shape changes required for processes such as phagocytosis of apoptotic cells (Lundquist et al., 2001), cell migration and axon outgrowth (Wu et al., 2002).
Other types of signal transduction pathways induced by PS are those directly upstream of intracellular events that suppress inflammatory responses by the macrophage. Apoptotic cells are known to directly suppress pro-inflammatory responses in engulfing phagocytic cells (Fadok et al., 1998), triggering the release of anti-inflammatory cytokines such as interleukin (IL)-10 (Voll et al., 1997) and transforming growth factor beta (TGF-β1) (Huynh et al., 2002). While the mechanisms that determine anti-inflammatory mediator release have yet to be detailed, the process is known to be PS-dependent, and it has been suggested that the PSR may play an important signaling role in this pathway, particularly with respect to TGF-β1 (reviewed in Gregory et al., 2004).

1.6 Human Immunodeficiency Virus (HIV)

Human immunodeficiency virus (HIV), the etiological agent of the acquired immune deficiency syndrome (AIDS) has the capability of selectively infecting and ultimately incapacitating the immune system whose function is to protect the body against pathogenic invaders. HIV was first described in the early 1980s, and has since caused the death of millions of individuals world-wide. The most recent epidemiological studies of the virus present evidence of a global pandemic; over 40 million people are estimated to be living with HIV. In 2004 alone, more than 3 million people died from AIDS related complications and there were over 5 million new cases of HIV infection.
The disease burden is heaviest in Sub-Saharan Africa and South and Southeast Asia with a high incidence of infections and fatalities occurring in these regions (UNAIDS).

HIV-induced immunosuppression results in a host defense defect that renders the body highly susceptible to opportunistic infections and neoplasms (Fauci, 1988). The virus is spread by sexual contact, by infected blood or blood products, and perinatally by mother to infant (Fauci et al., 1985), causing a slowly progressive and inevitably fatal disease in its host. The immunodeficiency associated with AIDS results in the appearance of classical symptoms that include but are not limited to a rare skin cancer, Kaposi’s sarcoma caused by Kaposi’s sarcoma-associated herpes virus (KSHV), and a number of opportunistic infections caused by, for example, *Pneumocystis carinii* and *Candida albicans*. These microbes that are normally kept under control in an immunocompetent individual ultimately overwhelm the AIDS patient’s failing immune system, resulting in premature death.

HIV is an enveloped RNA virus of the viral family Retroviridae, genus Lentivirus. As visualized by electron microscopy, HIV has a dense cylindrical core whose structural elements are coded by the viral gene gag, and encase two molecules of the viral RNA genome (Rabson and Martin, 1985) and two molecules of reverse transcriptase (RT). In total, HIV has nine genes (Figure 1.6), expressing proteins with diverse functions. The gag, pol and env gene products are prototypic proteins that ensure efficient replication of retroviruses in general. The six additional genes, Nef, Tat, Rev, Vif, Vpr, and Vpr encode auxiliary proteins with important functions specific to HIV.

Tat protein is an ~86 amino acid (aa) protein expressed shortly after infection of cells that functions as a potent transcriptional activator of the HIV long terminal repeat
(LTR) promoter element. Tat is crucial to viral replication and acts via an RNA structure termed transactivation response (TAR) element. The role of TAR is to recruit Tat, as well as associated cellular proteins, CycT1 and Cdk9, to the HIV LTR promoter, which then activates transcription by promoting the processivity of initiated RNA polymerase II (Pol II) molecules (Kao et al., 1987; Jones et al., 1994; reviewed in Cullen, 1995).

Nef is the largest auxiliary protein (206 aa) and is expressed in high levels throughout HIV infection. Functionally, Nef mediates the internalization of the CD4 receptor and MHC class I molecules in T lymphocytes (Schwartz et al., 1996; Piguet et al., 1999). Nef interacts with tyrosine kinases of the Src family (Saksela et al., 1995) with serine/threonine kinases (Sawai et al., 1994) and with signaling molecules such as c-cbl (Yang et al., 2005). Nef has also been shown to enhance the apoptotic process by affecting multiple components of the apoptotic machinery. For instance, Nef has been shown to promote the transcription of Fas ligand and Fas in T cells, thereby increasing their sensitivity to Fas-mediated apoptosis (Xu et al., 1999).

Rev (116 aa), also expressed shortly after infection, is a sequence-specific nuclear RNA export factor, that is able to induce the efficient nuclear export, and hence expression, of the various incompletely spliced viral transcripts (Malim et al., 1989). Tat, Nef and Rev are expressed shortly after infection of cells because these proteins are encoded by fully spliced HIV mRNAs. In contrast, the Gag, Pol, Env, Vif, Vpr and Vpu proteins are all dependent on Rev for the nucleocytoplasmic transport of their cognate mRNAs and are therefore expressed with delayed kinetics (Cullen, 1998).

Vif is about 192 aa in length, and the most well defined biological activity of Vif is to enhance the infectivity of HIV virions produced in primary T cells and in non-
permissive cell lines (Gabuzda et al., 1992) by inducing the polyubiquitination and proteasomal degradation of APOBEC3-F (Zheng et al., 2004) and –G (Sheehy et al., 2002), members of the apolipoprotein B mRNA-editing enzyme catalytic (APOBEC) polypeptide family. APOBEC3-F and –G have been shown to inhibit HIV infection by causing cytidine deamination of HIV negative strand DNA (Goff et al., 2003), and inducing hypermutations that block HIV replication (KewalRamani et al., 2003). Much remains to be discovered about HIV’s less understood auxiliary proteins Vpr protein and Vpu protein. Vpr has been associated with a role in nuclear import of the HIV pre-integration complex particularly in non-dividing cells (Heinzinger et al., 1994), whereas, Vpu, which is thought to be unique to HIV and simian immunodeficiency virus (SIV), has been associated with enhancing virion release from infected cells (Cullen, 1998).

### 1.7 HIV Life-cycle

HIV gains entry into susceptible cells by fusion of the viral membrane with the cell plasma membrane (Chan et al., 1998). The CD4 molecule was identified as the receptor for HIV in 1984, hence helping to identify T cells and macrophages, both of which express CD4, as the major targets of HIV (Miedema et al., 1994). T cells are known to be particularly susceptible to the cytopathic effect of HIV infection, and this contributes to the dramatic depletion of helper T cells that is characteristic of AIDS. Macrophages on the other hand, are relatively immune to the cytopathic effect of the virus, and are known to serve as long-term reservoirs (Fauci 1988), rendering infection of macrophages largely instrumental to the development of the disease.
In 1996 researchers were able to provide an explanation for the observed cellular bias that the virus seemed to exhibit, i.e. that individual isolates of HIV displayed markedly distinct tropisms for infection of primary macrophages as compared with CD4+ T cell lines. It was discovered that a second receptor was required for HIV binding to the target cell, and that these secondary receptors were chemokine receptors. This led to the characterization of CXCR4 and CCR5, seven transmembrane G-protein coupled receptors, also described as members of the chemokine receptor family. These studies showed that recombinant chemokine proteins allowed HIV env-CD4-mediated fusion and infection, and further that antibodies raised against the chemokine receptors, or their natural ligands, which for CXCR4 is SDF-1 and for CCR5 include RANTES, MIP-1α and MIP-1β, were able to block infection with the respective viral strains. These results provided conclusive evidence to define CXCR4 and CCR5 as fusion and infection co-factors for T cells (Feng et al., 1996) and macrophages (Alkhatib et al., 1996) respectively.

The chemokine co-receptors are extremely crucial to the establishment of infection as studies performed on individuals who, in spite of repeated exposure to HIV were resistant to infection, illustrated. Mutations in the M-tropic co-receptor, a 32-base pair deletion in the gene encoding CCR5, were identified that conferred resistance to infection on these highly exposed persistently seronegative individuals (Liu et al., 1996). These observations suggest possible therapies through receptor alteration, which by way of influencing CCR5-mediated signaling might offer protection against infection (Fauci, 1996). The initial phase in HIV-1 infection involves binding of the virus to target cells. Once bound, the virus enters through fusion into the cytoplasm of its host cell where it uncoats, exposing its RNA genome. This viral RNA is then reverse transcribed into
double-stranded DNA, which then translocates into the nucleus of the cell and randomly integrates into the host cell’s chromosomes. Upon integration, the virus hijacks the host’s cellular transcription machinery, which it uses in addition to Tat and Rev to generate viral messenger-RNA (mRNA) and translate viral proteins that are assembled at lipid-enriched regions of the host cell plasma membrane, which also represent the site of exit of mature virions (Figure 1.7).

HIV binding to its host cell is facilitated via an interaction between CD4 molecule on the target cell and the HIV-1 envelope (env) glycoprotein (gp) 120. Once gp120 binds, the protein undergoes a conformational change, which allows it to interact with either CXCR4 for R4 (T cell) tropic virus strains and CCR5 for R5 (macrophage) tropic virus (Kedzierska et al., 2003). Upon binding of CD4 and the co receptors, an additional conformation change is induced in the transmembrane protein gp41 that results in the exposure of the protein’s fusogenic domain, which ultimately mediates the fusion of virus particles to host cell membranes (Binley et al., 1997), thus permitting entry of the virus into the cell. Experiments using cell lines have indicated that the fusion event is facilitated by the high expression of chemokine receptors and CD4 molecules on the cell surface; therefore suggesting that the existence of an active mechanism for receptor clustering is of particular importance (Viard et al., 2002).

Other receptors and co-factors that are necessary for viral infection of target cells have also been identified. HIV is known to use dendritic cells for transport from genital mucosa to its major target, CD4⁺ T cells in the draining lymph nodes. In order to interact with immature dendritic cells at sites of infection, studies have emphasized the importance of mannose C-type lectin receptors, particularly the dendritic cell specific
ICAM-1-grabbing noningetrin (DC-SIGN) for HIV gp120 binding. DC-SIGN bound HIV is protected from intracellular degradation (Geijtenbeek T et al., 2000) and this interaction also enhances the infection of T cells at low virus titers (Kwon et al., 2002). DC-SIGN might also function as a cis-receptor for HIV, as co-expression of DC-SIGN with CD4 and CCR5 increases infection of target cell (Lee et al., 2001). Similarly, HIV is capable of utilizing high affinity attachment molecules such as macrophage mannose receptor (MMR), which binds to the carbohydrate moieties of gp120 (Robinson et al., 1987; Lifson et al., 1986; Nguyen et al., 2003) to enhance its capture by macrophages and transmission to permissive T cells. In addition, adhesion receptors such as CD44 (Guo et al., 1995), lymphocyte function-associated antigen-1 (LFA1), intracellular adhesion molecule-1 (ICAM1) (Liao et al., 2000), and heparin-sulphate proteoglycan (HSPG) (Patel et al., 1993) have been shown to promote HIV adsorption and infectivity. Notably, macrophage annexin II was recently shown to support HIV infection of these cells. Ma and colleagues demonstrated that soluble annexin II can bind directly to HIV, and that this binding is sufficient to inhibit infection of monocytes, thus identifying annexin II as a novel macrophage specific co-factor for HIV infection (Ma et al., 2004).

Retroviruses use a virus-associated RNA-dependent DNA polymerase termed reverse transcriptase (RT) that is able to transcribe viral RNA into double-stranded linear DNA. After the virus generates its double-stranded linear DNA, a pre-integration complex (PIC), consisting of viral DNA and associated proteins, is assembled that translocates into the nucleus. PIC nuclear import is facilitated by a nuclear localization signal. In order for viral DNA to be transcribed and viral proteins synthesized using both host cell and viral factors, the DNA is permanently integrated into the host cell
chromosome by the action of the HIV integrase enzyme, establishing a provirus. Transcription of HIV genes in all permissive cell types is directed by the HIV LTR promoter element. Transcription of early HIV genes Nef, Tat and Rev is regulated by cellular transcription factors, whereas the transcription of late HIV genes, is under the control of Tat associated with cellular transcription factors. Studies on Tat have indicated the indispensable role played by this viral protein on HIV infectivity; Tat is a participant in several cellular complexes that modulate viral transcription. One such complex involves Tat binding to the TAR element in association with a complex of cyclin T1 and cyclin dependent kinase 9 (CDK9), a component of positive transcription-elongation factor b (P-TEFb). P-TEFb in turn phosphorylates RNA polymerase II (Pol II) leading to the successful elongation of viral message (Fujinaga K et al., 1998). Numerous cellular transcription factors have been found to support the transcription process following infection and cellular activation. These include members of Sp-1 (Harrich et al., 1989), NFκB (Jacque et al., 1996), NF-AT (Cron et al., 2000) and C/EBP (Henderson et al., 1996; Tesmer et al., 1996) families of transcription factors that have been shown to act individually or in synergy at the LTR to activate HIV transcriptional activation. Some of these factors have a broad, non-cell type specific effect on transcription, whereas others are more restricted in the cell types in which they exert their transcriptional modulation e.g. NF-AT in primary CD4+ T cells (Cron et al., 2000), and C/EBP family during monocyte/macrophage infection (Henderson et al., 1996).

The translation of viral proteins is facilitated by the activity of the viral protein Rev. Following translation, the maturation phase of the viral life cycle is marked by protein assembly and packaging into virus particles, promoted by the viral structural
protein Gag. In many cell types, viral assembly takes place at the plasma membrane; however, in macrophages, particle assembly occurs primarily in intracellular compartments identified as multi-vesicular bodies (MVB) (Ono et al., 2004) (Figure 1.7). Subsequently, virus particles are released from the host cell through a budding mechanism.

1.8 HIV and apoptosis

HIV induces apoptosis, which is primarily responsible for the rapid depletion of immune cells that is one of the hallmarks of HIV infection. Unlike macrophages, T cells are susceptible to the cytopathic effect of HIV infection. The death of infected T cells is due in part to cell lysis and to syncytium formation; however, these forms of direct cell death alone are not sufficient to account for the massive immune cell depletion that is characteristic of AIDS. In addition to the death of infected cells, uninfected cells are also known to succumb to secondary effects of HIV infection. This bystander death is attributed to CD4-crosslinking by HIV gp120, which primes T cells for activation-induced apoptosis, via the upregulation of Fas (CD95) and Fas ligand (FasL; CD95L) on uninfected T cells (Banda et al., 1992). Nef expression in infected cells, and Tat secreted by infected cells have both been linked to this Fas-mediated cell death in both infected and uninfected cells (Zauli et al., 1996; Westendorp et al., 1995). In addition to these Fas-FasL interactions, TNFα secreted by activated macrophages has also been reported to trigger apoptosis in uninfected T cells during HIV infection (Bradley et al., 1997).
1.9 Role of lipids during HIV infection

Considerable interest and effort have been directed toward understanding the lipid composition of the viral envelope, and the role these lipids might potentially play during the infection process. Like all biological membranes, the HIV envelope consists of a lipid bilayer embedded with proteins. A large proportion of the proteins and lipids that constitute the viral lipid bilayer are derived from the host cell upon budding. There is evidence to suggest that the viral envelope has a lipid composition similar to the cell plasma membrane, however, with elevated cholesterol to lipid molar ratio. The phospholipid class ratios indicate decreased levels of PC and phosphatidylinositol (PI) by 50% and 80% respectively, whereas Sph is enriched 3-fold and PS is elevated by 40% (Aloia et al., 1993). These observations strongly support the hypothesis that HIV selectively evaginates the host cell plasma membrane at sphingolipid- and cholesterol-enriched microdomains termed lipid rafts, which eventually constitute the viral coat (Raulin et al., 2001).

Some of the lipid molecules that have generated interest in HIV biology include cholesterol, glycosphingolipids and phospholipids. Cholesterol-enriched microdomains in the plasma membrane serve as sites for recruitment of gp120-gp41-CD4 co-receptor complexes (Simons et al., 1997) thus increasing HIV infection. Furthermore, depletion of cholesterol from HIV particles results in a decrease in env-mediated fusion (Liao et al., 2001) and a general loss of infectivity and the permeabilization of otherwise intact virions (Graham et al., 2003). Because progressive depletion of CD4+ T cells due to apoptosis is a characteristic feature of HIV infection, it was not surprising therefore that
PS is one of the lipids found on the HIV envelope (Aloia et al., 1993). This virus associated PS is a co-factor that mediates infection of monocytes/macrophages (Callahan et al., 2003a), through its interaction with annexin II on the macrophage surface (Ma et al., 2004) or through its engagement of the PSR. These observations provide evidence of a more prominent role for lipids during HIV infection. It is apparent therefore, given the principal role played by PS during phagocytosis that the mechanisms through which PS is contributing to infection of monocytic cells deserves greater scrutiny. It is the aim of the latter part of this work to elucidate the role of PS, and PS-induced signals in regulating HIV pathogenesis.
Chapter 2

Identifying plasma membrane aminophospholipid translocase candidate genes

2.1 Introduction

Apoptosis or programmed cell death is the central mechanism that has been perfected over evolutionary time to effectively eliminate any sick or senescent cells without harm to neighboring healthy cells. Loss of phospholipid asymmetry and the subsequent appearance of PS on the surface of cells undergoing apoptosis have been attributed to a down regulation in the activity of the aminophospholipid translocase. The translocase belongs to subfamily IV of the P-type ATPases, and includes representatives that have been identified in fungi, plants, nematodes, protozoans, and mammals. In yeast a single cell expresses multiple members of subfamily IV genes simultaneously. In mammals in situ hybridization has been used to determine expression patterns in whole tissue; however, studies examining the expression patterns in a single cell type had not been undertaken until this study.

There are 14 different mammalian genes that are proposed to be amphipath transporters; however, definitive studies have yet to be carried out on many of them to determine their biochemical and physiological functions. These genes have been placed into classes and sub-classes based on sequence similarity and class and sub-class consensus sequences, with mammals expressing genes from four of the six classes. At the time when northern blot and in situ hybridization studies were used to determine
expression patterns in tissues of the mouse, the cDNA probes of only seven genes (1a, 1b, 1c, 1h, 2a, 2b and 5a) in the subfamily were available. These studies were carried out on both embryonic and adult mouse tissue, and revealed that multiple genes were widely expressed in a range of tested tissue, which included digestive track to the central nervous system (CNS). Most of the genes tested showed a broad expression pattern, giving a positive signal in most of the tissue types tested whether embryonic or adult; whereas other genes had a more exclusive distribution, being expressed in tissues where no other subfamily gene was found and at unique stages during development (Halleck et al., 1999).

Because the natural cellular substrates of the different enzymes are not known, it remains to be elucidated whether the different classes of the subfamily IV P-type ATPases code for aminophospholipid transporters, or whether these gene products transport some other amphipathic molecules (Halleck et al., 1999). The first objective of this study was to establish the expression patterns of the subfamily IV P-type ATPase genes in various individual cell types in order to determine whether single cells were capable of expressing more than one subfamily IV P-type ATPase gene at a time. In addition, we were interested in distinguishing expression patterns between cells of different lineage. The second objective was to identify possible candidate genes encoding the plasma membrane aminophospholipid translocase, the rationale being that since all cells transport PS across their plasma membrane, any gene found expressed in all cell types would be a good candidate for the plasma membrane aminosphopholipid translocase, assuming that there is only one that all cells employ.
2.2 Materials and Methods

2.2.1 Cell Lines. J774 mouse macrophage cells and NIH 3T3 mouse fibroblast cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin/ml, 100 µg of streptomycin/ml and 2 mM glutamine at 37°C in 5% CO₂. PC12 rat adrenal cells were cultured in DMEM supplemented with 10% FBS 100 U of penicillin/ml, 100 µg of streptomycin/ml and 2 mM glutamine and incubated at 37°C in 5% CO₂. Liver and brain tissue were isolated from 6 – 8 week old mice.

2.2.2 Library Preparation. An immobilized mouse cDNA library was prepared from whole liver tissue, brain tissue, J774 cells, NIH 3T3 cells and PC12 cells using the SOLIDscript Solid Phase cDNA Synthesis Kit (CPG Inc) according to the manufacturer’s instructions. Briefly, tissue or cells were suspended in 1X tissue extraction/hybridization buffer and homogenized using about ten strokes of a dounce homogenizer. Total RNA was isolated from the homogenate using the guanine thiocyanate phenol/chloroform method (Ausubel et al., 1997). Messenger RNA (mRNA) was immobilized on biotinylated oligo (dT)₂₅ complexed to streptavidin-coated magnetic porous glass beads. Reverse transcriptase was then used to generate the immobilized cDNA library. The selection of the genes to be tested was based on the availability of templates that could be used to test the specificity of the primers. Gene-specific PCR primers (shown in Table 2.1) were designed for ten genes based on selecting regions of the genes that exhibited least consensus and greatest variation between classes and within sub-classes (Halleck M, personal communication). These gene-specific primers were then used to probe the different immobilized cDNA libraries for the expression of the different subfamily IV P-type ATPase genes.
<table>
<thead>
<tr>
<th>Subfamily Member</th>
<th>Template</th>
<th>Source of Template</th>
<th>Forward Primer 5'-3'</th>
<th>Reverse Primer 5'-3'</th>
<th>Expected Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a (Ap8a1)</td>
<td>Clned gene</td>
<td>Pradhan D., Blackman C.</td>
<td>5'-GGAACAGCGTTCGGAAAC-3'</td>
<td>5'-AGCGGCAATATACTTAGATG-3'</td>
<td>295</td>
</tr>
<tr>
<td>1b (Ap8b2)</td>
<td>Cloned gene</td>
<td>Hacker C.</td>
<td>5'-AGAGAGAATGACACCGACAGATCA-3'</td>
<td>5'-GCTGCGCTTCTGCAGGATCT-3'</td>
<td>903</td>
</tr>
<tr>
<td>1c (Ap9b1, EST)</td>
<td>ATP8b1 EST</td>
<td>Research Genetics Inc.</td>
<td>5'-GGTCCTTTCGGTGGACATTT-3'</td>
<td>5'-GGAAGTAGAGTGGCTGCGGTACCC-3'</td>
<td>297</td>
</tr>
<tr>
<td>1d (Ap9b2)</td>
<td>ATP8b2 EST</td>
<td>Research Genetics Inc.</td>
<td>5'-TCACACACGAGGAGTTCTTT-3'</td>
<td>5'-GCTGGCCGACAGTGGACCTTT-3'</td>
<td>892</td>
</tr>
<tr>
<td>2a (Ap10a)</td>
<td>Cloned gene</td>
<td>Gas L., Blackman C.</td>
<td>5'-GGAAGAGCGGCTGACAGTGGG-3'</td>
<td>5'-GGTCGACAGAGCCGACATCTTAC-3'</td>
<td>850</td>
</tr>
<tr>
<td>2b (Ap10b)</td>
<td>Cloned gene</td>
<td>Nagatani P.</td>
<td>5'-GACCCATAGCTACGGTACG-3'</td>
<td>5'-GACTCCATGCTGGAAGATCC-3'</td>
<td>381</td>
</tr>
<tr>
<td>5c (Ap10c, sB10a)</td>
<td>Cloned gene</td>
<td>Pyle S.</td>
<td>5'-GACCACTAGCGCCACATCAGC-3'</td>
<td>5'-GAACGCGCCAGCCACATCAGC-3'</td>
<td>900</td>
</tr>
<tr>
<td>6f (Ap11a)</td>
<td>ATP11a EST</td>
<td>Research Genetics Inc.</td>
<td>5'-GCTGAGAGAATAAAGATGGA-3'</td>
<td>5'-GCTGAGAGAATAAAGATGGA-3'</td>
<td>400</td>
</tr>
<tr>
<td>6g (Ap11b)</td>
<td>ATP11b EST</td>
<td>Research Genetics Inc.</td>
<td>5'-GCTGAGAGAATAAAGATGGA-3'</td>
<td>5'-GCTGAGAGAATAAAGATGGA-3'</td>
<td>400</td>
</tr>
<tr>
<td>7h (Ap11c)</td>
<td>ATP11a EST</td>
<td>Research Genetics Inc.</td>
<td>5'-GCTGAGAGAATAAAGATGGA-3'</td>
<td>5'-GCTGAGAGAATAAAGATGGA-3'</td>
<td>381</td>
</tr>
</tbody>
</table>

Table 21: Gene-specific primer pairs with optimal annealing temperatures used for PCR amplification of subfamily genes, templates used to test primers, and predicted PCR product sizes in base pairs.
2.2.3 PCR amplification and sequencing. PCR was performed in a 50 µl reaction mixture containing 2.5 µl of the immobilized cDNA library (~5 µg of template), 5 µl of 10X PCR buffer (Gene choice), 5 µl of 2 mM deoxynucleoside triphosphates (dNTPs), 1 µl each of sense and antisense primers (Table 2.1) from a 10 µM stock, and 1 µl of Taq DNA polymerase (Gene choice). Samples were cycled as follows: Denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 1 min, annealing at the optimal annealing temperature for each primer (Table 2.1) for 1 min, 72°C for 2 min, and a final extension step at 72°C for 10 min. PCR products of the predicted molecular weight (Table 2.1) were resolved on a 1% low melting point agarose gel, purified by phenol/chloroform extraction, and cloned into the pGEM-T Easy Vector (Promega) according to the manufacturer’s instructions. White colonies were selected after overnight growth on LB Ampicillin plates, and plasmid DNA was isolated and sequenced at the Penn State University Nucleic Acid Facility.

2.3 Results

2.3.1 PCR analysis of the expression of mammalian subfamily IV P-type ATPase subfamily members in mouse liver and mouse brain. Halleck and colleagues previously reported that multiple subfamily IV genes are simultaneously expressed in a single tissue (Halleck et al., 1999). To test the utility of the immobilized cDNA library and PCR methods in establishing gene expression, we analyzed the expression patterns of the subfamily genes 1a, 1b, 1c, 1d, 2a, 2b, 5c, 6f, 6g, and 6h in mouse liver and mouse brain tissue using cDNA libraries generated from the two types of tissue.
PCR analysis of these genes in mouse liver tissue indicated that all the genes, except 1a and 1b yielded a PCR product as a single distinct band at the predicted molecular weight (Fig 2.1; Table 2.2). Likewise, analysis of mouse brain tissue revealed that all of the ten subfamily genes tested are expressed in the brain (Table 2.3). These results, summarized in Table 2.2 and Table 2.3, confirm that a single tissue is capable of expressing multiple subfamily IV genes at a time.

The data generated from PCR analysis of mouse tissue were compared to, and found to be consistent with, the expression patterns observed in mouse liver and mouse brain tissue examined in the earlier study using northern blot and in situ hybridization methods (Halleck et al., 1999). An evaluation of the seven genes previously analyzed in mouse liver yielded a similar pattern of expression with PCR as compared to northern and in situ hybridization methods. The following subfamily IV genes, 1c, 1d, 2a and 2b were expressed as previously determined, whereas 5c and 6h were newly identified using PCR (Table 2.2). However, previously detected 1a and 1b were undetected using PCR, raising the likelihood that in-situ hybridization, which relies primarily on visual interpretation of data, may have given false positives. PCR is a more sensitive method of determining gene expression, and hence provides more accurate data. Similarly, in brain tissue, the PCR data generated was consistent with northern blot and in situ hybridization data for a subset of genes; namely, 1a, 1b, 2a, 2b, 5c and 6g, while 1c, which northern blot and in situ had indicated was not expressed in the brain, was detected using PCR (Table 2.3). The PCR results were additionally compared to expression data available on the database, which are based on quantifying ESTs. Mouse liver PCR data was consistent with the EST expression data available for 1a, 1b, 1c, 2a, 6g and 6h; and varied
for the remaining genes. On the other hand, the mouse brain PCR data was consistent with EST expression data for all the genes (Table 2.2; Table 2.3).

Based on these results, PCR proved not only to comply with these methods that had been utilized before, but proved to be a more sensitive method, given that the expression of a number of genes that had not been previously observed in either mouse liver (5c and 6h) or mouse brain (1c) preparations gave positive results with PCR. Accordingly, the data collected from our PCR analysis validated the use of the immobilized cDNA library system

### 2.3.2 Expression of subfamily IV P-type ATPase genes in J774 cells, NIH 3T3 cells and PC12 cells

Tissue is defined as an aggregate of cells that carry out specific functions. Tissue is made up of different cell types; therefore, looking at gene expression profiles on a tissue basis represents the expression pattern over a number of cell types. For instance, liver, the largest internal organ, functions in the manufacture, storage and secretion of a number of substances involved in metabolism. About 80% of liver mass is contributed by hepatocytes, the principal parenchymal cells of this organ. Hepatocytes are exceptionally active in the synthesis of bile salts, proteins and lipids, the manufacture of clotting factors and the detoxification of various endogenous and exogenous compounds. The liver is also composed of Kupffer cells, which constitute an important part of the phagocytic system, removing particulate material such as dead and damaged red blood cells, and microbes from the hepatic circulation.

In order to determine the expression pattern of the subfamily IV genes on a single cell type, a homogenous population of cells (tissue culture cells) would accurately
determine whether a single cell is capable of expressing multiple subfamily IV genes concurrently. The expression of the different subfamily members in cell types of different lineage was next tested. PC12 cells, derived from rat adrenal glands, were selected because the 1a gene product was first isolated from bovine adrenal chromaffin granules (Zachowski et al., 1989), and used to clone the 1a gene (Tang et al., 1996). As the 1a gene should be expressed in these cells, PC12 cells would enable us to determine whether multiple members of the same class (class 1) can be expressed in a single cell. Also, the issue of redundancy of expression and/or function of the subfamily IV genes would be automatically addressed. An immobilized cDNA library generated using PC12 cells was analyzed using PCR and the gene specific primers for 1a, 1b, 1c, 1d, 2a, 2b, 5c, 6f, 6g and 6h. In addition to the 1a gene at 325bp, PC12 cells also expressed the following genes: 1c at 297bp, 1d at 692bp, 2a at 856bp, 2b at 381bp, 5c at 497bp, 6f at 400bp, 6g at 449bp, and 6h at 381bp (Fig 2.2C; Table 2.4), each gene product appearing as a single predominant band. Therefore, these data indicate that besides the 1a gene, PC12 cells express multiple subfamily IV genes of class 1 in addition to members of the different classes.

The second cell type examined was J774, a mouse macrophage-like cell line. Macrophages are professional phagocytes that engulf apoptotic cells and other pathogens, which they deliver to intracellular vesicles or vacuoles that secrete digestive enzymes that eventually degrade the contents of these cellular compartments. Macrophages are also known to secrete cytokines that facilitate their various tasks. These innate immune macrophage functions illustrate the importance of secretion and the need for membrane cycling. Yeast Drs2 has been implicated in budding of clathrin coated vesicles from the
Figure 2. Multiple subfamily IV genes are expressed in mouse liver. PCR was performed using 5 μg of mouse liver template and gene specific primers. These results are representative of three similar experiments.
### Expression of type IV P-ATPases in Murine Liver

<table>
<thead>
<tr>
<th>Subfamily Member</th>
<th>Northern Analysis (Halleck et al., 1999)</th>
<th>In Situ Analysis (Halleck et al., 1999)</th>
<th>PCR Analysis</th>
<th>Database**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1b</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1c</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1d</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>1k</td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>1m</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>2a</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2b</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3b</td>
<td>ND</td>
<td>++</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>5c</td>
<td>ND</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5d</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>6f</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>6g</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6h</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Not Determined  ** Expression based on quantifying EST.

**Table 2.2** Comparison of subfamily IV gene expression patterns in mouse liver determined by PCR, northern blot and in situ analysis.
<table>
<thead>
<tr>
<th>Subfamily Member</th>
<th>Northern Analysis (Hallock et al., 1999)</th>
<th>in situ Analysis (Hallock et al., 1999)</th>
<th>PCR Analysis</th>
<th>Database **</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1b</td>
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<td>+</td>
</tr>
<tr>
<td>1c</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1d</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1k</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>1m</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
</tr>
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<td>2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5b</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>5c</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5d</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>6f</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6h</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Not Determined  **Expression based on quantifying EST

**Table 2.3** Comparison of subfamily IV gene expression patterns in mouse brain determined by PCR, northern blot, and in-situ analysis.
late Golgi (Chen et al., 1999), whereas members of the Drs2-family are critical for the budding of endocytic vesicles (Pomorski et al., 2003). It would not be surprising; therefore, if macrophages could express multiple subfamily IV members as their functions are enabled by membrane flexibility and protein secretion. Therefore, J774 cells were an attractive cell type for examining subfamily gene expression. J774 cells expressed the following genes: 1a at 325bp, 1c at 297bp, 1d at 692bp, 2a at 856bp, 2b at 381bp, 5c at 497bp, 6f at 400bp, and 6h at 381bp, which produced a single distinct band at the predicted molecular weight. Additionally, the 6g gene was expressed as two splice variants migrating at 449bp and 550bp (Fig 2.2A; Table 2.4). These observations illustrate a similar pattern of subfamily IV gene expression in PC12 cells and J774 cells, indicating that cells of different lineage are capable of expressing the same subfamily IV genes.

Because PC12 cells and J774 cells both utilize secretion in order to perform their respective functions, we can hypothesize that cells that have similar function may display a similar pattern of subfamily IV P-type ATPase gene expression. Determining whether these genes transport the same substrate in the two different cell types will require further biochemical and physiological characterization of these genes.

NIH3T3 cells represent mouse fibroblast cells that have been described as pre-adipocytes or fat cells. NIH3T3 cells were selected because this cell line was used in an earlier study that characterized 5c, also dubbed p-locus fat associated ATPase (pfatp), that is presumably responsible for modulating body fat content in rodents (Dhar et al., 2002). Analyzing subfamily IV gene expression in these cells would thus further address the question of redundancy of expression within the same cell and between cell types.
Figure 2.2 Multiple subfamily IV genes are expressed in A) J774 cells, B) NIH 3T3 cells and C) PC12 cells. PCR was performed using 5 μg of mouse liver template and gene specific primers. These results are representative of three similar experiments.
<table>
<thead>
<tr>
<th>Subfamily Member</th>
<th>Expected Size (bp)</th>
<th>J774 Cells</th>
<th>3T3 L1 Cells</th>
<th>PC12 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>335</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1b</td>
<td>900</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1c</td>
<td>297</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1d</td>
<td>690</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1k</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1m</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2a</td>
<td>856</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2b</td>
<td>381</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>-</td>
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<td>+</td>
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<td>+</td>
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<td>6g</td>
<td>449,550</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6h</td>
<td>381</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Table 2.4* Comparison of subfamily IV gene expression patterns in J774 cells, NIH 3T3 cells and PC12 cells determined by PCR, northern blot and in-situ analysis.
The NIH3T3 immobilized cDNA library generated PCR products of 1c at 297bp, 1d at 692bp, 2a at 856bp, 2b at 381bp, 5c at 497bp, 6f at 400bp, 6g at 449bp, and 6h at 381bp. 1a and 1b are not expressed in NIH 3T3 cells (Fig 2.2B; Table 2.4). In comparison to the PC12 cells and J774 cells, the NIH 3T3 cells showed a unique pattern of expression.

2.4 Discussion

While northern blot and in situ hybridization methods of analysis are relatively sensitive, investigations performed using either method require large amounts of whole tissue, plus most relevant to our hypothesis, these methods are not very useful when analyzing the simultaneous expression of multiple genes. Polymerase chain reaction (PCR) was an attractive option for determining gene expression profiles within a cell type for a combination of factors. Firstly, PCR exhibits a great ease with which multiple genes can be analyzed because a small quantity of DNA or RNA is required to amplify the desired gene. Therefore, the same DNA or RNA preparation can be used to amplify numerous genes using the respective gene-specific primers. Additionally, PCR is known for its relatively high probing sensitivity; minuscule amounts of DNA being detectable using PCR.

This study demonstrates that most of the subfamily IV genes, specifically 1c, 1d, 2a, 2b, 5c, 6f, 6g and 6h, are broadly expressed; whereas 1a shows a more restricted expression pattern. The fact that the 1a gene, which transports aminophospholipids in vesicles is not expressed in NIH3T3 cells suggests that multiple aminophospholipid
translocase genes exist since all cells have a plasma membrane aminophospholipid translocase, further suggesting a redundant role for some of the subfamily genes. Furthermore, because 1b was not expressed in the three cell lines tested, we must consider the possibility that the expression of this gene is highly restricted, and may be limited to cells with a particular function.

Based on the observations that were made with the three individual cell line libraries, we can conclude that there are several subfamily genes that are expressed in a single cell type. This study in itself is insufficient to determine why this may be the case; however, one may speculate that some of these broadly expressed genes might serve to transport general cellular substrates i.e. those that are fundamental in all cells, thus serving as housekeeping genes. On the other hand, expression of some of the subfamily genes, 1a and 1b for instance, might be governed by cellular function. In cells whose tasks depend on the active transport of required substrates into the cell, or on the secretion of various cellular cytokines or other proteins, these cells might be expected to express other transporters, in addition to housekeeping genes, depending on the specialized cellular function they perform.

That yeast cells express subfamily IV genes from class 1 (DRS2), class 2 (NEO1), class 3 (DNF2, DNF3) and class 4 (DNF3) supports our observation and hypothesis, i.e. that mammalian cells express multiple genes from different classes; and further, that these genes likely have overlapping (housekeeping) or unique functions within the same cell. Of the ten genes that were tested, some were expressed in a non cell-type specific manner as is seen with either the PC12 cells or the J774 cells, which show similar patterns of expression. Alternatively, the data generated with the NIH3T3 cells shows a
unique pattern of expression. Consequently, although 1c, 1d, 2a, 2b, 5c, 6f, 6g and 6h were found in all the cDNA libraries tested, 1d, 2a, 2b, 6f, 6g and 6h, which unlike 1a, have not been linked with the transport of any substrate or unlike 1c, and 5c, linked with any physiological disorders (although the precise anomalies in cell function associated with diseases linked with mutations in 1c and 5c are yet unknown), stand as the strongest candidates for the plasma membrane aminophospholipid translocases.

The characterization of the subfamily members will necessitate the analysis of a greater number of different cell types, in order to constitute a representative sample that will facilitate the confirmation of some of these tentative conclusions. This in turn will serve as a key tool in the identification of important players in the translocation of amino phospholipids across the plasma membrane, a venture that defines healthy versus dying in the cellular milieu.
Chapter 3
Signal transduction events induced by apoptotic cells inhibit HIV transcription in monocytes/macrophages

3.1 Introduction

Macrophages are professional phagocytes that are able to identify and engulf apoptotic cells using specific receptors that recognize modifications on the apoptotic cell surface. The appearance of the aminophospholipid phosphatidylserine (PS) in the external leaflet of the plasma membrane is one of the earliest features used to distinguish apoptotic cells (Martin et al., 1995; Verhoven et al.; 1995). The plasma membrane of viable, healthy cells is characterized by an unequal distribution of phospholipids across the bilayer, with PS being restricted to the inner leaflet of the plasma membrane (Williamson and Schlegel, 1994). The expression of PS in the outer leaflet of apoptotic cells serves as a signal for triggering their recognition, and subsequent engulfment by macrophages and other phagocytes (Fadok et al., 1992; Schlegel and Williamson, 2001). Evidence that PS is a key player in the recognition of apoptotic cells by macrophages has been demonstrated by pre-treating apoptotic cells with annexin V, a Ca^{2+}-dependent PS-binding protein, which blocks the uptake of apoptotic cells (Krahling et al., 1999). Pre-treating macrophages, which constitutively express low levels of surface PS, with
annexin V also blocks engulfment, indicating that macrophage PS is required for engulfment (Callahan et al., 2000; Callahan et al., 2003b).

Numerous macrophage receptors are known to interact with ligands on the apoptotic cell surface, resulting in the tethering of apoptotic cells to the macrophage, which constitutes the initial stage of phagocytosis (Hoffman et al., 2001; Lauber et al., 2004). The engagement of phagocytic receptors also triggers essential signaling pathways, which induce cytoskeletal changes in the macrophage that are required for the engulfment of apoptotic cells. Studies in the nematode, *Caenorhabditis elegans*, identified genes in two redundant pathways that are involved in the recognition and engulfment of cell corpses. In the first pathway, two membrane proteins, ced-1 and ced-7 (mammalian homologs, LDL receptor-related protein (LRP) and ABC transporter ABCA1) have been shown to function upstream of intracellular adaptor protein ced-6 (GULP) (Zhou et al., 2001; Liu et al., 1998). In the second pathway, ced-2, -5 and -12 (CrkII, Dock180 and ELMO) function to activate ced-10 (Rac1), thereby regulating the actin cytoskeleton and controlling cell shape changes required for processes such as phagocytosis of apoptotic cells, cell migration and axon outgrowth (Lundquist et al., 2001; Wu et al., 2002; deBakker et al., 2004).

Human immunodeficiency virus (HIV), the etiological agent of acquired immune deficiency syndrome (AIDS), has the capability of selectively infecting and ultimately incapacitating the immune system. The progressive depletion of both CD4$^+$ and CD8$^+$ T cells, one of the hallmarks of HIV-1 infection, is caused in part by a general increase in apoptosis of both infected and uninfected T cells (Fauci, 1993). HIV also targets macrophages and alters their production of inflammatory cytokines (Breen et al., 1990;
Merril and Chen, 1991), expression of surface receptors (Kedzierska et al., 2002) and phagocytic function (Kedzierska et al., 2000; 2001; Biggs et al., 1995), thus crippling key innate immune functions. Given the documented increase in the incidence of apoptosis during HIV infection, it is probable that these dying cells, and features associated with apoptosis, might create a microenvironment that influences HIV infection and replication. Consistent with this hypothesis, it has been demonstrated that HIV infected cells, as well as the HIV virions, have PS exposed on their surface, and that PS is a cofactor for establishing HIV infection in monocytic cells (Callahan et al., 2003a; Ma et al., 2004).

The PS expressed on HIV virions, and/or the PS expressed on macrophages is functional since PS inhibitors are able to reduce HIV infection of U937 monocytic cells and monocyte-derived macrophages (Callahan et al., 2003a). Although PS might influence the early stages in the interaction between viral and target cell membrane, virus binding/attachment is not impaired in the presence of PS inhibitors (Callahan et al., 2003a). Therefore, it remains unclear precisely how this membrane phospholipid impacts the establishment of HIV-1 infection. In this chapter, we explore the role of PS during various stages of HIV-1 infection of monocytic cells that occur downstream of virus binding. In addition, we test whether HIV is able to utilize certain features of apoptosis, such as PS display, in order to increase its infectivity, and in particular, how the signaling events initiated by apoptotic cells might influence HIV-1 replication in infected macrophages.

3.2 Materials and Methods
3.2.1 **Cell Lines.** U937 human monocytic cells, U1 chronically infected human monocytic cells, and Jurkat human T lymphocytes were cultured in complete RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin/ml, 100 µg of streptomycin/ml and 2 mM glutamine at 37°C in 5% CO₂. 293T human embryonic kidney cells were cultured in complete DMEM medium supplemented with 10% FBS and incubated at 37°C in 5% CO₂. Ghost cells stably expressing CD4 and the chemokine receptor CCR5 and a Tat-dependent reporter construct consisting of the HIV-2 long terminal repeat enhancer-promoter directing the expression of green fluorescent protein (GFP) as previously described (Morner et al., 1999) were cultured in complete DMEM supplemented with 500 µg/ml G418, 1.0 µg/ml puromycin and 100 µg/ml hygromycin. Ghost cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr Vineet N KewalRamani and Dr Dan R Littman. Peripheral blood mononuclear cells were isolated from whole blood obtained from healthy donors according to institutional guidelines. Mononuclear cells were obtained by differential centrifugation using a Ficoll/Hypaque gradient (Sigma Chemicals) as previously described (Henderson et al., 1997). The cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS, 100 U of penicillin/ml, 100 µg of streptomycin/ml and 2 mM glutamine at 37°C in 5% CO₂; macrophages were separated from lymphocytes by adherence to plastic flasks overnight. After removal of the non-adherent cells, monocytes were differentiated by culturing in plastic tissue culture plates for 5 to 7 d prior to infection. Reactivation of latent proviral HIV in U1 cells was done by stimulating cells with 10ng/ml of phorbol ester or 10U TNFα.
3.2.2 Preparation of HIV and infections. Virus was generated by transfecting 293T cells with 15 µg of pHXBnPLAP Nef+ DNA (Chen et al., 1996) or HIV Bal DNA, and 3 µg of Rev in a Rous sarcoma virus expression construct (RSV) by CaPO₄ transfection (Pear et al., 1993). Replication incompetent virus was similarly generated, except 15 µg of pNL43-Luc(+)-Env(-) (HIV-luc) DNA (Henderson et al., 1995), and 3 µg of HXB2 or JRFL envelope DNA were used with 3 µg of Rev DNA. 293T transfection efficiency was monitored by luciferase activity. For infections, 1.0 ml of undiluted viral stocks was added to 3.0 X 10⁵ U937 cells or primary macrophages in the absence or presence of either 0.01 µM recombinant annexin V protein (Callahan et al., 2003a), 15 nM PC vesicles or PS vesicles prepared from egg PC or brain PS respectively (Pradhan et al., 1994), purchased from Avanti Polar Lipids. Cells were harvested 24 h post infection and used in the indicated assays. Expression of HIV-luc was measured using the Promega Luciferase Assay System kit whereas HIV release was monitored by p24 ELISA (Perkin Elmer).

3.2.3 Expression plasmids and transfections. Plasmids expressing GFP-tagged ELMO1 (Gumienny et al., 2001) were a gift from Dr. Kodi Ravichandran, University of Virginia. The siRNA for ELMO designed to target 19 nucleotides in human ELMO2 (Katoh et al., 2003) was a gift from Dr. Hironori Katoh, Kyoto University. 293T cells were transiently transfected either with no DNA, 3 µg of HIV-luc DNA or with 3 µg of SV2-luc DNA serving as a control, and 3 µg of Elmo DNA. Cells were lysed 24-h post transfection and assayed for luciferase activity. An MSCV-Elmo1GFP construct was generated by subcloning ELMO1 from pEBB ELMO1GFP via blunt-end ligation into the
Hpa1 site of MSCV2.1 vector (MSCV2.1 vector was a gift from Dr. G Nolan, Stanford University). Virus for transduction was generated by transfecting 293T cells with 10 µg of MSCV-Elmo1GFP, 2 µg of Tat DNA, 2 µg of pEco and 2 µg of LVSVG, by CaPO4 method. The supernatant collected from these cells 48 h post transfection was used to transduce 3.0 X 10⁵ U937 monocytic cells or 10⁶ monocyte-derived macrophages for 4 d. Transduction efficiency was monitored by FACS analysis for GFP positive cells. For nucleofection, 10⁶ U937 cells were resuspended in 100 µl Nucleofector solution (Amaxa Biosystems) along with 7 µg of Elmo specific or control RNAi plasmid and transfected using the Amaxa Nucleofector system (Amaxa Biosystems, Cologne Germany).

3.2.4 Virus free fusion assay. 293T cells were transfected with 2 µg of M-tropic JRFL envelope DNA, and 2 µg of Tat DNA by CaPO4 method. CD4⁺CCR5⁺ Ghost cells were added at an equal density to the transfected 293T cells, and co-cultured for 24 h. Following the 24 h incubation, GFP expression was read on a Perkin Elmer spectrophotometer. Fusion was also assayed by determining syncitia formation.

3.2.5 PCR detection of reverse transcription intermediates and integrated provirus. For the detection of reverse transcription products, extrachromosomal DNA was isolated from Hirt supernatant extractions as originally described (Hirt B, 1967). The primer sets for newly synthesized strong stop DNA were (5’GGCTAACGAGGAACCCACTG3’) and (5’CTGCTAGAGATTTTCACACTGAC3’) (Passati et al., 2001). PCR was performed in a 50 µl reaction mixture and cycled as follows: Denaturation step at 94°C for 3 min, followed by 29 cycles of 94°C for 1 min, 46.8°C for 2 min, 72°C for 3 min; and a final
extension of 72°C for 10 min. Integrated viral DNA was detected by nested Alu-PCR amplification (Butler et al., 2001). Briefly, a 22-cycle first-round used primers corresponding to a consensus sequence found in the Alu repetitive elements (5’TCCCAGCTACTCGGGAGGCTGAGG3’) and the U3 region of the 3’ long terminal repeat (5’AGGCAAGCTTTATTGAGGCTTAAGC3’). PCR products from the first amplification were then subjected to a 29-cycle second round using primer set (5’CACACACAAGGCTACTTCCCT3’) and (5’GCCACTCCCCGCTCCCGCCC3’) located within the Nef gene. For normalization, β–actin primer sets (5’CCTAAGGCCAACCGTGAAAAG3’) and (5’TCTTCATGGTGTAGCAGCCA3’) were used. PCR was performed in a 50µl reaction mixture and cycled following standard PCR conditions. Products were resolved on a 1% agarose ethidium bromide gel. The identity of the PCR products was confirmed by southern blot using an internal probe (data not shown).

3.2.6 Cell death induction and analysis. Apoptosis was induced by treatment of 5 x 10⁵ target cells/ml with 4 µg/ml of the topoisomerase inhibitor camptothecin for 15 h at 37°C (Callahan et al., 2003b). Cells were washed 3 x in PBS, and apoptotic cells were determined by pre-incubating 10⁶ cells in 2 µg/ml of annexin V-FITC in 100 µl of staining buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2 mM CaCl₂) containing 1% FBS (+ FBS) for 15 min on ice. Cells were resuspended to 500 µl in binding buffer and analyzed immediately by flow cytometry. This treatment produced apoptosis in 60% of the cells.
3.3 Results

3.3.1 HIV-1 proviral integration in macrophages requires PS. As reported (Callahan et al., 2003a), PS is a co-factor for HIV infection of monocyctic cells, although binding or attachment is unaffected by PS inhibitors leaving open the question of the stage of viral lifecycle at which this lipid acts. Because PS mediates homotypic recognition between muscle cells in the process of myotube formation through fusion (van den Eijnde et al., 1997), PS could enhance fusion between viral envelope and the target cell membrane. Using a virus-free fusion assay to test this possibility, 293T cells displaying surface PS as a result of transfection with M-tropic JRFL envelope DNA, and Tat DNA were overlain with Ghost cells stably expressing CD4, CCR5 and a Tat-dependent reporter construct consisting of the HIV-2 LTR directing the expression of green fluorescent protein. Successful fusion of the two membranes would result in fluorescence as well as syncytia formation. Following the 24 h incubation, fusion was similar in the absence and in the presence of annexin V (Figure 3.1), consistent with the observations of Ma and colleagues (Ma et al., 2004). This observation implies that PS effects are exerted at stages of the viral lifecycle beyond binding and fusion.

PS interacts with specific receptors on the phagocytic cell surface and downstream signals initiated by these interactions could affect the establishment of HIV infection. Therefore, the ability of PS to influence the generation of early reverse transcriptase (RT) products, specifically strong stop DNA, which occurs after viral entry and uncoating in the cytoplasm, was tested. U937 monocytes were infected with HIV-1 in the absence or presence of annexin V protein, which inhibits infection, and RT
Figure 3.1 PS does not influence fusion. 10⁴ 293T cells were transduced with 2 μg of M-tropic JRFL envelope DNA, and 2 μg of Tat DNA and overlaid with 10⁵ CD4⁺CCR5⁺ Ghost cells. After 24 h fusion was assayed by A) determining syncytia formation or B) GFP expression.
products were monitored using PCR. In the presence of annexin V, the production of strong stop DNA remained unaffected (Figure 3.2A). A nested PCR assay that utilized primers to genomic Alu sequences and HIV LTR sequences (Butler et al., 2001) was employed to assure that amplified sequences were HIV provirus that successfully integrated into the host genome. As shown in Figure 3.2A, there was a substantial decrease of HIV provirus in monocytic cells infected with HIV in the presence of annexin V compared to controls infected in the absence of annexin V, indicating that PS was influencing proviral integration in these cells.

To validate that PS had similar effects on the ability of HIV-1 to establish infection in primary cells, monocyte-derived macrophages (MDM) were infected with HIV-BaL in the absence or presence of annexin V. Similar to what was observed with U937 cells, annexin V had no effect on the generation of strong stop DNA but did block HIV-1 provirus integration (Figure 3.2B). Furthermore, infecting monocytic cells in the presence of PS vesicles, which also inhibit infection, diminished HIV-1 provirus (Figure 3.2C). Taken together, these data indicate that PS or signaling pathways initiated by PS, does not alter early stages of HIV binding, entry and reverse transcription, but is required for establishing HIV provirus integration.

3.3.2 Apoptotic cells inhibit HIV-1 transcription. Because HIV infection of lymphocytes has been associated with an increase in apoptosis, and because apoptotic cells engage a variety of receptors on macrophages, apoptotic cells might regulate HIV replication in infected monocytic cells. To test this possibility, U937 cells were infected with the replication defective HIV-1 NL4-3.Luc clone, and after 24 h, infected cells were
Figure 3.2 PS is required for the establishment of HIV provirus in monocyte cells. A) $1 \times 10^6$ U937 cells or B) $1 \times 10^5$ MDM were infected with HXB2 and HIVBal strains, respectively, at a multiplicity of infection of 0.5 - 1.0, in the presence or absence of annexin V (AV). After 24 h, viral DNA and genomic DNA were harvested, and the generation of strong stop DNA and integrated provirus were determined by PCR. β-actin was used as a DNA loading control for provirus. C) $1 \times 10^5$ MDM were infected with HIVBal strain in the presence of PC or PS vesicles. These data are representative of three independent experiments.
Figure 3.3 Inhibition of HIV-1 transcription in macrophages by apoptotic cells. A) Apoptosis was induced in Jurkat cells by treatment with camptothecin for 15 h at 37°C. This treatment consistently yielded about 60% apoptotic cells, as determined by labeling with annexin V-FITC. B) 3 x 10^6 MDM infected with an HIV-Luc virus, were co-cultured with viable or apoptotic Jurkat cells at a ratio of 1:20. Cells were lysed after 24 h, and luciferase activity was analyzed. The same experiment was repeated in U937 cells. C) 3 x 10^5 U937 cells infected with an HIV-Luc virus, were co-cultured with different ratios of viable or apoptotic Jurkat cells. Cells were lysed after 24 h, and luciferase activity was analyzed. Each data point represents three independent infections and error bars show the standard deviation. * p < 0.05 (TTEST). These data are from a single experiment that was repeated three times.
co-cultured with either viable Jurkat cells or apoptotic Jurkat cells. Jurkat cells treated with camptothecin to induce apoptosis, resulted in a population of cells that was over 60% apoptotic, using FITC labeled annexin V to detect surface PS (Figure 3.3A). HIV transcription as measured by luciferase activity in U937 cells was decreased by 70% in the presence of apoptotic cells as compared to viable cells (Figure 3.3 B and C).

To determine whether signals delivered by apoptotic cells regulate the induction of latent proviral HIV-1, U1 cells, a pro-monocytic cell line derived from U937 cells that harbor latent HIV-1 provirus that can be activated by various chemical stimuli or cytokines (Folks et al., 1987), were used. Cells were stimulated either with phorbol myristate acetate (PMA) or TNFα and co-cultured in the absence or presence of either viable or apoptotic cells for 24 h. In the presence of apoptotic cells, U1 cells activated by PMA produced 80% less HIV compared to U1 cells co-cultured in the presence of viable cells, as measured by p24 ELISA (Figure 3.4A). TNFα-induced HIV expression in U1 cells was similarly inhibited in the presence of apoptotic Jurkat cells (Figure 3.4B).

In order to determine whether PS alone was sufficient for the inhibitory effect seen, infected cells were co-incubated with PS vesicles (Figure 3.5A); however, this treatment did not reproduce the observations made with apoptotic cells. Furthermore, when the PS displayed on the apoptotic cell surface was blocked with annexin V, there was no effect on HIV-1 transcription (Figure 3.5B), suggesting that apoptotic cells are inhibiting HIV transcription through a mechanism that is not solely dependent on PS. These effects were reproducible in U1 cells, which were similarly insensitive to the addition of PS vesicles after the stimulation of cells with PMA, and the blocking of PS on the apoptotic cell surfaces with annexin V (data not shown).
These results suggest that apoptotic cells either through direct cell-cell contact or via a secreted factor initiate signals in monocytic cells and monocyte-derived-macrophages that directly influence HIV transcription and subsequent replication. Since apoptotic cells have been demonstrated to produce soluble mediators that influence macrophage function (Lauber et al., 2003), transwell chambers of 0.4 µm pore size were used to determine whether apoptotic cells required contact with infected cells to inhibit HIV-1 transcription. As shown in Figure 3.5C, when apoptotic and infected cells were separated in a transwell, HIV-1 transcription was significantly reduced, suggesting that direct cell contact was not required, and further implying that apoptotic cells secrete a factor that partially inhibits HIV transcription.

3.3.3 CrkII/Dock180/ELMO inhibit HIV transcription. The recognition of apoptotic cells by macrophages triggers signaling cascades that are necessary for engulfment of apoptotic targets. For example, over expression of Elmo, a key player in the CrkII/Dock 180 pathway is sufficient to induce actin cytoskeleton re-organization associated with phagocytosis of latex beads (Gumienny et al., 2001). In addition, phagocytosis of apoptotic cells is regulated by the RhoG signaling pathway, mediated through Elmo (deBakker et al., 2004). In order to determine whether signals associated with recognition and clearance of apoptotic cells could inhibit HIV transcription, 293T cells were transiently transfected with an HIV-luciferase cDNA construct in the absence or presence of CrkII, Dock 180 or Elmo. As shown in Figure 3.6A, over expression of CrkII, Dock 180, or Elmo specifically inhibited HIV transcription as compared to cells transfected with HIV-luc in the absence of either CrkII, Dock 180 or Elmo. The
inhibition produced when CrkII, Dock 180 and Elmo were used in combination was not significantly greater than that produced when either protein was used alone, suggesting that all three proteins participate in the same step that is inhibitory to HIV transcription. This response is specific to HIV since transfection with the SV2 promoter (pSV2-Luc) resulted in no difference when co-transfected with or without Elmo (Figure 3.6B). To determine whether Elmo influenced HIV transcription in a physiologically relevant context, HIV-luc infected U937 cells were transduced either with MSCV-Elmo1GFP or MSCV empty vector; harvested 5 days post infection and luciferase activity measured to monitor provirus transcription. The expression of Elmo was confirmed in these cells by flow cytometry (Figure 3.7A). Over-expression of Elmo1 reduced HIV transcription in U937 cells by about 50% compared to controls (Figure 3.7B). Significantly, these observations were reproduced in primary MDMs transduced with MSCV-Elmo1 (Figure 3.7C).

The role of Elmo was further substantiated in experiments in which Elmo specific siRNA was used to reduce endogenous Elmo expression. We initially confirmed that the siRNA strategy could reduce Elmo expression using an Elmo-GFP fusion protein. Co-transfecting the Elmo-GFP construct into 293T cells with Elmo siRNA drastically reduced expression when compared to the control siRNA (Figure 3.8 A and B) confirming that Elmo siRNA was functional. When Elmo was knocked-down in infected U937 cells (Figure 3.8 C) that were subsequently co-incubated with apoptotic cells there
Figure 3.4 Apoptotic cells inhibit induction of latent HIV. 3 x 10^5 U1 cells activated using A) 10 ng/ml of PMA or B) 10U of TNFα were cocultured with viable or apoptotic Jurkat cells at a ratio of 1:20. Culture supernatants were collected after 24 h, and analyzed by HIV-1 p24 ELISA. Results expressed as mean +/- standard deviation of three replicate samples. This experiment was repeated three times. * p < 0.05.
Figure 3.5 PS is not required for inhibition of HIV transcription. 3 x 10^6 infected U937 cells were co-incubated with A) 15nM PC vesicles or 15nM PS vesicles, B) viable Jurkat cells or apoptotic Jurkat cells in the absence or presence of annexin V. C) 3 x 10^6 infected U937 cells were plated in transwell plates and co-incubated with viable or apoptotic Jurkat cells either directly or with the Jurkats in transwell insert. After 24 h, cells were lysed and analyzed for luciferase activity. Results represent three separate experiments. * p < 0.05 compared with HIV-luc.
was a 3-fold rescue in HIV transcriptional activity (Fig 3.8 D) indicating that the ability of apoptotic cells to inhibit HIV transcription requires Elmo.

3.4 Discussion

In this study we demonstrate that signals induced by PS block infection of monocytic cells early in the virus’ life cycle. In cells that have an established infection, apoptotic cells displaying surface PS inhibit HIV transcription and replication through a PS-independent mechanism. Furthermore, we show that Elmo, which regulates the Rac signaling pathway during engulfment of apoptotic cells, suppresses viral transcription. Apoptosis is an important and natural process that animal cells utilize to shape organs and tissues during development, and to maintain tissue homeostasis throughout life; however, apoptosis is also associated with disease. Apoptotic cells are physically characterized by a fragmented nucleus, condensed chromatin and by the exposure of the aminophospholipid PS in the outer leaflet of their plasma membrane, a general feature used to distinguish healthy cells from their apoptotic counterparts. We have described the role of PS during the early stages of HIV infectivity, where this aminophospholipid affects events after binding and fusion but prior to integration. Our observations are consistent with those reported by Ma et al., who describe a role for annexin II during HIV infection of macrophages (Ma et al., 2004). Annexin II, like annexin V, is a member of the annexin family of Ca\(^{2+}\)-dependent phospholipid-binding proteins, which is present on the surface of viable macrophages and functions during phagocytosis (Fan et al., 2004). As annexin II is not bound to PS on the macrophage surface, its PS binding site is
Figure 3.6 CrkII/Dock180/Elmo inhibit HIV transcription. A) 293T cells were transiently transfected with HIV-luc and either CrkII, Dock180 or Elmo or B) with SV2-luc and Elmo. Cells were lysed 24-h post transfection and assayed for luciferase activity. Each data point represents three independent transfections. These data are from a single experiment and are representative of three experiments.
Figure 3.7 Elmo inhibits HIV transcription in monocytes. A) $3 \times 10^5$ U937 cells transduced with MSCV or MSCV-ELMO-GFP and analyzed via flow cytometry. B) $3 \times 10^5$ U937 cells and C) $1 \times 10^6$ MDM were infected with HIV-Luc virus. Twenty-four h post-infection, cells were transduced with MSCV alone or with MSCV-Elmo-GFP. Cells were harvested 4 d post-transduction, and luciferase activity was measured. Each data point represents three independent infections. These data are from a single experiment that is representative of three independent experiments.
available to bind PS on the virion. Indeed, blocking annexin II on the macrophage surface using antibodies inhibits infection by principally influencing a step prior to proviral integration (Ma et al., 2004).

Our data indicate that recognition of apoptotic cells by infected macrophages suppresses HIV provirus transcription, and that soluble factors produced by apoptotic cells contribute to this activity. A soluble lipid factor, lysophosphatidylcholine (LPC), produced by apoptotic cells has been shown to attract monocytes and macrophages (Lauber et al., 2003) although preliminary data from our laboratory indicates that LPC is not sufficient to inhibit HIV transcription (Figure 3.9A). Other studies have shown that ingestion of apoptotic cells actively suppresses immune and inflammatory responses (reviewed in Savill et al., 2002), in part due to the secretion of anti-inflammatory mediators such as TGF-β (Huynh et al., 2002), although in our system, TGF-β alone is not sufficient for inhibiting HIV transcription (Figure 3.9B). On the contrary, HIV infection has been associated with macrophage activation and an increase in the levels of TNFα and IL-6 (Merrill et al., 1989; Breen et al., 1990). That LPC is not responsible for the downregulation of HIV transcription does not preclude the involvement of lipid moieties secreted by apoptotic cells. These lipid formations may be in the form of micelles or membrane blebs, which may be of sizes small enough to penetrate a 0.4µ filter, and further be sufficient to inhibit HIV transcription. We hypothesize that the anti-inflammatory signals delivered by apoptotic cells through multiple and redundant effectors, mediate the suppression of HIV transcription. Furthermore, that the transmembrane receptor that activates the CrkII/Dock180/Elmo pathway in higher
Figure 3.8 Rescue of HIV transcription upon diminishing Elmo expression. A) $5 \times 10^5$ 293T cells were transfected with control siRNA and ElmoGFP or B) Elmo specific siRNA and ElmoGFP. After 48h, cells were viewed under fluorescent microscope and Elmo expression was determined as a measure of GFP expression. C) $3 \times 10^5$ U937 cells were nucleofected with Elmo specific siRNA. Level of Elmo expression was assessed after 24 hr and 48 hrs by Western blot. D) $3 \times 10^5$ U937 cells were nucleofected with control siRNA or Elmo specific siRNA and co-incubated with apoptotic Jurkat cells. After 48 hr, cells were lysed and luciferase activity was measured.
Figure 3.9 LPC and TGFβ do not influence HIV transcription. 3 x 10⁶ infected U937 cells were treated with A) 20μM LPC or B) 13ng/ml TGFβ. After 24h, cells were lysed and analyzed for luciferase activity.
organisms is unknown, further potentiates the involvement of a soluble factor/factors as playing a role i.e. as ligand for the transmembrane receptor.

The engulfment of apoptotic cells is characterized by cytoskeletal changes in the phagocyte that are mediated through a conserved signaling pathway involving Rho-GTPase activation. Elmo functions with CrkII and Dock 180 upstream of Rac1 during engulfment and over expression of Elmo alone significantly inhibited HIV transcription in both U937 cells and MDMs. Although it is unclear what signal transduction pathways and transcription factors downstream of activated Rac 1 may be targeted during engulfment, the Elmo-induced suppression of HIV transcription does not seem to be influencing NF-κB or CEBPβ activity (data not shown).

Our data suggest that apoptotic cells are part of a suppressive microenvironment, which may help to establish reservoirs of latent macrophage populations; however, macrophages remain an important source of infectious virus in tissues such as the central nervous system. This indicates that HIV has mechanisms to overcome the anti-inflammatory signals associated with recognition of apoptotic cells. Furthermore, macrophages from HIV infected patients have impaired phagocytic function (Kedzierska et al., 2003) indicating that HIV is able to alter the immunological function of macrophages to improve its pathogenesis. HIV accessory proteins including Nef and Tat have been demonstrated to have multiple functions including those associated with disrupting cellular signaling pathways (Garza et al., 1995; Renkema et al., 2000; Yang et al., 2005; Kalantari P, unpublished data). Indeed, Nef has been shown to associate with signaling pathways involving small GTPases (Lu et al., 1996) and it was recently reported that Nef binds the Dock 2-Elmo-Rac complex downstream of the T cell receptor
consequently disrupting chemotactic responses of T cells (Janardhan et al., 2004).

Identifying which viral proteins might be responsible for regulating macrophage functions such as phagocytosis of apoptotic cells; in order to favor HIV pathogenesis is currently in progress.
Chapter 4

Conclusions

The clinical manifestations of AIDS, which include the development of characteristic infections and various cancers caused by opportunistic microbes, become most apparent when a patient’s CD4 T cell count drops below 200 cells/ml of blood (CDC). HIV induced apoptosis is predominantly responsible for the lymphopenia that is distinctive of AIDS. In their role as professional phagocytes, macrophages therefore become crucial players in the body’s ability to combat the progression of disease. Firstly, macrophages are primarily responsible for recognizing and eliminating opportunistic pathogens such as *Mycobacterium tuberculosis*, *Pneumocystis carinii* and *Candida albicans*, which tend to overwhelm an HIV infected individual’s compromised immune system. Macrophages fulfill this role by secreting anti-microbial agents such as NO, superoxide and inflammatory cytokines such as TNFα (Mosser et al., 2003). Secondly, macrophages recognize and engulf cells that are undergoing apoptosis, thereby preventing inadvertent inflammation. The uptake of apoptotic cells is facilitated by the interaction between so called ‘eat me signals’ on the apoptotic cells surface, of which PS is one, and specific receptors found on the macrophage surface.

Due to the mode of egress employed by HIV, virus particles collected from infected cells have been shown to bear PS on their surface as well (Callahan et al.,
We initially hypothesized that HIV is able to utilize certain features of apoptosis, such as PS display, in order to facilitate its internalization by macrophages and other phagocytes, thus increasing its infectivity. Through the PS found on the envelope, HIV might be able to exploit the PS receptor (PSR) on the macrophage surface to influence viral fusion or other early entry events. Our results indicate that PS influences neither HIV binding (Callahan et al., 2003a) or fusion; however, PS was found to affect integration of provirus in host cells. We were unable to determine the mechanism through which PS on the viral surface is influencing events that occur in the macrophage nucleus; however, our observations suggest a model in which virus associated PS might cause changes in downstream cytoskeletal events involved in intracellular trafficking and cell motility. As an apoptotic cell surface ligand for the PSR on macrophages, PS is able to initiate signaling cascades in the phagocyte that induce modifications in the actin cytoskeleton that manifest as membrane extensions that facilitate engulfment. This was confirmed by studies in C. elegans showing that over expression of ced-2, ced-5, ced-10, ced-12 or the PSR gene itself (psr-1) rescued an engulfment defect observed in worms mutant in psr-1, suggesting that the PSR likely acts upstream of ced-2, ced-5, ced-10 and ced-12 to control the engulfment of cell corpses (Wang et al., 2003). It is possible therefore, that virus associated PS induced alterations in the actin cytoskeleton influence the assembly and migration of the pre-integration complex (PIC) from the cytoplasm into the nucleus, thereby having an overall effect on the rate of integration (Figure 4.1).

Macrophages present an interesting paradox in their capacity as targets for HIV infection and in their function as phagocytes responsible for clearing the trail of apoptotic debris that is known to accumulate following HIV infection (Muro-Cacho et al., 1993).
Figure 4.1 A model for PS-dependent effects on HIV integration
The contradiction behind this ambiguous relationship between HIV and macrophages is hinged on the fact that the activation of macrophages that encounter apoptotic cells is antagonistic to the productive infection of macrophages. Whereas HIV infection is enhanced by elevated levels of inflammatory cytokines such as TNFα and IL-6 (Merrill et al., 1989; Breen et al., 1990), the uptake and subsequent activation of macrophages following phagocytosis of apoptotic cells triggers the secretion of suppressive cytokines such as TGFβ–1 (Huynh et al., 2002) that prevent macrophages from mounting an inflammatory response. Taken together, it becomes apparent that phagocytosis of apoptotic cells creates a microenvironment that is unfavorable to HIV replication. Our results support this model as our data demonstrate that apoptotic cells inhibit HIV transcription and replication in monocytes and macrophages. Most significantly, we show that the ability of apoptotic cells to inhibit transcription and replication are independent of PS. In addition, we demonstrate that HIV transcription is inhibited in cells over expressing proteins that function during the phagocytic pathway, specifically CrkII, Dock180 and Elmo. In spite of this, macrophages remain susceptible to HIV infection; moreover, due to their resistance to the cytopathic effects of HIV infection, macrophages serve as reservoirs for long-term infection. This ability of HIV to persistently infect macrophages suggests that the virus has developed mechanisms to overcome the ‘protection’ from infection that could presumably be conferred to macrophages as a consequence of their function. HIV proteins Nef and Tat, have been shown to alter the cell cycle (Garber et al., 1999), and sway cellular signaling pathways (Garza et al., 1995; Renkema et al., 2000) to favor HIV replication. It is expected therefore, that mechanisms exist that enable HIV to counter the suppressive effects of
apoptotic cells and phagocytosis. The mechanism through which the virus is able to do this is not known, and elucidating the process is complicated by the fact that the extracellular stimuli that precede the activation of CrkII/Dock180/Elmo leading to Rac activation during phagocytosis remain elusive; however, as illustrated in Figure 4.2, we can speculate that certain HIV auxiliary proteins might deter signaling events that are associated with the recognition and engulfment of apoptotic cells, subsequently inhibiting the expression of anti-inflammatory cytokines and preventing the activation of macrophages down an adverse path.

Overall, it is important to consider how the temporal nature of signaling events influences various stages in the HIV lifecycle. It is conceivable that early PS-induced cytoskeletal changes might tamper with the translocation of the PIC into the nucleus, hence the rate of integration. On the contrary, once there is an established infection, any effects on the virus appear to be PS-independent, and yet susceptible to other features of the phagocytic process, such as the signaling cascades that are involved. It is imperative to bear in mind that these signaling pathways can be initiated by the engagement of other recognition signals on apoptotic cells with other macrophage receptors. There is clearly a parallel between what our data show and the events that define the recognition of apoptotic cells and phagocytosis proper. Proficient recognition of apoptotic cells is attributed primarily to early detection of PS; however, engulfment as a whole is known to be the result of a combination of events and factors that are not entirely contingent on PS. Our findings illustrate the complexity of an innate macrophage function, i.e. phagocytosis, and offer some evidence supporting the idea that the virus is able to overcome cellular barriers to its successful infectivity.
Figure 4.2 A model for phagocytic pathways that are altered by HIV
Given this interpretation, one may speculate that the body may develop novel defense strategies using apoptotic cells and their engulfment to counter debilitating pathogens and conditions as are perpetuated by HIV-1.
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