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Circumferin: A biochemical and immunochemical investigation of a nuclear envelope protein in mammalian cells

by

Jennifer Lynn Lee, B.Sc. (Hons.), B.Ed. (Concurrent)

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the degree of**

Master of Science

**Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario
October 2001
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**“Circumferin: A biochemical and immunochemical investigation of a nuclear envelope
protein in mammalian cells”**

**submitted by Jennifer Lynn Lee, B.Sc. (Hons.), B.Ed. (Concurrent)
in partial fulfillment of the requirements for
the degree of Master of Science**

Thesis Supervisor

Chair, Department of Biology

Carleton University

October, 2001

ABSTRACT

The biochemistry and immunochemistry of circumferin were studied in mouse splenocytes, through 48 h of stimulation with concanavalin A. Immunoblotting revealed four bands with molecular masses of about 72, 75, 78, and 81 kDa. After 24 h of stimulation, the amount of circumferin per cell increased 1.9 times, and it increased 5.6 times after 48 h of stimulation. After 24 h of stimulation, the amount of circumferin present as a proportion of total cellular protein decreased to 0.62 times that of resting lymphocytes, and to 0.27 times that of resting cells after 48 h. Circumferin was detected by immunoblotting and immunofluorescence microscopy in apoptotic cells that had undergone significant protein degradation. Circumferin was partially extracted by treatment with detergent and nucleases. Circumferin was enriched in the pellet fraction through nuclear fractionation. Circumferin was found to possess both N- and O-linked glycosylation, but phosphorylation was not detected.

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I have said on many occasions that I wanted to write this thesis as a “Choose Your Own Adventure” story. Although on several occasions it seemed as though all routes led to trouble, I’m glad I stuck with it to “the end”. I could never have found my way without the help of so many...

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ABBREVIATIONS

a.a.	amino acid
BSA	bovine serum albumin
Con A	concanavalin A
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
ER	endoplasmic reticulum
LAP	lamina-associated protein
LBR	lamin B receptor
mAb	monoclonal antibody
MAR	matrix-attachment region
Nups	nucleoporins
O-GlcNAc	O-linked <i>N</i>-acetylglucosamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PNase F	peptide N glycosidase F
p.s.	post-stimulation
RIPA	radio-immunoprecipitation assay
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
Topo II	DNA topoisomerase II

Chapter 1: LITERATURE REVIEW

1A. The Nuclear Envelope

The nuclear envelope serves to compartmentalize eukaryotic cells into nucleus and cytoplasm, thereby segregating various cellular processes such as transcription and translation. The major structural components of the nuclear envelope are the nuclear membranes, nuclear pore complexes, and the nuclear lamina.

1A1. The Inner Nuclear Membrane

There are two nuclear membranes - an inner and an outer, with the space between them continuous with the lumen of the endoplasmic reticulum (ER). The nuclear membranes are themselves continuous with the membranes of the ER, and frequently, there are also ribosomes bound to the outer nuclear membrane (Gerace and Burke, 1988). Despite this continuity, there are several proteins, such as the lamina-associated proteins (LAPs), that are localized specifically at the inner nuclear membrane. Functions of these integral membrane proteins include allowing for attachment of chromatin and of the lamina (Collas and Courvalin, 2000).

1A1i. Lamina-Associated Proteins

Proteins of the inner nuclear membrane are presumed to play an important role in the spatial and temporal organization of the nucleus throughout the cell cycle. These proteins give the inner nuclear membrane a biochemical identity unique from that of the

outer nuclear membrane and the ER, and help maintain the spatial arrangement of the nuclear envelope through binding to chromatin and the lamina. Proteins of the lamina-associated protein (LAP) family are, with one exception, known or predicted to be type II integral membrane proteins (Georgatos, 2001; Chu *et al.*, 1998). LAP1 is a family of proteins with three isoforms (LAP1A, -B, and -C) (Martin *et al.*, 1995), while LAP2 has at least three major (LAP2 α , - β , and γ) and four minor (LAP2 β ', - δ , - ϵ , and - ζ) isoforms that are highly conserved among mammals (Gant *et al.*, 1999; Berger *et al.*, 1996). All except LAP2 α have a single predicted transmembrane domain near the carboxy terminal and a large nucleoplasmic region (Furukawa *et al.*, 1998). LAP2 α does not possess this domain, and is intranuclear (Gant *et al.*, 1999).

Other differences in the LAP2 isoforms include differential expression. LAP2 β is consistently expressed in proliferating cells, while the α and γ , but not β isoforms are expressed at high levels in non-proliferating cerebellar tissue (Ishijima *et al.*, 1996). Spermatogenesis also presents a difference in LAP2 expression, with the α isoform being the only one detectable (Alsheimer *et al.*, 1998). The isoforms have also been shown to interact differentially with lamins. LAP2 β was shown to bind lamin B, while LAP2 α interacts with the A-type lamins in newly formed nuclei after mitosis, as shown by confocal immunofluorescence microscopy (Dechat *et al.*, 2000).

Inner nuclear membrane proteins that have been shown to bind chromatin include LAP2 (Foisner and Gerace, 1993) and the lamin B receptor (LBR) (Ye and Worman, 1996, Foisner and Gerace, 1993). Those proteins that bind lamins include LAP1, LAP2 and LBR (Foisner and Gerace, 1993). Using the yeast two-hybrid system, Furukawa *et*

al. (1998) have shown that the chromatin-binding and lamin-binding domains of LAP2 are different. During mitosis, LAP1, LAP2 and LBR become dispersed throughout the ER (Furukawa *et al.*, 1998). They found the 76-amino acid (a.a.) lamin-binding region of LAP2 to be sufficient for localizing it to the nuclear envelope and postulated that it is lamin binding that prevents the diffusion of inner nuclear membrane proteins through the ER during interphase. LAP2 binding to both lamins and chromatin is regulated by mitotic phosphorylation (Furukawa *et al.*, 1998).

Using deletion mutants in HeLa cells, an approximately 350 a.a. region in the C-terminal of the α -specific domain of LAP2 α was identified that is required and sufficient for retention of LAP2 α in interphase nuclei and association with the mitotic chromosomes (Vlcek *et al.*, 1999). The association of LAP2 α with chromosomes was found to occur before the association of the membrane-bound isoform, LAP2 β , although both isoforms contain the same putative chromatin-interaction domains (Vlcek *et al.*, 1999).

LBR is an inner nuclear membrane protein expressed in all metazoans that interacts with lamin B, DNA, and human chromodomain proteins (Georgatos, 2001; Vlcek *et al.*, 1999; Worman *et al.*, 1988). It is predicted to have eight membrane-spanning regions (Furukawa *et al.*, 1998). LBR occurs in a complex with lamins, a specific kinase (LBR kinase) and other proteins (Georgatos, 2001).

Other proteins of the inner nuclear membrane include emerin (Bione *et al.*, 1994), MAN1 (Lin *et al.*, 1998) and nurim (Rolls *et al.*, 1999). LAP2, emerin, and MAN1 share a 43-residue motif called the LEM (LAP2-emerin-MAN1) domain (Lin *et al.*, 2000). It

has been suggested that emerin interacts with lamin A, since loss of lamin A expression leads to a variant of Emery-Dreifuss muscular dystrophy, for which emerin was named (Sullivan *et al.*, 1999). Immunofluorescence labelling of emerin in mouse embryonic fibroblasts mutated so that they do not express lamins A/C displays a cytoplasmic pattern more consistent with ER proteins. This suggests that lamin A may serve to fix the localization of emerin in the inner nuclear membrane (Sullivan *et al.*, 1999). Through their interactions with chromatin and the lamina, the inner nuclear membrane proteins thus contribute to the overall organization of the nucleus.

1A2. Nuclear Pore Complexes

The inner and outer membranes are continuous with one another at the nuclear pore complexes. Nuclear pore complexes serve as channels for nucleocytoplasmic trafficking across the nuclear membranes. They also may contribute to the regulation of gene expression through the selectivity by which proteins and RNAs are allowed to cross the nuclear envelope (Gerace and Burke, 1988). These gates between the nuclear and cytoplasmic compartments of cells were first described by Callan *et al.* (1949) and are present in all eukaryotic cells. The structure of the pore complex exhibits both bilateral and radial symmetry and has been well conserved throughout evolution. The nucleoplasmic and cytoplasmic sides of the pore complex are lined by two rings (inner and outer annuli, respectively) each with a diameter of approximately 120 nm. Cytoplasmic filaments and particles line the cytoplasmic ring. Between the two rings, a central core, formed by eight spokes, surrounds a central plug (Wente, 2000). The spokes

project into the interior of the pore, and reduce the channel diameter from about 80 nm to a functional diameter of about 10 nm (Talcott and Moore, 1999). Nuclear filaments form a basket which projects into the nucleus, connected to a distal ring (Akey and Radermacher, 1993). The nucleoplasmic side of the pore complex is believed to be attached to the lamina (Gerace and Burke, 1988).

The density of pores ranges from 2-4 pores/ μm^2 in chick erythrocytes and unstimulated mammalian lymphocytes to over 60 pores/ μm^2 in mature *Xenopus* oocytes and *Acetabularia mediterranea* (Maul, 1977), with an average of 10-20 pores/ μm^2 or 2000-4000/nucleus (Gerace and Burke, 1988). The broad range of pore densities appears to reflect the metabolic activity level of the cell types, with mature, essentially non-proliferative cells such as erythrocytes and resting lymphocytes exhibiting low densities of pore complexes, and highly active cells demonstrating pore complexes at much higher densities (Maul, 1977).

Structural proteins of the nuclear pore complexes are termed nucleoporins (Nups). Recently, mass spectrometry was used to try to identify all detectable Nups in a purified preparation of yeast nuclear pore complexes (Rout *et al.*, 2000). It was found that there are only around 30 yeast Nups, and it is predicted that the larger vertebrate nuclear pore complex will possess about 50 Nups. Immunoelectron microscopy further indicated that the complex consists of a basic subunit that is repeated 16 times (Rout *et al.*, 2000).

Through binding to wheat germ agglutinin, some nuclear pore proteins have been found to possess O-linked N-acetylglucosamine (O-GlcNAc) residues (Snow and Hart, 1998). This modification is attached directly to the polypeptide backbone via serine or

threonine (Holt *et al.*, 1987). The nuclear pore complex contains the highest concentration of proteins with the *O*-GlcNAc modification (Holt *et al.*, 1987). Functions of this modification are thought to include influencing nuclear import, and targeting of proteins to the nucleus. Depletion of *O*-GlcNAc-bearing nucleoporins from *Xenopus* nuclear extracts has been shown to prevent binding and nuclear import of proteins, although nuclear pore complexes are still able to form (Snow and Hart, 1998). *O*-GlcNAc can also act as a nuclear localization signal (NLS), as shown when the sugar was conjugated to fluorescein-tagged BSA, causing the protein to be translocated to the nucleus (Snow and Hart, 1998). Interestingly, although yeast Nups possess the same amino acid motif as mammalian Nups that are modified by *O*-GlcNAc, the yeast Nups are not glycosylated (Miller *et al.*, 1999).

1A3. The Nuclear Lamina

The nuclear lamina is a proteinaceous layer 10 to 50 nm thick lying subjacent to the inner nuclear membrane. Nuclear architecture is organized in part by the anchoring of chromatin to the lamina. The major proteins of the lamina, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), are the lamins. Other proteins of the lamina, which include circumferin, statin, and Young Arrest (YA) are much less well-known. The lamins appear to serve numerous functions, including conferring strength on the nuclear envelope, attaching chromatin to the nuclear envelope, targeting of membrane vesicles to the chromosome surface after mitosis, and interaction with the inner nuclear membrane (Chaly and Stochaj, 1999). Lamins are synthesized

throughout the cell cycle. They are initially soluble in the cytoplasm, and are transported to the nucleus where they are incorporated into the lamina (Lehner *et al.*, 1986).

1A3i. The Lamins

Although lamins are present in most vertebrate and some invertebrate species, they have not been identified in lower eukaryotes such as yeast (Stuurman *et al.*, 1998). The lamins are Type V intermediate filaments, the only nuclear members of the intermediate filament family of proteins (Stuurman *et al.*, 1998). Like other intermediate filaments, the lamins possess an N-terminal head domain and a C-terminal tail domain. They also possess an alpha-helical rod domain, which is 42 amino acids longer than the rod domain of cytoplasmic intermediate filaments (Stuurman *et al.*, 1998). The end segments of this rod domain are highly conserved among intermediate filaments (Stuurman *et al.*, 1998). Like other intermediate filaments, the rod domain of lamins is characterized by a heptad repeat pattern. Hydrophobic residues in some positions and hydrophilic residues in others allow coiled-coil dimers to be formed when two α -helices interact with each other in a parallel, unstaggered manner, as shown by *in vitro* studies (Stuurman *et al.*, 1998). The N- and C-terminal domains possess conserved segments that promote polymerization of lamins. Lamin dimers form head-to-tail polymers, and a meshwork is also formed by lateral, anti-parallel, half-staggered associations with a 24-25 nm repeat (Stuurman *et al.*, 1998).

Post-translational modifications allow the different types of lamins to exhibit different behaviour throughout the cell cycle. All lamins except lamin C of mammals and

Drosophila possess a Ras-like CaaX box (cysteine, two aliphatic residues, any residue) at the C-terminal tail (Stuurman *et al.*, 1998). Post-translational modifications at this sequence, including isoprenylation, carboxy-methylation, and proteolytic cleavage, occur shortly after synthesis and allow B-type lamins to remain attached to the nuclear membrane (Hennekes and Nigg, 1994). Since this sequence is absent in lamin C and is cleaved in lamin A, the A-type lamins are solubilized more easily.

Lamins are broadly characterized as being A-type or B-type. The A-type lamins have an isoelectric point of close to 7 while the B-type lamins are acidic with an isoelectric point of about 5.6 (Gerace and Burke, 1988). All lamins known have molecular weights between 60 and 80 kDa (Cohen *et al.*, 2001). Mammals possess four major variants: lamins A, B₁, B₂, and C. Lamins A and C are alternate splicing products of the same gene, while the B lamins are encoded by separate genes. The B-type lamins are constitutively expressed, but the expression of the A-type lamins is developmentally regulated, generally occurring during or after differentiation (Stuurman *et al.*, 1998). For example, early chick embryos express only lamin B. Lamin A expression increases during development, while lamin B expression decreases (Stuurman *et al.*, 1998). It is suggested that the changes in lamin expression may reflect the transcription state of the chromatin (Moir *et al.*, 1995).

By immunofluorescence microscopy, lamin labelling appears as a thin, even band around the periphery of the nucleus (Chaly and Stochaj, 1999). However, the distribution of lamins is not regular over the whole lamina. By electron microscopy, it was shown that the concentration of lamins is higher in areas of high chromatin concentration, while

nuclear pore complexes occur in areas with a lower concentration of lamins (Belmont *et al.*, 1993). This observation is consistent with the role of lamins in binding chromatin. Lamins bind DNA at specific sequences called matrix attachment regions (MARs). Motifs for attachment to MARs occur in the lamin rod and tail domains (Stuurman *et al.*, 1998).

Invaginations of the nuclear envelope occur throughout the nucleus, and these invaginations possess all the features of the peripheral envelope, such as nuclear pore complexes and lamins (Fricker *et al.*, 1997). Lamins have also been observed in the interior of the nucleus in foci, although it is not clear whether or not the foci are associated with the envelope invaginations (Stuurman *et al.*, 1998). Foci of lamin A were more commonly detected during G1 and were noted to be found in close proximity with condensed chromatin (Bridger *et al.*, 1993). Lamin B foci have also been observed, although these were seen more frequently during S phase, and occurred at sites of DNA replication, as seen by bromodeoxyuridine (BrdU) incorporation (Moir *et al.*, 1994). More recently, cells transiently transfected with LBR fused to green fluorescent protein (GFP) (Ellenberg *et al.*, 1997) and lamin A-GFP (Broers *et al.*, 1999) have shown intra- and transnuclear tubules regardless of cell cycle stage.

1A3ii. Dynamics of the nuclear envelope throughout mitosis

Lower eukaryotes, such as yeast, exhibit a closed mitosis where the nuclear envelope remains intact, but in higher eukaryotes, open mitosis occurs, and the nuclear envelope is reversibly disassembled. During prophase, the nuclear envelope is

disassembled, and pore complexes disappear. It has long been held that small vesicles were formed from the envelope fragments, but recent evidence suggests that it is more likely that the nuclear membrane simply becomes indistinguishable from the ER, with membrane proteins diffusing freely (Collas and Courvalin, 2000). This has been shown by immunofluorescence, confocal, and immunogold electron microscopy, where LAP1 and LAP2, as well as an integral membrane protein of the nuclear pore membrane, gp210, were seen to disperse in the ER during mitosis (Yang *et al.*, 1997). Redistribution to the ER rather than to membrane vesicles has also been shown for LBR in COS-7 cells transfected with LBR-GFP (Ellenberg *et al.*, 1997).

Much of lamin B remains membrane-associated during mitosis, while lamins A and C are solubilized and dispersed throughout the cytoplasm. While in the cytoplasm, the lamins exist in monomeric form (Gerace and Blobel, 1980). The difference in solubility arises due to a difference in post-translational modifications and mode of association with the inner nuclear membrane (Hutchison *et al.*, 2001). At mitosis, lamins show a dramatic increase in phosphorylation on serine and threonine residues - about three-fold for lamins A and C, six-fold for lamin B (Gerace and Blobel, 1982). This phosphorylation, by the mitotic CDC2 kinase, occurs at "P sites" at the ends of the α -helical rod domain (Stuurman *et al.*, 1998). The morphology of the lamina is disrupted as a result of this phosphorylation, contributing to the disassembly of the nuclear envelope at mitosis. When these P sites are mutated, disassembly of the lamina is severely impaired (Heald and McKeon, 1990). Dephosphorylation of lamins occurs at the end of mitosis, allowing the lamins to repolymerize. Another modification exhibited by lamin B is

demethylation during mitosis (Chelsky *et al.*, 1987).

Ellenberg *et al.* (1997) have proposed that during late anaphase, before the decondensation of chromatin, LBR in the ER associates with chromatin, resulting in wrapping of the ER membranes, which are now the new nuclear membranes, around the chromatin. During late anaphase and telophase, segments of the nuclear membrane gradually fuse to form the new interphase nuclear envelope. GFP-tagged Lamin A and B1 have been used to demonstrate the reincorporation of lamins into the lamina (Moir *et al.*, 2000). Lamin B1 was found to begin to associate with the chromosomes as they reached the spindle poles during late anaphase and telophase, and to begin to polymerize almost immediately, as demonstrated by fluorescence recovery after photobleaching (FRAP) and insolubility after treatment with detergent and high ionic strength buffer (Moir *et al.*, 2000). The association of lamin A with the chromosomes was shown to occur later in telophase, through mid-late cytokinesis, after the chromosomes had begun to decondense. Later still, during early G1, lamin A became incorporated into a stable structure in the lamina (Moir *et al.*, 2000). During late anaphase through telophase, pore complexes reappear in the envelope, by sequential relocation of Nups to the chromosome periphery, although it is not known precisely how they are reassembled (Bodoor *et al.*, 1999). Nuclear components are reapportioned to the nucleus by selective import through nuclear pore complexes. After telophase and cytokinesis, expansion and invagination of the nuclear membranes occurs (Ellenberg *et al.*, 1997).

1A3iii. Non-lamin Proteins of the Lamina

Lamins are by far the most abundant proteins of the lamina, and there are only a few non-lamin proteins of the lamina that have been described. Circumferin, the focus of this study, is one of these. Others include statin and the *Drosophila* young arrest (YA) protein. YA is only expressed in ovaries and pregastrulation embryos (Chaly and Stochaj, 1999). It binds chromatin, and interacts with lamin Dm₀, the *Drosophila* homologue of lamin B (Chaly and Stochaj, 1999). Statin is a protein found only in senescent or non-proliferating cells. It disappears when non-proliferating cell cultures are induced to begin dividing again, and it appears when dividing cultures are differentiated or otherwise prevented from proliferating (Chaly and Stochaj, 1999). Its function is not known.

1B. *Circumferin*

As reported in Chaly *et al.* (1984), four hybridomas were produced from mice immunized with mouse or bovine lymphocyte nuclear matrices. Monoclonal antibodies (mAb) PI1 and PI2 labelled both the periphery and the interior of the nucleus, with some characteristic differences. PI1 was later shown to be p62, a protein of the nuclear pore complex (Dabauvalle *et al.*, 1988). MAb PI2 detected antigens at 35, 70, and 140 kDa, and is currently under further study. I1 labelled the nuclear interior, but remains otherwise uncharacterized. Antibody P1, a mAb of the IgM class, was produced against bovine retropharyngeal lymphocyte nuclear matrix. The designation "P" arose from the labelling of the periphery of the interphase nucleus and mitotic chromosomes by this antibody, as observed by indirect immunofluorescence microscopy (Chaly *et al.*, 1984).

The P1 antigen was later briefly referred to as peripherin (Brown *et al.*, 1989; Chaly *et al.*, 1989; Dardick *et al.*, 1989; Chaly *et al.*, 1988) but to avoid confusion with a cytoskeletal protein of the same name, has since been renamed “circumferin”.

Most work on circumferin to date has examined its distribution by immunofluorescence and electron microscopy. In mouse 3T3 fibroblasts at interphase, the staining pattern was similar to that seen for lamins, but mAb P1 revealed some uneven, apparently randomly distributed projections toward the interior of the nucleus which were not seen with lamins. At the beginning of prophase, staining intensity with mAb P1 increased, and the antibody labeled the portions of the chromosomes which were at the nuclear periphery. Throughout mitosis, P1 continued to delineate closely the chromosome masses, showing the two sets of daughter chromosomes by anaphase. At telophase, some staining was also observed in the cell cytoplasm. By G1, staining with P1 was again localized exclusively to the periphery of the nucleus (Chaly *et al.*, 1984).

The distribution of the P1 antigen as seen by electron microscopy of interphase nuclei using immunoperoxidase labelling supported that seen by immunofluorescence. Again, the P1 antigen was seen to occur in a layer underlying the inner nuclear membrane, with some internal projections. The P1 layer was found to be thicker and more irregular than that of lamins (Chaly *et al.*, 1989). Immunogold labeling of interphase nuclei confirmed the localization identified by immunoperoxidase staining (Chaly and Stochaj, 1999). Electron microscopy of immunogold-labelled mitotic cells showed circumferin closely outlining the irregular periphery of the chromosomes (Chaly and Stochaj, 1999).

1B1. Evolutionary conservation of the P1 antigen

The P1 antigen was detected by immunofluorescence labeling with mAb P1 in a wide variety of species and tissues, including mouse embryos up to and including blastocysts, mouse adult somatic cells in culture, mouse 3T3 fibroblasts and rat L6E9 cells, sea urchin embryos up to and including plutei, and adult sea urchin cells (Chaly *et al.*, 1996; 1984; Schatten *et al.*, 1985). It has also been detected in HeLa, Chinese hamster ovary (CHO), rat kangaroo PtK2, and *Drosophila melanogaster* KcA311 cells, as well as in the unicellular alga *Polytomella agilis*, the fungus *Entomophthora aulicae*, and the bean *Vicia hajistana* (Chaly *et al.*, 1986), and in the yeast *Saccharomyces cerevisiae* (Chaly and Stochaj, 1999). The only cell type assayed in which circumferin has not been detected is mature mouse sperm (Schatten *et al.*, 1985). It is not clear whether circumferin is absent from these cells, or is simply undetectable, perhaps due to antibody inaccessibility. The high degree of conservation of the P1 labelling pattern has been interpreted as evidence that circumferin plays an important role in cellular function. Interestingly, although the nuclear envelope does not break down during mitosis in *P. agilis*, the distribution of circumferin in the alga is similar to that seen in the other species tested (Chaly *et al.*, 1986). This has also been shown with yeast, which also has a closed mitosis (Chaly *et al.*, 1999). The implication of these observations is that the association of circumferin with the chromosomes during mitosis does not depend on the breakdown of the nuclear envelope.

1B2. Biochemistry of circumferin

Part of the initial characterization of the P1 antibody included immunoblotting, which revealed a triplet of polypeptides with mobilities between 27 and 31 kDa (Chaly *et al.*, 1984). This was subsequently shown to be artefactual, as it was no longer detected after more stringent blotting conditions (Chaly *et al.*, 1996). Because of the difficulty until recently in obtaining reliable immunoblotting results with mAb P1, little is known of the biochemistry of the P1 antigen. However, the behaviour of circumferin during preparation of nuclear matrices *in situ* has been monitored by immunofluorescence microscopy (Chaly *et al.*, 1985). Nuclei were examined at each step of the nuclear matrix preparation, which consisted of sequential treatment with 0.5% Triton X-100, 10 µg/mL DNase I, low magnesium buffer (0.2 mM MgCl₂), 1 M and 2 M NaCl, and 50 µg/mL each of DNase I and RNase A. For the first three steps, the distribution of circumferin was unaffected compared to intact interphase nuclei (Chaly *et al.*, 1985). After extraction of the samples with 1 M NaCl, however, the distribution of circumferin ceased to be peripheral, and instead was found throughout the interior of the nucleus (Chaly *et al.*, 1985). These results suggest that circumferin may be involved in mediating chromosome order through the cell cycle (Chaly *et al.*, 1985).

1B3. Behaviour of circumferin during changes in cell metabolism

Several studies have investigated changes in circumferin organization as a function of changes in cell physiology. The distribution of circumferin in lymphocytes is not affected by stimulation with concanavalin A, as shown by immunofluorescence

microscopy (Chaly *et al.*, 1988; Setterfield *et al.*, 1985). Since resting lymphocytes are in G₀, whereas stimulated cells pass through G₁, S, and G₂, this suggests that circumferin distribution is conserved at all stages of interphase. However, an investigation of various subtypes of non-Hodgkin's lymphomas revealed that staining of circumferin varied in intensity from one subtype to another (Dardick *et al.*, 1989). Therefore, changes in the quantity of circumferin may reflect other changes in interphase nuclear organization which occur with malignancy.

Another instance in which the organization of circumferin is affected is in heat-shocked cells. In both HeLa cells and in yeast, heat shock results in the redistribution of circumferin into the nucleoplasm, and out of the nucleus into the cytoplasm (Chu *et al.*, 1999). Furthermore, when cells were treated with cycloheximide throughout the heat shock, circumferin still redistributed. These observations suggest that the accumulation of circumferin in the cytoplasm was not a consequence of the effects of heat shock on nuclear import, and did not depend on protein synthesis (Chu *et al.*, 1999).

When rat myoblast L6E9 cells were induced to differentiate into multinucleated myotubes, the intensity of labeling with P1 gradually decreased and was almost undetectable by day 7 (Chaly *et al.*, 1996). By this point, most of the cells had fused to form multinucleated, elongated myotubes. Although the quantity of lamins detected increased during the differentiation process, these changes do not appear to be responsible for the changes in labeling with mAb P1. Circumferin distribution has been observed to be constant in cells of differing lamin composition (Chaly *et al.*, 1996). For example, circumferin has been detected in a conserved distribution in cells which constitutively

express A- and B-type lamins, such as HeLa, as well as in cells which only contain lamin B, such as lymphocytes (Chaly *et al.*, 1996). Interestingly, when myotube nuclei were isolated prior to cell fixation, the characteristic peripheral labelling pattern of P1 was observed (Chaly *et al.*, 1996). It was suggested that a differentiation-specific factor prevented detection of circumferin in intact myotubes. The process of isolating the myotube nuclei may have solubilized this factor, thus allowing circumferin to be detected (Chaly *et al.*, 1996).

1C. *Nuclear organization during lymphocyte stimulation*

The resting lymphocyte is a small cell, less than 10 μm , made up almost entirely of nucleus, with only a thin rim of cytoplasm. The chromatin is highly condensed and is distributed in large aggregates. The interchromatinic volume is also very small, which is reflected in a poorly developed nuclear matrix. Resting cells appear terminally differentiated, are in G_0 and exhibit very low levels of transcription and protein synthesis (Setterfield *et al.*, 1983). In the absence of antigen challenge or some other method of activation such as mitogen stimulation, lymphocytes will remain in this essentially inactive state throughout their lifespan.

Upon stimulation with a mitogen, the lymphocyte nucleus undergoes extensive structural remodelling. Concanavalin A (Con A), a lectin isolated from Jack bean, is capable of inducing blastogenesis in T lymphocytes, resulting in complete restructuring of the nucleus 18 to 36 hours after stimulation (Bladon *et al.*, 1988; Setterfield *et al.*, 1983). Electron microscopy and analysis of nuclear protein content by microspectrophotometry

have shown that the response of lymphocytes to mitogen is asynchronous (Setterfield *et al.*, 1985).

Lymphocyte nuclei in stimulated cell populations have been categorized based on their degree of stimulation. Electron microscopy of human peripheral blood lymphocytes stimulated with phytohemagglutinin resulted in three morphotypes being identified: unstimulated (Type I), partially stimulated (Type II), and fully stimulated (Type III) (Setterfield *et al.*, 1983). Morphotype I nuclei are small with large masses of condensed chromatin distributed mainly around the periphery of the nucleus (Fig. 1). Nucleoli are rarely visible and there is little granular interchromatinic material. Morphotype III nuclei are up to six times larger in volume than unstimulated nuclei, and are also rounder and more regular in shape. The volume of condensed chromatin, however, is only about 1.5-fold that of unstimulated nuclei. Stimulated lymphocytes exhibit smaller aggregates of condensed chromatin, which are found scattered throughout the nucleus (Fig. 1). The chromatin aggregates in morphotype I nuclei, in contrast, are nearly all in close contact with the nuclear membrane. Complex nucleoli with lacunae are often visible in fully stimulated cells, as are other fibrils and granular material. Morphotype II nuclei represent an intermediate stage between the resting lymphocyte and the lymphoblast (Setterfield *et al.*, 1983; Fig. 1). The general response of an increase in the volume of the interchromatinic region and reorganization of condensed chromatin was later shown by light microscopy in other lymphocytes, including human T lymphocytes *in vitro*, mouse splenic B lymphocytes *in situ*, and human lymphocytes in germinal centres of intact lymph nodes (Setterfield *et al.*, 1985).

Figure 1: Lymphocyte morphology during stimulation

Electron micrographs of mouse splenocytes stimulated with Con A for 36 h, showing morphotype I (a), II (b), and III (c). The three micrographs are shown at the same magnification. Bar = 1 μ m. (From Brown *et al.*, 1989.)

Lymphocytes have a relatively low frequency of nuclear pore complexes per surface area, which may be due to their metabolically inactive state (Maul, 1977). When human lymphocytes were stimulated with phytohemagglutinin, the total number of nuclear pore complexes was approximately double in the stimulated cells (1100) compared to unstimulated cells (490) (Maul *et al.*, 1971). Synthesis of new pore complexes in stimulated lymphocytes occurs in two phases: first, six hours after stimulation, correlating with an increase in the rate of RNA synthesis, and second, correlating with the onset of DNA synthesis (Maul *et al.*, 1972).

Although the response of lymphocytes to mitogen stimulation is asynchronous, a general time course can be described. Freshly isolated lymphocytes are all morphotype I. By 12 to 18 h after stimulation, many morphotype II nuclei can be seen, and by 24 to 30 h after stimulation, many lymphocytes attain the morphotype III characteristics (Bladon *et al.*, 1988; Setterfield *et al.*, 1985). DNA synthesis, however, does not begin until about 36 hours post-stimulation (p.s.), as evidenced by incorporation of [3 H]thymidine (Setterfield *et al.*, 1983). This was also shown by pulse-labelling experiments on human peripheral blood lymphocytes stimulated with Con A, where S phase was found to begin at about 40 h p.s. (Konstantinović and Ševaljević, 1983). Nuclear remodelling is not dependent on the synthesis of DNA, as shown when DNA synthesis was inhibited by hydroxyurea or cytosine arabinoside but structural changes to the nucleus were observed to continue as usual (Setterfield *et al.*, 1983). Protein synthesis, on the other hand, is a requirement for the remodelling of the nucleus. The addition of cycloheximide so that incorporation of [3 H]leucine was blocked resulted in inhibition of the usual

morphological changes to the nucleus (Setterfield *et al.*, 1983). Inhibition of RNA synthesis by the RNA polymerase II inhibitor, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole, resulted in the disruption of the organization of chromatin and other components of transcription domains in Con A-stimulated mouse splenocytes (Davis *et al.*, 1993). This organization returned to normal after removal of the drug and resumption of transcription.

DNA topoisomerase II (Topo II) is an enzyme which is important in regulating the organization of chromatin during both interphase and mitosis. During interphase, Topo II is thought to anchor chromatin to the nuclear matrix at MARS (Daev *et al.*, 1994). The topology of DNA is regulated by Topo II through transient enzyme-linked double strand breaks. There are two isoforms of Topo II, encoded by separate genes (Chung *et al.*, 1989). Topo II β is present in quiescent cells such as unstimulated lymphocytes, while the α isoform is expressed after stimulation and is essential for progression through S phase (Chaly *et al.*, 1996; Daev *et al.*, 1994). When mouse lymphocytes were treated with low doses of a Topo II inhibitor, VM26, throughout stimulation with Con A, DNA and RNA synthesis were inhibited, but cell viability and the size increase expected with stimulation were largely unaffected (Daev *et al.*, 1994). Larger doses of VM26, however, inhibited stimulation and resulted in apoptosis (Daev *et al.*, 1994). Treatment of unstimulated mouse lymphocytes with VM26 also induced apoptosis and prevented stimulation from proceeding when Con A was added later (Roy *et al.*, 1992). These experiments illustrate the importance of maintaining the order of the spatial organization of chromatin.

The interchromatinic region is the most dramatically affected part of the nucleus during stimulation. There is a roughly ten-fold increase in the volume of this region, largely due to an increase in proteins of the nuclear matrix. This has been shown by examining isolated lymphocytes throughout the various stages of nuclear matrix preparation. Unstimulated cells exhibited mostly space where the chromatin had been removed, while morphotype III cells had smaller spaces and more residual fibrogranular material, nuclear bodies, and nucleoli (Setterfield *et al.*, 1983). Extensive RNP elements were accumulated, as were non-histone proteins which were synthesized *de novo* during mitogenesis, as shown by pulse-chase experiments with [^3H]leucine (Setterfield *et al.*, 1983). Non-histone proteins were also phosphorylated at a high level, as shown by 2D gel electrophoresis, indicating preparation for S phase (Bladon *et al.*, 1988). The number and type of nuclear bodies present also changes with stimulation. Chaly *et al.* (1983a) have shown that nuclear bodies of types IVa and V decrease in number with stimulation, while nuclear bodies of types II and III increase in number, possibly suggesting differentiation from one type to another. The overall number of nuclear bodies was shown to increase with stimulation, a phenomenon which is known to be correlated with high levels of cell activity (Chaly *et al.*, 1983a). The increase in number of nuclear bodies represents a reorganization of existing cellular material, rather than *de novo* synthesis, as shown by pulse-chase experiments using radioactive precursors (Chaly *et al.*, 1983b).

1D. *Apoptosis*

Apoptosis is a tightly controlled form of cell death, important in maintaining cell number, controlling tissue shape and size, and protecting the organism from uncontrolled cell division (Hengartner, 2000). It differs from necrosis in regulation, appearance, cause, and perhaps most importantly, in disposal of the dead cells. Necrotic cells exhibit increased membrane permeability, leading them to swell and burst, releasing their contents into the extracellular space (Vaux, 1993). *In vivo*, this generally leads to inflammation and tissue damage. Apoptotic cells, in contrast, are compacted and broken down into membrane-bound packages which are then phagocytosed and recycled (Raff, 1998). By depleting Jurkat cells of ATP and treating with staurosporin or anti-CD95 antibodies, both of which are known to induce apoptosis, apoptosis has been shown to be an energy-dependent process (Leist *et al.*, 1997). This was later confirmed in Burkitt's lymphoma cells in which apoptosis was triggered by the etoposide VP-16, where ATP-depletion was induced with H₂O₂ (Lee and Shacter, 1999).

One of the hallmarks of apoptosis is the translocation of phosphatidylserine from the inner to the outer plasma membrane. This property is commonly exploited for identifying apoptotic cells. Annexin V is a phospholipid-binding protein that binds phosphatidylserine. It can bind to apoptotic cells without the need for the cells to be permeabilized or otherwise treated. When conjugated to a fluorochrome, biotin, or magnetic beads, Annexin V can be used to identify or separate apoptotic cells from a population. Recently, it has also been shown that exposure of phosphatidylserine occurs on the surface of phagocytic cells as well as apoptotic cells (Marguet *et al.*, 1999).

Phosphatidylserine exposure is a signal that triggers clearance of apoptotic cells by phagocytes. Other signals include changes in surface sugars leading to recognition by phagocytes, and binding of bridging molecules in extracellular fluid by components of complement, or other molecules (Savill and Fadok, 2000).

Apoptosis is characterized by a number of well-conserved events, particularly changes in the nucleus. Caspases are cysteine proteases that become activated in apoptotic cells, leading to proteolysis of specific proteins. While this generally leads to a loss of function, in some cases, the result of caspase proteolysis is activation (Hengartner, 2000). DNA is cleaved in successively smaller fragments by caspase-activated DNase (CAD), producing a “ladder” when electrophoretically separated. CAD is normally inactive, in complex with an inhibitory subunit, ICAD. When ICAD is cleaved by caspase-3, CAD becomes activated (Hengartner, 2000). Chromatin collapse is also observed.

Biochemical and morphological changes in the nuclear envelope during apoptosis have been observed in lymphoid and HeLa cells stimulated to undergo apoptosis with actinomycin D or etoposide (Buendia *et al.*, 1999). Lamin B, LAP2 and Nup153 were found by immunoblotting to be proteolytically cleaved regardless of cell type or inducing agent. The rate of cleavage, however, was greater in the lymphoid cells than in the HeLa cells. Conversely, LBR, p62 (a nuclear pore complex protein) and gp210 exhibited little or no degradation (Buendia *et al.*, 1999). It was shown by immunofluorescence and electron microscopy that apoptosis led to a detachment of chromatin from the nuclear envelope and to the clustering of nuclear pore complexes (Buendia *et al.*, 1999). These

morphological changes may be facilitated by the cleavage of lamin B, LAP2 and Nup153, all of which interact with chromatin (Buendia *et al.*, 1999). Lamin cleavage occurs early in apoptosis, before DNA fragmentation, and is important in the collapse of the nucleus (Weaver *et al.*, 1996). When cells depleted of ATP were stimulated to undergo apoptosis, it was found that lamin B degradation was reduced, as shown by immunoblotting, and cells were forced to succumb to necrosis rather than apoptosis (Leist *et al.*, 1997). This suggests that lamin cleavage is specific to a proteolytic pathway leading to apoptosis. Lamin B is phosphorylated by protein kinase C α before it is degraded, as seen when HL60 cells were stimulated to undergo apoptosis with camptothecin, a DNA topoisomerase I inhibitor (Shimizu *et al.*, 1998).

1E. *Post-translational modifications*

The functions of many proteins are modulated by their structures, which are frequently influenced by post-translational modifications. With disassembly and reassembly of the nuclear envelope occurring with each cell cycle, it is critical that nuclear proteins, and particularly proteins of the nuclear envelope, be able to alter their interactions with other proteins in order for these changes to take place. In many cases, the dynamic properties of nuclear proteins are regulated by means of post-translational modifications.

1E1. Glycosylation

Although it has long been assumed that glycosylation plays an important role in

protein function, elucidating this potential function has proven difficult. Glycosylation is thought to play a role in interactions between cells, and between cells and the extracellular matrix, trafficking between and within cells, cell signalling, binding of microorganisms, protein folding, and antibody recognition (Snow and Hart, 1998; Varki, 1998). It was long thought that only secretory proteins or cell-surface proteins were glycosylated, but many nuclear and cytoplasmic glycoproteins have now been identified, although the pathways to these modifications are not as well known (Snow and Hart, 1998).

There are two broad categories of glycosylation: asparagine- or N-linked, and serine/threonine- or O-linked. While all eukaryotes exhibit N-linked glycosylation, the modification is not present in prokaryotes. In the classical understanding, most proteins that are made in the ER are glycoproteins with N-linked glycosylation. On the other hand, the few cytosolic proteins that are glycosylated tend to exhibit O-linked glycosylation (Snow and Hart, 1998). In N-linked glycosylation, a 14-sugar oligosaccharide is added in the ER lumen to the amino group of an asparagine side chain. This oligosaccharide is frequently cleaved in the Golgi apparatus to a core of only five sugars, with three mannose and two N-acetylglucosamine residues (Helenius and Aebi, 2001). O-linked oligosaccharides have sugars attached to the hydroxyl groups of serine or threonine side chains and are also produced in the Golgi apparatus (Varki, 1998).

The best-studied modification of nuclear and cytoplasmic proteins is *O*-linked *N*-acetylglucosaminylation, or *O*-GlcNAcylation. In contrast with secreted proteins, in which the oligosaccharide residues are stable, the addition of *O*-GlcNAc is reversible

(Snow and Hart, 1998). The modification in cytosolic and nuclear proteins was discovered in mouse lymphocytes when it was noted that detergents aided in detection of the sugar (Snow and Hart, 1998). *O*-GlcNAc has since been found in all eukaryotic cells studied, and modifying diverse proteins such as nucleoporins, RNA polymerase II, chromatin proteins, heat shock proteins, cytoskeletal proteins and oncoproteins (Snow and Hart, 1998). The modification is dynamically added and removed by an *O*-GlcNAc transferase and an *O*-GlcNAcase, respectively (Snow and Hart, 1998). In this manner, *O*-GlcNAcylation is used in response to cell signals. For instance, during stimulation of mouse lymphocytes with mitogen, there is a decrease in levels of *O*-GlcNAc on cytosolic proteins, and a transient increase in *O*-GlcNAc levels on nuclear proteins (Kearse and Hart, 1991).

All known proteins possessing *O*-GlcNAc are also phosphoproteins (Snow and Hart, 1998). In fact, many sites of *O*-GlcNAcylation are also phosphorylation sites. *O*-GlcNAc also acts as an antagonist of phosphorylation, and the two modifications compete for attachment (Snow and Hart, 1998).

1E2. Phosphorylation

A key regulator of the reorganization of cellular components during entry and exit from mitosis is phosphorylation. In particular, mitotic disassembly and reassembly of the nuclear envelope has been shown to depend on reversible phosphorylation of a number of proteins. Functions of phosphorylation include regulation of interactions between lamins and transport into the nucleus. At the onset of mitosis, lamins become

hyperphosphorylated, leading to disassembly of the nuclear envelope as the lamin dimers depolymerize (Gerace and Blobel, 1980). One possible cause for the depolymerization is thought to be charge repulsion due to the negative charges conferred by the phosphate groups (Fields and Thompson, 1995). The increase in phosphorylation at mitosis is about five-fold compared to interphase, with two phosphates added to each lamin molecule (Moir *et al.*, 1995). At the end of mitosis, the lamins are dephosphorylated, allowing their repolymerization and reformation of the nuclear lamina (Gerace and Blobel, 1980).

Phosphorylation of lamins is possible at many serine or threonine residues in sites flanking the rod domain. The kinase responsible for lamin mitotic dynamics is CDC2, in complex with cyclin B (Moir *et al.*, 1995). This kinase, which is active exclusively in mitosis, is also responsible for phosphorylation of many other proteins including histone H1 and nucleoporins (Fields and Thompson, 1995). It has been shown that phosphorylation of sites at both ends of the rod domain is necessary for proper mitotic depolymerization/polymerization of lamins. When phosphorylation was prevented at the C-terminal site of lamin B, head-to-tail polymerization of lamins was inhibited (Peter *et al.*, 1990). The CDC2/cyclin B kinase is sufficient for lamin depolymerization at the beginning of mitosis, but complete nuclear envelope breakdown requires additional factors, including phosphorylation of other proteins (Moir *et al.*, 1995).

Mitosis also sees phosphorylation of inner nuclear membrane and nuclear pore proteins (Collas, 1998). LBR is phosphorylated during mitosis by RS kinase, which releases it from its interaction with chromatin (Chu *et al.*, 1998). The phosphorylation of histone H1 contributes to chromosome condensation at the beginning of mitosis (Fields

and Thompson, 1995).

The changes brought on by multiple phosphorylation events are reversed at the end of each mitosis. One requirement for exiting mitosis is inactivation of the CDC2/cyclin B kinase. This is accomplished by ubiquitin-dependent degradation of cyclin B late in mitosis (Fields and Thompson, 1995).

1E3. Prenylation

Prenylation is a common post-translational modification, frequently occurring on membrane proteins. Isoprene groups are carbon chains with alternating double and single bonds. Prenylation involves the addition of either a farnesyl (15 C) or a geranylgeranyl (20 C) group to a cysteine residue near the C-terminus (Casey and Seabra, 1996). These groups are attached via thioether linkages.

A conserved motif, a *CAAX* box (see section 1A3ii), occurs at the C-termini of proteins that undergo prenylation. Prenylation is carried out by enzymes termed prenyltransferases, which fall into two categories. The first category includes farnesyltransferase (FTase) and geranylgeranyltransferase type I (GGTase-I) while Rab geranylgeranyltransferase, or GGTase-II, is in the second category (Casey and Seabra, 1996). FTase was the first prenyltransferase to be identified (Reiss *et al.*, 1990).

Enzymes in the first category act on proteins with a cysteine residue in the fourth position from the C-terminus. The Rab family of Ras-related GTPases, which terminate in Cys-Cys or Cys-X-Cys, are modified by GGTase-II, at both cysteine positions (Casey and Seabra, 1996). Whether a protein is farnesylated or geranylgeranylated is determined by

the terminal residue, the *X* in the *CAAX* box. If the terminal residue is Ser, Met, Ala, or Gln, the protein will be modified by FTase, while a terminal residue of Leu will result in modification by GGTase-I (Casey and Seabra, 1996).

Prenylation is a lipid modification, and as such, along with carboxymethylation, acts to increase the hydrophobicity of proteins bearing the modification (Hennekes and Nigg, 1994). It follows, therefore, that a function of prenylation is mediation of membrane association or of specific protein-protein interactions. Other functions include signal transduction and regulation of intracellular trafficking pathways (Casey and Seabra, 1996).

Lamins A and B are prenylated (farnesylated) and methylated, while lamin C and other cytoplasmic intermediate filament proteins are not (Moir *et al.*, 1995). Subsequently, lamins A and B undergo proteolysis, which removes the three terminal residues (*AAX*) and the cysteine is then carboxymethylated (Moir *et al.*, 1995). A second proteolytic step occurs for lamin A only, in which 15 terminal residues, including the prenylated and methylated cysteine, are removed (Moir *et al.*, 1995).

It has been shown that prenylation of lamins is essential for their association with the nuclear membrane. Lamin A was found by immunofluorescence to accumulate in the nucleoplasm when prenylation was inhibited by treatment with lovastatin. This demonstrated that the targeting of lamin A to the nuclear envelope is dependent upon farnesylation (Lutz *et al.*, 1992). Release of the block allowed the proteins to become incorporated into the lamina as usual, suggesting that the nucleus is a site of farnesylation (Lutz *et al.*, 1992). In further evidence that the association of lamins with the envelope is

modulated by prenylation, transfection of HeLa or P19 cells with cDNAs for either mature lamin A or for a mutant lacking the *CAAX* box resulted in a nucleoplasmic distribution of the lamins (Hennekes and Nigg, 1994). However, some of the proteins were noted to associate with the lamina after the cells underwent mitosis, suggesting that association with the membrane may be dependent on farnesylation in interphase cells, but that a farnesylation-independent pathway exists that allows the association following mitosis (Hennekes and Nigg, 1994).

The difference in prenylation may explain the differing solubility properties exhibited by lamins A and B. A point mutation substituting arginine for valine prevented lamin A constructs from undergoing the usual second proteolysis. This mutation left the prenylation modification intact, and conferred membrane-binding ability on the constructs (Hennekes and Nigg, 1994). This may explain why lamins A and C appear soluble at mitosis, while lamin B remains in association with the fragmented nuclear membrane (Moir *et al.*, 1995). It is still unclear whether prenylation results in a direct association with the nuclear membrane, or if the interaction is between the prenyl group and proteins such as LAPs or LBR (Moir *et al.*, 1995).

1E4. Ubiquitination

Ubiquitin is a 76 amino acid protein which may exist independently or in complex with other proteins. In most cases, proteins which carry ubiquitin tags have been damaged by denaturing, misfolding, or oxidation, and are destined to be degraded in proteasomes. Ubiquitin is added by covalent attachment to lysine residues. Three

enzymes are needed for this process: a ubiquitin activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin protein ligase (E3) (Yang *et al.*, 2000).

Although ubiquitin-independent pathways for degradation exist, particularly for ER-associated degradation (Werner *et al.*, 1996), the ubiquitin pathway is more common and has been better characterized.

Ubiquitination is common on proteins that have a high rate of turnover, but the modification is also seen on some stable proteins such as actin and histones (Delic *et al.*, 1993). In these cases, the function of ubiquitination is thought to be related to signalling or transcriptional regulation. While chains of at least four ubiquitin subunits are required for degradation, single or short ubiquitin chains appear to serve this regulatory function (Hicke, 2001). Ubiquitin also plays a role in apoptosis. Ubiquitin gene transcription is activated early in apoptosis, and many nuclear proteins are ubiquitinated in apoptotic cells (Delic *et al.*, 1993). The ubiquitin gene also confers resistance to stress such as high temperature and starvation in yeast (Finley *et al.*, 1987).

Chapter 2: MATERIALS AND METHODS

2A. Isolation of mouse splenocytes

Mouse splenic lymphocytes were isolated according to previously described methods (Setterfield *et al.*, 1983). Male Balb/c mice (≥ 8 wks) were sacrificed by cervical dislocation and thoroughly wetted with 70% ethanol. The spleen was removed and placed on a sterile metal screen in a sterile glass petri dish containing 4 mL of RPMI 1640 medium (Gibco-BRL) with no additives. The spleen was pushed through the screen with the flat end of a sterile syringe plunger until no large pieces remained. Another 4 mL of RPMI 1640 medium (no additives) was added to the dish and the cell suspension was passed through a 20-gauge needle four times. Using a Pasteur pipette with a rubber bulb, the cell suspension was layered onto 4 mL of ice-cold Cool Calf (Sigma) in a sterile 15-mL screwcap tube and left to stand on ice for 5 min. Using a Pasteur pipette and rubber bulb, the cell suspension (upper phase) was transferred to a fresh sterile 15-mL screwcap tube and centrifuged for 5 min at 1500 rpm in a clinical centrifuge (IEC Centra-4). The supernatant was discarded and the red pellet was resuspended in 4 mL of ice-cold 0.83% NH_4Cl in order to lyse red blood cells. The suspension was left for 5 min on ice. Using a Pasteur pipette as above, the suspension was then underlaid with 4 mL of ice-cold Cool Calf, and the tube was centrifuged for 5 min as before. The supernatant was discarded and the whitish pellet was resuspended in 10 mL of complete RPMI 1640 medium (including 10% CPSR II (Sigma), 2 mM glutamine, and 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 0.25 $\mu\text{g/mL}$ fungizone).

To determine the total number of cells in the suspension, an aliquot of the suspension was diluted 1:5 in Trypan Blue (one part 0.2% (w/v) Trypan Blue in four parts 4.25% (w/v) NaCl) and delivered to a Reicher haemocytometer for counting. Live cells appeared clear in colour and were birefringent, while dead cells had taken up the Trypan Blue and were therefore blue in colour. Live and dead cells were counted separately. After determining the total number of cells present, the cells were resuspended in complete RPMI 1640 at a density of 2×10^6 live cells/mL. To stimulate splenocytes, Con A was added 1 h after isolation to a final concentration of 4 $\mu\text{g/mL}$ for up to 48 h.

2B. Labelling of splenocytes with fluorescent Annexin V

A 0.5 mL aliquot of splenocytes (i.e. about 1×10^6 cells) was centrifuged for 5 min at 1500 rpm in the IEC Centra 4. The supernatant was discarded and the pellet was resuspended in 1 mL of Annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4), to about 1×10^6 cells/mL. The suspension was centrifuged for 5 min at 1500 rpm in an Eppendorf microfuge and 800 μL of the supernatant was discarded. The pellet was resuspended in the remaining 200 μL of medium and mixed with 10 μL of Alexa 568-conjugated Annexin V (Molecular Probes)(5 μL per 100 μL cell suspension) and 2 μL of Hoechst 33342 (Sigma, 15 mg/mL in distilled water)(1 μL per 100 μL of cells suspension. After incubating at room temperature for 5 min, the cell suspension was layered onto a poly-L-lysine-coated coverslip and incubated for 10 min in a humid chamber at room temperature. The cells were then fixed in 3% paraformaldehyde in phosphate buffered saline (PBS)(0.13 M NaCl, 5.1 mM Na_2HPO_4 , 1.6 mM KH_2PO_4 , pH

7.0) for 5 min, washed 3 x 4 min in PBS and mounted in Vectashield mounting medium (Vector Labs, Burlingame, CA). The samples were visualized using the Zeiss Axiophot epifluorescence microscope equipped with a 100 Watt mercury burner, using a 63 X, 1.35 N.A. oil immersion objective. Images were digitally captured using the Hamamatsu integrating chilled CCD camera and MetaMorph® v.2.75 software (Universal Imaging Corporation, Downingtown, PA). Tagged image file format (TIFF) images were processed in MetaMorph® v.2.75 and Adobe Photoshop v.4.01.

2C. Immunofluorescent labelling of splenocytes with antibody P1

About 5×10^5 cells concentrated in 250 μ L were layered onto a poly-L-lysine-coated coverslip and the coverslips were incubated for 10 min in a humid chamber at room temperature. The cells were then fixed in 3% paraformaldehyde in PBS for 5 min, washed 2 x 30 sec in PBS and permeabilized in 0.2% Triton X-100 (Pierce) in PBS for 20 min. After washing 3 x 4 min in PBS, the cells were incubated in primary antibody P1 (1:100) (a mouse monoclonal IgM, Chaly *et al.*, 1984; see Section 1B) for 45 min in a humid chamber. The P1 mAb had been prepared from hybridoma supernatant, then partially purified by ammonium sulphate precipitation, as described by Harlow and Lane (1988). The cells were washed 3 x 4 min in PBS as above, and then were incubated in FITC-conjugated goat anti-mouse IgM (μ chain-specific) (1:200) (Jackson) in a humid chamber as above. Finally, the cells were again washed 3 x 4 min in PBS, incubated in 2×10^{-4} μ g/mL 4',6-diamidino-2-phenylindole (DAPI)(1:5000)(Sigma) in PBS for 2 min, mounted in Vectashield mounting medium, and examined as above.

Samples double-labelled with Annexin V and monoclonal antibody P1 were first processed for annexin labelling as above, but in the absence of Hoechst 33342. There were then fixed in 3% paraformaldehyde, and processed for immunolabelling with monoclonal antibody P1 as above.

2D. Separation of live and apoptotic cell fractions

Splenocytes stimulated for 24 or 48 h were separated into living and dead/dying populations using the Dead Cell Removal Kit and Mini-Mac system (Miltenyi Biotec, Auburn, CA). An aliquot of the cell suspension was first taken to determine the starting proportion of living and dead cells, using Trypan Blue staining and a haemocytometer, as above. Cultures were then centrifuged at 300 g (2000 rpm) in the IEC Centra 4 for 10 min. The supernatant was discarded, the pellet was resuspended in Annexin V-conjugated Dead Cell Removal Microbeads using 100 μ L of beads per total of 10^7 cells, and was incubated for 15 min at room temperature. An MS+ column mounted on a Mini-Mac magnet was rinsed with 500 μ L of 1X Binding Buffer (Miltenyi Biotec) prewarmed to 37°C and the effluent was discarded. The cell suspension was added to the column and the effluent was collected as the live cell fraction. The column was rinsed four times with 500 μ L of 1X binding buffer, with the entire amount of the buffer allowed to flow through the column before the next volume was added. The effluent was added to the live cell fraction. The column was then removed from the magnet, 1 mL of 1X binding buffer was added to the column, and the dead cell fraction was pushed out using a syringe plunger into a fresh tube. Live and dead cell numbers before and after separation were

monitored by Trypan Blue staining. Aliquots of the live and dead cell fractions were prepared for SDS-PAGE, flow cytometry, and/or microscopy.

2E. Preparation of samples for flow cytometry

At least 2×10^6 cells in culture were centrifuged for 5 min at 1500 rpm in the IEC Centra 4. The supernatant was discarded and the pellet resuspended in 1 mL of PBS. After addition of ice-cold absolute ethanol (4 mL), the sample was mixed well by shaking and stored at -20°C for up to two weeks.

Samples were then centrifuged for 5 min at 1500 rpm and the supernatant was removed by aspiration, taking care not to disturb the fluffy white pellet. The pellet was resuspended in 1 mL of PBS and mixed by vortexing. After addition of 100 μL of 100 $\mu\text{g/mL}$ RNase A (Sigma) in PBS to each sample, the tubes were incubated at 37°C for 30 min. The samples were then stained by adding 100 μL of a stock solution of propidium iodide (1 mg/mL in PBS, Sigma) and incubating at least 10 min in the dark. The side scatter and forward scatter were then measured using a Coulter Elite flow cytometer, counting 20 000 events per sample, to determine the size, granularity, and DNA content of the cells.

2F. Preparation of cell lysates for SDS-PAGE

After magnetic separation, the numbers of live and dead cells in the samples were determined by Trypan Blue staining and a haemocytometer and the cultures were centrifuged for 5 min at 1500 rpm in the IEC Centra-4. The supernatant was discarded

and the pellet resuspended in 1 mL of PBS. The suspension was then transferred to a microfuge tube and centrifuged for 5 min at 1500 rpm in the Eppendorf microfuge. The supernatant was discarded and the pellet frozen at -80°C until needed for lysis.

For lysis, the cell pellets were suspended in high radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% sodium deoxycholate, and 1% Triton X-100 with protease inhibitors (1 mM Pefabloc, Boehringer-Mannheim; 1 mM benzamidine, Sigma; 50 µg/mL leupeptin, Sigma; and 10 µg/mL aprotinin, Sigma). The tube was incubated on ice for 15 min, and centrifuged at 14, 000 rpm in the Eppendorf microfuge, at 4°C, for 20 min. The supernatant (lysate) was transferred to a new microfuge tube and the pellet was discarded. The protein concentration in the lysate was determined using the bicinchoninic acid (BCA) assay (Sigma) according to the supplier's instructions.

2G. Nuclear matrix preparation

At each stage of nuclear matrix preparation, aliquots from the pellet and supernatant were saved separately for assay by SDS-PAGE. Protease inhibitors (as above) and 50 mM sodium tetrathionate (NaTT, Sigma) were added to each solution just before use.

Freshly isolated mouse splenocytes (about 2×10^8 cells) were centrifuged for 10 min at 2000 rpm in an IEC Centra 4 centrifuge and the pellet was resuspended in 1 mL of sucrose-Tris-magnesium (STM) buffer (0.25 M RNase-free sucrose, 10 mM Tris-HCl, pH 7.4, 25 mM KCl, and 2 mM $MgCl_2$). The sample was transferred to a microfuge tube,

incubated for 5 min on ice, and centrifuged for 10 min at 2000 rpm in an Eppendorf microfuge, providing the “STM” pellet and supernatant. The pellet was resuspended in 1 mL of 0.5% Triton X-100 in STM buffer. After 15 min on ice, the sample was centrifuged for 10 min at 2000 rpm in an Eppendorf microfuge, providing the “Tx100” pellet and supernatant. The pellet was resuspended in 1 mL of 10 µg/mL DNase I (Boehringer Mannheim) in STM and incubated for 10 min at room temperature. The cells were centrifuged as above, providing the “DNase I” pellet and supernatant. The pellet was resuspended in 1 mL of low magnesium buffer (LMB, 10 mM Tris-HCl, pH 7.4 and 0.2 mM MgCl₂), incubated for 20 min on ice. An aliquot (160 µL) was removed for treatment with NaCl. The remainder (840 µL) was centrifuged as above, providing the “LMB” pellet and supernatant. To the saved 160 µL aliquot, 160 µL of 2 M NaCl in LMB was added slowly. The sample was incubated for 20 min on ice. The sample was centrifuged as above, providing the “NaCl” pellet and supernatant.

Supernatants from each stage of the matrix preparation were dried down in the Speed Vac for at least eight hours. Pellets and dried supernatants were then dissolved in high RIPA buffer and assayed for protein concentration as described above.

2H. SDS-PAGE and immunoblotting

Gel electrophoresis was performed using the Bio-Rad Mini Protein II apparatus according to standard protocols, with a 3% stacking gel and an 8% separating gel. Cell lysates were diluted in sample buffer (4X Laemmli buffer: 0.25 M Tris-HCl, pH 6.8, 8% SDS, 7.5% glycerol, 0.005% bromophenol blue, 22.5% β-mercaptoethanol) and loaded

on the gels directly without heating. Samples were loaded by equal cell number, usually 2.5×10^5 total live and dead cells as determined by counting with Trypan Blue, or by equal amount of protein, usually 2.5 μg as determined by the BCA assay. The gels were run at 125 volts until the dye front had run off the bottom of the gel. The samples were then transferred onto nitrocellulose using the Bio-Rad Mini Protein II blotting apparatus. The transfer buffer consisted of 25 mM Tris base, 192 mM glycine, 20% (v/v) methanol, pH 8.3. Transfer was carried out at 30 volts overnight, to a maximum of 18 hours, at 4°C on a magnetic stirrer.

2I. Probing with Antibody P1

All washing and blocking steps were performed with agitation on a rotary shaker.

The nitrocellulose membrane was rinsed several times with distilled water and stained with Ponceau Red (0.5% Ponceau Red, 3% trichloroacetic acid) for 5 min with agitation. The membrane was then rinsed several times with distilled water, and air-dried. After scanning with a Hewlett-Packard DeskScan 6300C, the membrane was destained in PBS for 10 min with agitation, rinsed in distilled water, and washed 3 x 10 min in TBST (Tris-buffered saline with Tween 20)(50 mM Tris base, 150 mM NaCl, pH 7.5, 0.12% Tween 20) to remove the methanol from the transfer buffer. Blocking was then carried out for 1 h in 6% bovine serum albumin (BSA, fraction V, Sigma) in TBST; the BSA solution was filtered through a 0.45 μm syringe filter before use. The membrane was then incubated in approximately 1 mL of mAb P1 (1:7500 in TBST), in a sealed plastic bag for 1 h on a nutator. The nitrocellulose membrane was then washed 8 x 5 min

in TBST, and incubated in horseradish peroxidase-conjugated goat anti-mouse IgM (μ chain-specific) (Sigma) (1:7500 in TBST) as above. The membrane was then again washed 8 x 5 min in TBST and processed for chemiluminescence using the Lumi-Light Plus kit (Roche). The membrane was taped into a Kodak Bio-Max intensifying screen and exposed to Kodak X-OMAT Blue film for 1-30 sec. The film was then developed with the GBX developing kit and air-dried. The films were scanned for printing and quantification.

2J. Slot blotting

Slot blotting was carried out using a 64-slot apparatus from Schleicher and Schuell (Keene, NH) according to the manufacturer's instructions. After the nitrocellulose was clamped between the plates, each well was washed with 500 μ L of transfer buffer (25 mM Tris base, 192 mM glycine, 20% (v/v) methanol, pH 8.3), and low vacuum was applied. The samples used were whole cell lysates of resting and stimulated lymphocytes, prepared as for SDS-PAGE (Section 2F), loaded by ranges of cell number (2.5×10^5 - 0.25×10^5 cell equivalents) or protein amount (5 - 0.5 μ g). The samples were introduced into the wells, and allowed to drain through completely. Each well was washed with 500 μ L of transfer buffer. When all liquid had run through the wells, the vacuum was stopped and the apparatus disassembled. The nitrocellulose membrane was then probed with antibody P1 as above.

2K. Quantification using Sigma Gel

Quantification of slot blots was performed using the software Sigma Gel (version 1.0, Jandel Scientific Corporation). Blots were scanned and saved in JPEG format. The software was run in Lane mode. The lane with the best exposure of all bands (*i.e.* bands were neither too faint nor overloaded) was selected and defined. The detection threshold was set to just above background. The areas under the resulting peaks were integrated, thus giving an intensity value for each band. All values were then normalized to the Day 0 band. Means and standard deviations were calculated by sampling the same blots at least six times.

2L. Dephosphorylation

Resting and stimulated splenocyte lysates (2.5 μ g) were incubated with 200 U of λ phosphatase and protease inhibitors (see “Nuclear matrix preparation”, above) for 30 min at 30°C in a water bath, according to the manufacturer’s instructions (Lambda Phosphatase Dephosphorylation Kit, New England Biolabs). The samples were then processed for SDS-PAGE and immunoblotting as above.

2M. Deglycosylation

Deglycosylation was carried out using the Glycopro Deglycosylation and prO-link Extender Kit (Prozyme, San Leandro, CA). The Deglycosylation Kit includes peptide N glycosidase F (PNGase F), Sialidase A, and Endo-O-glycosidase. The prO-link Extender Kit includes β (1-4)galactosidase, and glucosaminidase. For deglycosylation, 2.5 μ g of

each splenocyte lysate was heated with one or more enzymes in the presence of protease inhibitors (see "Nuclear matrix preparation", above) for 24 h at 37°C in a water bath. The samples were then processed for SDS-PAGE and immunoblotting as above.

Chapter 3: RESULTS

3A. Fractionation of mouse splenocyte populations

Studies have shown that populations of mouse splenocytes in culture undergo considerable apoptotic cell death, whether in the presence (Roy *et al.*, 1992, Daev *et al.*, 1994) or absence (Roy *et al.*, 1992, Sodja *et al.*, 1998, Sodja *et al.*, 1997) of mitogen. In order to examine the behaviour of circumferin during lymphocyte stimulation, it was therefore important to fractionate the splenocyte cultures into living and dead populations. This was accomplished by incubating splenocyte populations with magnetic beads coated with Annexin V, which bound to apoptotic cells, and then separating live and dead fractions by passing the suspension through a column mounted in a magnet. Immediately after isolation (Day 0), the cells exhibited >90% viability (Fig. 2). The proportion of living and dead cells was monitored by cell counts using the vital dye Trypan Blue (Fig. 2) and by flow cytometry of samples in which the DNA was stained with propidium iodide (Fig. 2). Trypan Blue is excluded from living cells which have intact plasma membranes. Propidium iodide labels nucleic acids, but samples were treated with RNase before flow cytometry (see Section 2E) so that only DNA would be labelled. Both cellular morphology and efficiency of separation were also monitored by fluorescent labelling with Alexa 568-conjugated Annexin V (Figs 3-5). The percentages of living and dead cells in each fraction were statistically the same by Trypan Blue counting and by flow cytometry (Fig. 1, $p < 0.01$ in every case). There was consistently little or no cell death on Day 0, with a large and increasing number of dead cells on Days 1 and 2. The trend was similar for all experiments, but the extent of cell death in

Figure 2: Percentage of living cells in stimulated populations before and after magnetic separation.

Histogram showing the percent of living cells in Total (1T, 2T) splenocyte populations, and in the Living (1L, 2L) and Dead (1D, 2D) fractions prepared by magnetic separation on Day 1 (1T, 1L, 1D) and Day 2 (2T, 2L, 2D) after addition of mitogen. Light boxes show data obtained by flow cytometry, and dark boxes show data from Trypan Blue staining. Error bars represent the standard deviation of the average of eight experiments.

Figure 3: Fluorescence microscopy of splenocyte populations at 24 h p.s.

Total (A-A''), Living (B-B'') and Dead (C-C'') fractions of splenocyte populations stimulated for 24 h with Con A were labelled with Hoechst 33342 (A, B, C) and Annexin V-Alexa 568 (A', B', C'). The respective merged images are shown in A'', B'', and C''. Magnification = 2000 X

Figure 4: Fluorescence microscopy of splenocyte populations at 48 h p.s.

Total (A-A''), Living (B-B'') and Dead (C-C'') fractions of splenocyte populations stimulated for 48 h with Con A were labelled with Hoechst 33342 (A, B, C) and Annexin V-Alexa 568 (A', B', C'). The respective merged images are shown in A'', B'', and C''. Cells of morphotypes I, II, and III are visible, as indicated beside representative cells. Magnification = 2000 X

Figure 5: Immunofluorescence microscopy of splenocytes labelled with mAb P1.

Splenocytes 24 h p.s. were labelled with mAb P1 (A), Alexa 568-conjugated Annexin V (B), and Hoechst 33342 (C). The upper two cells are labelled with Annexin V and are apoptotic; the other two cells are alive. Merged images are shown in the right hand column. Magnification = 2000 X

stimulated populations varied widely between experiments, accounting for the large error bars seen in Figure 2. For this reason, comparisons between samples from Day 1 and Day 2 were always made within the same experiment. By Day 1, only 50-80% of the cells remained living, with about 60% alive in most experiments. After magnetic separation, the Live fraction generally contained >80% living (Fig. 2). Day 2 cultures contained 30-50% living cells before separation, but increased only to about 60% in the Live fraction after separation (Fig. 2). The Dead fractions were more homogeneous, consisting mostly of >80% dead cells.

Immunofluorescent labelling with Alexa 568-conjugated Annexin V confirmed the results seen by flow cytometry. In Day 0 samples, lymphocytes were uniformly morphotype I (not shown) (Sodja *et al.*, 1997). They were small with little cytoplasm and highly compacted DNA. As has been previously described (Brown *et al.*, 1990, Setterfield *et al.*, 1983), Con A-treated populations contained some morphotype I cells, but also contained many which were significantly larger, with large nuclei and little condensed chromatin, *i.e.* of morphotype III. Morphotype II cells, intermediate in morphology between resting and fully-stimulated cells were also seen.

Morphotype I cells also predominated in Day 1 cultures, and morphotype III cells were rare (Fig 3). Some Annexin V-labelled, apoptotic cells were observed in Total populations (Fig. 3A-A'') but were less common in Living samples after magnetic separation (Fig. 3B-B''). Conversely, nearly all cells in the Dead fraction were apoptotic, as expected (Fig. 3C-C'').

By Day 2, all three morphotypes were readily visible in Total populations, as

shown in Figure 4A. As well, as is characteristic of stimulated lymphocyte cultures, the cells in these samples were often aggregated (Fig. 4A). The clumps often contained apoptotic cells, seemingly trapped among living ones. There was often extensive Annexin V labelling throughout the clump (Fig 4A'), associated both with cells which were clearly apoptotic and with cells otherwise apparently living. Unlabelled, morphologically intact cells were also observed. Perhaps as a result of this clumping, many cells labelled with Annexin V were visible in the Living fraction (Fig. 4B-B"). Once again, however, as for Day 1 samples, the Dead fractions consisted overwhelmingly of apoptotic cells (Fig. 4C-C").

To examine the behaviour of circumferin in apoptotic cells, Day 1 samples were double-labelled with mAb P1 and Alexa 568-conjugated Annexin V, and counter-stained with DAPI. As previously described (Chaly *et al.*, 1988), mAb P1 labelled the periphery of morphotype I, II, and III nuclei (Fig. 5). MAb P1 also labelled the periphery of unfragmented apoptotic nuclei, and outlined the periphery of each nuclear fragment in fragmented apoptotic nuclei (Fig. 5A, A+B, A+C). In addition, these samples showed that circumferin was retained in apoptotic cells (Fig. 5A+B, A+C). These results indicate that circumferin is not lost from nuclei during apoptosis, and also suggest that circumferin may not be degraded during this process.

3B. Apparent molecular mass of circumferin in lymphocytes

In the initial work describing the P1 antibody (Chaly *et al.*, 1984), immunoblotting with mAb P1 had identified a triplet of polypeptides migrating with

relative molecular masses between 27 and 31 kDa in samples of lymphocyte nuclear matrix. However, these results proved to be artefactual. Moreover, many subsequent attempts to identify the P1 antigen by immunoblotting in a variety of cell types, including rat L6E9 myoblasts, mouse 3T3 fibroblasts, and HeLa cells were unsatisfactory, with the blots showing varied arrays of ten or more bands (N. Chaly, personal communication). A marked increase in the specificity of the antibody reaction and in its reproducibility was achieved by serendipitous changes to the immunoblotting protocol in the mid-1990s (X. Chen and N. Chaly, unpublished data). These observations were the starting point for the immunoblotting protocol that I developed in the course of the current study. Several key changes were made in preparation of the cell lysates, in preparing samples for SDS-PAGE, and in the immunoblotting protocol. Reproducible results were obtained when High RIPA buffer was introduced to lyse the cells (see Materials and Methods), and it proved important that samples be loaded onto gels without heating at any stage. For immunoblotting, specificity greatly improved when blocking was carried out using BSA in TBST, rather than the more commonly used solution of skim milk in PBS. Optimal results were obtained by blocking in 6% BSA for 1 h and by very extensive washing of the nitrocellulose membrane with TBST at each step of the probing procedure.

When these changes were implemented, immunoblotting of Day 0 samples revealed that circumferin migrated most commonly as three bands: a doublet with a fainter lower band (band 1) and a strong upper one (band 2), and a single band with an even higher molecular mass (band 4) similar in intensity to band 1 (Fig. 6). An additional band, band 3, was seen occasionally between the doublet and band 4. The data showed

Figure 6: Immunoblot of circumferin in whole cell lysates.

Resting mouse splenocytes (lane 1), and Live cell fractions from populations stimulated for 24 h (1L, lane 2) and 48 h (2L, lane 3) with Con A, were loaded onto 10% SDS-PAGE gels and processed for immunoblotting with mAb P1. Each lane was loaded with 0.5×10^6 cells. The numbered arrows indicate the four bands normally detected in stimulated cells. Migration of molecular weight standards is indicated in kDa in the left-hand margin.

that bands 1, 2, 3, and 4 migrated with relative molecular masses of 72.1 ± 2.3 kDa, 75.1 ± 2.4 kDa, 78.0 ± 4.2 kDa and 80.8 ± 2.9 kDa, respectively (Fig. 6).

When mouse splenocytes were stimulated with Con A, the overall band pattern did not change, but the relative intensities of the bands did. In Day 0 samples, band 2 was most intense, bands 3 and 4 were of similar lesser intensity, and band 1 was of intermediate intensity (Fig. 6). In Live fractions on Day 1 (Day 1L), band 2 was still slightly more intense than the others, but the other bands were equal in intensity, and the bands were all of similar intensity in Live fractions on Day 2 (Day 2L) (Fig. 6).

3C. Circumferin in apoptotic cells

Although samples of the Dead fractions contained large numbers of cells as determined by cell counting, the protein concentrations routinely were below the detectable minimum. This is most likely due to the extensive degradation of proteins that typically occurs during apoptotic cell death. Nonetheless, immunoblotting of Dead fractions with mAb P1 detected bands 1, 2, and 4. In these blots, bands 1 and 2 were consistently significantly more intense than band 4 (Fig. 7). The difference in intensity between band 4 and bands 1 and 2 is not as prominent in live samples (Fig. 7). The enrichment of circumferin in dead samples made it necessary to use a short film exposure time (5 s). This avoided a large indistinct signal in the dead cell sample, although it resulted in a very faint signal for the Day 0 sample. The loading of $2.5 \mu\text{g}$ of protein is the same as in Figure 6. Significantly, there were no additional bands at lower molecular mass which might have been indicative of apoptosis-related degradation (Fig. 7).

Figure 7: Circumferin in unstimulated and Dead populations.

Immunoblotting with mAb P1 was carried out on membranes transferred from 10% SDS-PAGE gels loaded with 2.5 μ g of total protein per lane. Day 0 (0) samples were prepared immediately after isolation. After 48 h of stimulation with Con A, the cell population was fractionated to provide the Dead (2D) sample. The numbered arrows indicate the three bands generally detected in Day 0 samples (see Figure 6). Migration of molecular weight standards is indicated in kDa in the left-hand margin.

3D. Relative proportion of circumferin during stimulation

As discussed in the Introduction, the lymphocyte nucleus enlarges considerably during lymphocyte activation, with a concomitant increase in the surface area of the nuclear envelope. To examine the effect of these changes on the expression of circumferin, Day 0, Day 1, and Day 2 samples were processed for immunoblotting. For these experiments, an equal amount of protein was loaded for each sample (Fig. 8A).

Comparison of the overall band intensity in Day 0 and Day 1L samples indicated that there was a significant decrease in the amount of circumferin relative to the total cellular protein during the first 24 h of lymphocyte stimulation (Fig. 8A). The immunoblots suggested further that the relative amount of circumferin did not change significantly in the second 24 h of stimulation, as the overall reaction intensity for circumferin seemed similar in Day 1L and Day 2L samples (Fig. 8).

To test these observations, samples were processed for slot blotting. Slots were loaded with 5 - 0.5 μ g of total cellular protein, and the membrane was probed with mAb P1. The first lane of the slot blot, with 5 μ g of sample per well (Fig. 8B) is the same as the loading of the SDS-PAGE gel which was transferred and used for immunoblotting (Fig. 8A). In both the standard immunoblot and the slot blot, the Day 0 sample resulted in a very high signal intensity. The standard immunoblot shows a decrease in intensity from Day 0 to Day 1L (Fig. 8A) while the slot blot shows little difference between Day 0 and Day 1L, suggesting that at 5 μ g, the Day 0 signal is saturated in the slot blot. Loading lower amounts of total protein on the slot blots (2.5 μ g, 1 μ g, and 0.5 μ g), however, resulted in the signal intensity being highest on Day 0, less for Day 1L, and

Figure 8: Changes in the amount of circumferin present as a proportion of total cellular protein.

(A) Day 0 (0), Day 1 Live (1L) and Day 2 Live (2L) samples were loaded onto 10% SDS-PAGE gels at 5 μ g of total protein per lane, and processed for immunoblotting with mAb P1. Migration of molecular weight standards is indicated in kDa in the left-hand margin.

(B) Slot blot of samples, prepared as in (A) with an equal amount of protein in μ g loaded in each lane, as indicated, and probed with mAb P1.

A

B

even less for Day 2L (Fig. 8). This change was quantified, using the program Sigma Gel, for the slot blot lane with 1 μ g of total protein per well. Assigning a value of 1 to the Day 0 band, the relative quantities of circumferin in the Day 1L and Day 2L samples are 0.62 ± 0.03 and 0.27 ± 0.04 of Day 0, respectively. The results of the slot blot experiment of circumferin is consistent with the large changes in protein complexity and quantity during stimulation reported previously (Bladon *et al.*, 1988).

3E. Accumulation of circumferin during stimulation

Early studies have shown that nuclear volume increases by up to six-fold during stimulation (Setterfield *et al.*, 1983). To determine whether circumferin increases during mitogenesis, the amount of circumferin per cell was monitored by SDS-PAGE and immunoblotting, and by slot blotting (Fig. 9). By immunoblotting, with 2×10^5 cells loaded for each sample, there was no clear difference in band intensity between Day 0 and Day 1L samples, but there was a significantly greater intensity of all bands in Day 2L samples. The increase was most prominent for band 2 (Fig. 9A).

These results were confirmed by slot blotting, loading whole cell lysates equivalent to 2.5×10^5 to 0.25×10^5 cells per well. At 2.5×10^5 cells, band intensities were similar for the day 0, 1L and 2L samples. At each subsequent dilution, however, Day 0 and Day 1L samples showed little or no difference, and Day 2L samples were significantly more intense (Fig. 9B). Analysis of the relative intensities by Sigma Gel for the 0.5×10^5 slot blot lane, assigning a value of 1 to Day 0, gave an increase by a factor of 1.9 ± 0.3 for Day 1L, and a factor of 5.6 ± 1.4 for Day 2L. These results appear to

Figure 9: Changes in the amount of circumferin present per cell.

(A) Day 0 (0), Day 1 Live (1L) and Day 2 Live (2L) samples were loaded onto 10% SDS-PAGE gels at 2×10^5 cells per lane, and processed for immunoblotting with mAb P1. Migration of molecular weight standards is indicated in kDa in the left-hand margin.

(B) Slot blot of samples, prepared as in (A), with an equal number of cells ($\times 10^5$) loaded in each lane, as indicated, and probed with mAb P1.

A

B

indicate that circumferin is synthesized upon stimulation. However, the larger number of dead cells present in the Day 2L fraction is likely creating an overestimate of this synthesis, as dead cells were shown to produce a more intense signal than living cells (Fig. 7).

3F. Circumferin in nuclear matrix preparation

Through comparison of Coomassie Blue-stained gels with immunoblots of parallel lanes, it was hoped that the bands corresponding to circumferin could be identified on the gel. These bands could then be excised, and the sequence of circumferin obtained by mass spectroscopy. It was therefore necessary to try to remove as many of the other proteins in the samples as possible, so that the number of bands seen on the gels would be reduced. Modified nuclear matrices were prepared from Day 0 splenocytes, as described by Bladon *et al.* (1988). Aliquots were saved from both the pellet and the supernatant at each stage. The pellets and dried supernatants were dissolved with high RIPA buffer, and were processed for SDS-PAGE and immunoblotting. The buffers used apparently impair clear detection of the bands by mAb P1, as clearly defined bands could not be obtained after any stage of nuclear matrix preparation, although the signal was detected at the usual molecular weight. The results are shown in Figure 10, and indicate that circumferin was retained in the pellet at each stage up to treatment with NaCl. These results are consistent with those of Chaly *et al.* (1985), who found that circumferin was still visible by immunofluorescence after preparations of nuclear matrix. Some circumferin was solubilized after treatment with STM buffer, while a considerable

Figure 10: Circumferin in lymphocyte “nuclear matrix” preparation.

“Nuclear matrices” were prepared from Day 0 splenocytes, as described in the Materials and Methods. Aliquots of supernatants (SN) and pellets (P) from each stage of matrix isolation were loaded onto a 10% SDS-PAGE gel and then processed for immunoblotting with mAb P1. Each lane was loaded with 5 µg of protein, except for LMB P which was loaded with 1.4 µg, and NaCl P for which the protein concentration could not be determined. Migration of molecular weight standards is indicated in kDa in the left-hand margin. STM = Sucrose-Tris-Magnesium buffer; Tx100 = 0.5% Triton X-100 in STM buffer; DNase = 10 µg/mL DNase I in STM; LMB = low magnesium buffer; NaCl = 2 M NaCl in LMB buffer.

amount was detected in the supernatant after treatment with Triton X-100, and a smaller amount after treatment with DNase I. Circumferin was enriched in the pellet after treatment with Triton X-100, but did not appear to be further enriched at later stages of the preparation (Fig. 10). This is most likely due to the removal of proteins, resulting in a more concentrated sample of circumferin. After treatment with a high (1 M) concentration of salt, the signal could only occasionally be detected for the pellet sample and was absent for the supernatant sample (Fig. 10), even if samples were de-salted before SDS-PAGE.

Immunoblotting for circumferin in cell types other than mouse lymphocytes has consistently given either no bands, or too many bands. The enrichment of circumferin in lymphocyte “nuclear matrix” preparations suggested it might be similarly enriched, and thus detectable, in matrices of other cell types. Moreover, solubilization of many cellular proteins during matrix preparation might reduce the number of bands detectable by mAb P1 to a more reasonable number. Therefore, “nuclear matrices” of HeLa, mouse 3T3 fibroblasts (not shown), and mouse lymphoid EL4 cells were prepared and processed for immunoblotting with mAb P1. The results showed no significant improvements, however, with a large number of bands still detected in each cell type with matrices (Fig. 11)

3G. Deglycosylation

As discussed in the Introduction, many nuclear proteins including nuclear pore complex proteins, transcription factors, and chromatin proteins are glycosylated. The

Figure 11: Circumferin in fractionated EL4 and HeLa cells.

Nuclear matrices were prepared from (A) EL4 and (B) HeLa cells, as described in the Materials and Methods. Aliquots of pellets (lanes 1, 3, 5, and 7) and supernatants (lanes 2, 4, 6, and 8) from each stage of matrix isolation were loaded onto a 10% SDS-PAGE gel and then processed for immunoblotting with mAb P1. The gel in A was loaded with 10 μ g of protein per lane, except for lane 7 (LMB P) for which the protein concentration could not be determined. The gel in B was loaded with 20 μ g of protein per lane, except for lanes 2 and 3, for which the amounts of protein loaded were 1.5 and 3.2 μ g, respectively, and lanes 5 and 7, for which the protein concentration could not be determined. In Lane 9 (Day 0 splenocytes), 2.5 μ g were loaded. Migration of molecular weight standards is indicated in kDa in the left-hand margin. Samples were treated as follows:

Lanes 1 and 2	Sucrose-Tris-Magnesium buffer
Lanes 3 and 4	0.5% Triton X-100 in STM buffer
Lanes 5 and 6	10 μ g/mL DNase I in STM
Lanes 7 and 8	low magnesium buffer
Lane 9	Day 0 splenocytes.

A

B

detection of more than one band when immunoblotting suggested the possibility of multiple isoforms of circumferin. It was therefore postulated that circumferin might be glycosylated. Day 0 and Day 2 cell lysates were treated with various enzymes, alone and in combination, to remove sugars attached by both N- and O- linkages. The samples were then processed for immunoblotting.

No difference was observed between untreated samples (lane 1) and samples treated with deglycosylation buffer only (lane 2)(Figs 12, 13). Incubation of resting (Day 0) and stimulated (Day 2 Living) splenocytes with PNGase F, which removes N linkages, resulted in a dramatic shift in the mobility of circumferin (Figs. 12, 13, *c.f.* lanes 1 and 4). The overall pattern of four bands was retained after enzyme treatment, but at lower molecular weights of 59.8 ± 1.7 kDa, 63.2 ± 2.8 kDa, and 68.6 ± 2.6 kDa. This represents an overall shift in mobility of about 12 kDa (Figs 12, 13). For convenience, the fastest migrating band is still labelled Band 1, though the possibility that it is not necessarily derived from the Band 1 in untreated samples is recognized. Similarly, Bands 2, 3 and 4 are not necessarily derived from the similarly numbered bands in untreated samples. The lower band seen in lane 4 of Fig. 12 was not consistently detected, and its significance is unknown.

Endo-O-glycosidase removes O-linked sugars from proteins, but the enzyme is frequently not able to remove the sugar moiety until other side chains have been removed. Sialidase A cleaves non-reducing terminal sialic acids. Other side chains inhibiting the action of endo-O-glycosidase may be removed with $\beta(1-4)$ galactosidase or glucosaminidase. When splenocyte lysates were incubated with endo-O-glycosidase

Figure 12: Immunoblot of Day 0 mouse lymphocytes before and after deglycosylation.

Each lane was loaded with 2.5 µg of protein. Migration of molecular weight markers is indicated in kDa in the left-hand margin. Arrowheads in lanes 3 - 7 indicate the bands detected after enzyme treatment. See text for details. Samples were treated as follows:

Lane 1	Untreated; arrowheads indicate the three bands normally detected in Day 0 lymphocytes (see Figure 7)
Lane 2	Treated, deglycosylation buffer only
Lane 3	Treated with PNGase F, endo-O-glycosidase, sialidase A, $\beta(1-4)$ galactosidase and glucosaminidase
Lane 4	Treated with PNGase F
Lane 5	Treated with endo-O-glycosidase
Lane 6	Treated with endo-O-glycosidase and sialidase A
Lane 7	Treated with endo-O-glycosidase, sialidase A, $\beta(1-4)$ galactosidase and glucosaminidase.

Figure 13: Immunoblot of mouse lymphocytes stimulated for 2 days with Con A, before and after deglycosylation.

Samples were separated using Annexin V-conjugated magnetic beads, in order to obtain a predominantly living population. Each lane was loaded with 2.5 µg of protein. Migration of molecular weight markers is indicated in kDa. Arrowheads in lanes 3 - 7 indicate the bands detected after enzyme treatment. See text for details. Samples were treated as follows:

Lane 1	Untreated; arrowheads indicate Bands 1, 2, and 4 of those detected in Day 2 samples.
Lane 2	Treated, deglycosylation buffer only
Lane 3	Treated with PNGase F, endo-O-glycosidase, sialidase A, $\beta(1-4)$ galactosidase and glucosaminidase
Lane 4	Treated with PNGase F
Lane 5	Treated with endo-O-glycosidase
Lane 6	Treated with endo-O-glycosidase and sialidase A
Lane 7	Treated with endo-O-glycosidase, sialidase A, $\beta(1-4)$ galactosidase and glucosaminidase

alone (lane 5), the bands migrated as in untreated (lane 1) and buffer-treated (lane 2) controls (Figs 12, 13). However, band 4 was no longer visible when samples were treated with both endo-O-glycosidase and sialidase A (lane 6, Figs 12, 13). After incubating samples with β (1-4) galactosidase and glucosaminidase in addition to endo-O-glycosidase and sialidase A, two bands were detected at 72 and 73 kDa, suggesting a 2.12 ± 1.15 kDa increase in the mobility of band 2 (lane 7, Figs 12, 13).

When samples were treated with all five enzymes (PNGase F, endo-O-glycosidase, sialidase A, β (1-4) galactosidase, and glucosaminidase), the same banding pattern at the same molecular weight was seen as with treatment with PNGase F alone (lane 3, Figs. 12, 13).

As yet another approach to improving the specificity of mAb P1 blotting on samples of other cell types, lysates of the LMB pellet fractions of HeLa and EL4 cells were treated with a mixture of all the deglycosylating enzymes, and processed for immunoblotting (Fig. 14). As seen in Figure 13, there was no clear improvement in the specificity of mAb P1 on these samples. Similar results were obtained with 3T3 fibroblasts (not shown).

3H. Dephosphorylation

Phosphorylation is a common post-translational modification, and plays an important role in cell-signalling. Circumferin exhibits cell cycle-dependent changes in localization, detected at the nuclear periphery in interphase and at the chromosome periphery in mitosis. As such, it was a likely candidate for cell-cycle dependent

Figure 14: Immunoblot of LMB pellets of EL4 cells and HeLa cells.

Samples were run on 10% SDS-PAGE. Migration of molecular weights is indicated in kDa in the left-hand margin. Splenocyte samples (lanes 1 and 2) were loaded with 2.5 µg of total protein. The protein concentration of the LMB pellet fractions of EL4 and HeLa cells could not be determined, so each lane was loaded with 9 µL of sample.

Lane 1	Day 0 splenocytes, untreated
Lane 2	Day 0 splenocytes treated with PNGase F, endo-O-glycosidase, sialidase A, $\beta(1-4)$ galactosidase and glucosaminidase
Lane 3	EL4 LMB pellet
Lane 4	EL4 LMB pellet, treated as Lane 2
Lane 5	HeLa LMB pellet
Lane 6	HeLa LMB pellet, treated as Lane 2.

reversible phosphorylation. Lysates of Day 0 and Day 2 Living mouse splenocytes were treated with λ phosphatase to remove phosphate groups from serine/threonine residues. The lysates were then run on SDS-PAGE. Immunoblotting showed that bands of resting (Day 0) and stimulated (Day 2) splenocytes remained visible and migrated at their usual molecular masses (Fig. 15). Interestingly, the 72 and 75 kDa bands (Bands 1 and 2) were more intense after the sample preparation for dephosphorylation was completed, whether or not the enzyme was actually added, suggesting that the detection is enhanced by some component of the buffer used for the dephosphorylation.

Dephosphorylation of samples of other cell types, including mouse 3T3 fibroblasts, mouse lymphoid EL4 cells, and HeLa cells, resulted in the usual large number (>10) of bands being detected by immunoblotting (Fig. 16). With or without treatment by λ phosphatase, circumferin was not detectable by immunoblotting after 2D gel electrophoresis.

Figure 15: Immunoblot of dephosphorylated samples of resting and stimulated splenocytes.

Samples of resting (0) and 48 h-stimulated (2L) splenocytes were incubated with 200 units of λ phosphatase in buffer (λ PO₄ase), or in buffer alone (Buffer), as described in the Materials and Methods. Samples were loaded onto 8% SDS-PAGE gels at 5 μ g of total protein per lane. Migration of molecular weight standards is indicated in kDa in the left-hand margin.

Figure 16: Immunoblot of dephosphorylated EL4 whole cell lysate.

Samples of resting splenocytes (0) and EL4 whole cell lysate were incubated with 200 units of λ phosphatase in buffer (λ PO₄ase), or in buffer alone (Buffer), as described in the Materials and Methods. Samples were loaded onto 8% SDS-PAGE gels at 5 μ g of total protein per splenocyte lane, and 20 μ g of total protein per EL4 lane. Migration of molecular weight standards is indicated in kDa in the left-hand margin.

Chapter 4: DISCUSSION

4A. Behaviour of circumferin in stimulated lymphocyte populations

The first consistent immunoblots of circumferin were obtained using unstimulated mouse splenocyte samples. Changes to the standard blotting protocol, as described in the Materials and Methods and Results sections, resulted in the detection of three or four bands with apparent molecular weights of approximately 72, 75, 78, and 81 kDa (bands 1 through 4, respectively), with band 3 not always being detected. The detection of multiple bands led to the hypothesis that there are several isoforms of circumferin, due to post-translational modifications or splicing variants. Post-translational modifications of circumferin are discussed separately. The possibility of circumferin existing as multiple splicing variants cannot be ruled out.

In unstimulated lymphocytes, band 2 was significantly more intense than band 1, which was in turn more intense than bands 3 and 4. When lymphocytes were stimulated with Con A, however, the intensity of band 2 diminished and the intensity of the other three bands appeared to increase, until all bands were about equally prominent after 48 h of stimulation. It appears from these observations that stimulation of the lymphocytes led to a conversion of the 75 kDa isoform to the other three isoforms. Another possibility is that different splicing variants were expressed after stimulation.

Previous studies have shown an increase in nuclear and nucleolar volume of approximately 5.5-fold between unstimulated (morphotype I) and fully stimulated (morphotype III) lymphocytes (Setterfield *et al.*, 1985; Setterfield *et al.*, 1983). This

change is largely due to the ten-fold increase in the interchromatinic region of the nucleus (Bladon *et al.*, 1988). The volume of condensed chromatin, in contrast, was shown to increase only 1.5-fold (Setterfield *et al.*, 1983). Stimulation with Con A caused the mouse lymphocytes in this study to undergo a high amount of protein synthesis. When protein concentration was measured by the BCA assay, Day 2 samples were consistently found to have two to three times the amount of total protein per cell of Day 0 samples. With such a large increase in total protein, it was thought likely that the amount of circumferin present per cell would also increase, and this, in fact, was observed. By Sigma Gel analysis of slot blots, the amount of circumferin present per cell was found to double in the first 24 h p.s., and to undergo a further three-fold increase by 48 h p.s. However, since stimulated lymphocyte cultures still contain a significant proportion of morphotype I cells (Setterfield *et al.*, 1983), the increase in amount of circumferin per cell with stimulation may actually be larger than apparent by measuring a mixed population.

The intensity of P1 staining by immunofluorescence microscopy did not decrease with stimulation of mouse lymphocytes (Brown *et al.*, 1989). With the increase in nuclear volume and surface area that accompanies stimulation, this suggested that circumferin is synthesized during stimulation. This is also consistent with the observation that the amount of circumferin present per cell increased with stimulation. The change in the amount of circumferin, however, may have been different from the increase in total protein. When slot blots were analyzed using Sigma Gel, the amount of circumferin present as a proportion of total protein after 24 h of mitogen stimulation was found to be about 60% of that present in unstimulated (Day 0) samples. By 48 h p.s., the

relative amount of circumferin present was 30% of that present in Day 0 samples. The decrease observed in the amount of circumferin present relative to the total protein in the cells is likely due to a “dilution” of circumferin as the overall amount of protein per cell increases.

Circumferin remained detectable by mAb P1 in apoptotic lymphocytes by both immunoblotting and immunofluorescence microscopy. Despite the fact that extensive protein degradation occurs during apoptosis, circumferin was found to migrate with the same molecular mass in dead cells as in living cells, even when the cells had undergone so much degradation that the level of protein in the lysates was imperceptible by BCA assay. This implies that not only was the epitope still recognizable by the antibody, but that the protein had not been degraded to the point of losing mass. Fluorescent double-labelling with P1 and Annexin V also showed that circumferin was still peripheral both in early apoptotic cells and in cells that exhibited blebbing and other morphological signs of apoptosis. It therefore appears that circumferin is well maintained with little or no degradation during the apoptotic sequence of events. Although it is not unique for an antigen from apoptotic lymphocytes to migrate at the same molecular weight as in living samples (*e.g.* PI2) (Sodja *et al.*, 1997; Weaver *et al.*, 1996), many others, such as lamins, show degradation in the form of lower molecular weight bands in immunoblots of apoptotic samples (Sodja *et al.*, 1997; Weaver *et al.*, 1996). These observations support the hypothesis that circumferin is important in nuclear organization. Apoptosis is a highly regulated process, and circumferin may play a role in maintaining order during nuclear breakdown.

4B. Biochemical properties of circumferin and the P1 epitope

When SDS-PAGE and immunoblotting were conducted after “nuclear matrices” had been isolated from mouse splenic lymphocytes, circumferin was detected in the pellet (retained) fraction after treatment with detergent, partial chromatin extraction, and treatment with hypotonic buffer. Some of the antigen detected by immunoblotting with mAb P1, however, was also detected solubilized in the supernatant, after treatment with STM buffer. A larger amount of the antigen was solubilized after treatment with detergent (0.5% Triton X-100), and a small amount after partial solubilization of chromatin with DNase 1. Little or no circumferin was subsequently solubilized by hypotonic buffer (LMB). It is not clear whether all four of the bands detectable in untreated samples are present in either the retained or solubilized fractions during nuclear matrix preparation. The process, from the first step with STM buffer, has a negative effect on the quality of the blots obtained, so that the bands consistently appeared poorly defined and fuzzy.

It has previously been shown by indirect immunofluorescence microscopy of mouse 3T3 fibroblasts that the distribution of circumferin is largely unaffected by matrix preparation up to treatment with LMB, but that removal of chromatin by treatment with 1 M NaCl causes circumferin to be substantially redistributed (Chaly *et al.*, 1985). These observations are consistent with the hypothesis that circumferin is a chromosomal protein that serves to spatially organize the chromosomes during mitosis and binds chromatin to the nuclear envelope during interphase. Treatment with high salt and nucleases to remove chromatin appears to disrupt the interaction of circumferin with chromatin (Chaly

et al., 1985). Since microscopy of mouse 3T3 fibroblasts reveals that circumferin is still present, albeit redistributed, after nuclear matrix preparation is complete, failure to detect the antigen by immunoblotting beyond the LMB stage of the procedure is likely due to some other factor. It is possible that the high ionic strength of the salt buffer used in the final stages of matrix preparation changes the structure of the epitope so that it is no longer recognized on immunoblots.

Immunoblotting of circumferin has still not been carried out successfully in cell types other than mouse splenocytes. It had been hoped that treatments such as nuclear fractionation, deglycosylation, or dephosphorylation would “unmask” the epitope in other cell types. However, even in other murine lymphoid cells, such as mouse EL4 cells, and in mouse 3T3 fibroblasts, immunoblotting either showed no bands or a variable multiplicity of bands. The epitope detected by mAb P1 may be conformational, and this may be where the difference lies in other cell types. Other evidence suggesting a conformational epitope is the failure to detect circumferin in samples that had been boiled before SDS-PAGE. When the sequence of circumferin is obtained, it may be possible to produce another antibody that will recognize the protein in other cell types, or to assay circumferin by other methods, such as Southern blotting.

4C. Post-translational modifications of circumferin

Circumferin was shown to possess both N-linked and O-linked sugar moieties. N-linked glycosylation is an almost ubiquitous modification of proteins made in the ER. These proteins are destined to be trans-membrane proteins, or to be secreted. The N-

linked glycosylation of circumferin might therefore suggest that circumferin is a transmembrane protein of the inner nuclear membrane. However, immunoperoxidase labelling at the electron microscope level indicated that circumferin distribution more closely resembles that of lamins, underlying the nuclear membrane (Chaly *et al.*, 1989). Moreover, circumferin is localized around the periphery of the mitotic chromosomes during mitosis, as seen by immunofluorescence microscopy and by electron microscopy using immunogold labelling (Chaly *et al.*, 1984, 1989; Chaly and Stochaj, 1999). This change in localization would require some method for circumferin to become dissociated from the inner nuclear membrane since membranes are not detected at the chromosome surface at mitosis.

The function served by the glycosylation of circumferin also remains unknown. There was no difference observed in the glycosylation state of stimulated lymphocytes, compared to freshly isolated lymphocytes. All four forms of circumferin, represented by the four bands on immunoblots appear to possess similar amounts of N-linked glycosylation, since deglycosylation with PNGase F resulted in the same banding pattern, but at molecular weights 12 kDa lower than in the untreated samples. In contrast, the bands are apparently differentially modified by O-linked glycosylation. No band was detected at 81 kDa after treatment with endo-O-glycosidase and sialidase A, suggesting that the epitope was disrupted in Band 4 by the removal of the O-linked glycosylation. Treatment with endo-O-glycosidase, sialidase A, β (1-4) galactosidase, and glucosaminidase caused a slight mobility shift in one band, possibly Band 2. This suggests that the sugar moieties removed by these four enzymes are present in one form

of circumferin, but are not part of the epitope recognized by mAb P1.

After the basic N-linked oligosaccharide is added in the ER, many proteins are transferred to the Golgi apparatus where they undergo further processing. O-linked sugars are added to the hydroxyl groups of serine or threonine residues here. These proteins, like the N-linked glycoproteins, are destined for the lumen of organelles, or to be secreted. Circumferin is unusual in that it is neither secreted nor apparently membrane-associated, and yet it possesses both N- and O-linked glycosylations known only to be found on secreted or membrane-associated proteins. The relatively few known nuclear or cytoplasmic glycosylated proteins are modified by another form of O-linked glycosylation, O-GlcNAcylation (modification by *N*-acetylglucosamine) (Hart *et al.*, 1989). Other forms of glycosylation of nuclear and cytoplasmic glycosylation have been described but suspicion of contamination or lack of direct biochemical evidence have led these studies to be disregarded (Snow and Hart, 1998). The enzyme responsible for O-GlcNAcylation, O-GlcNAc transferase (OGT) was identified in rabbit reticulocyte membranes and rat liver, and has been found to be both cytosolic and nuclear (Haltiwanger *et al.*, 1990). Whether or not circumferin possesses this modification remains to be determined.

The glycosylation state of circumferin may explain why it was necessary to perform the blocking step of immunoblotting in BSA rather than skim milk. Skim milk is known to prevent detection of many glycosylated proteins.

Glycosylation is known to affect the mobility of proteins by SDS-PAGE. Therefore, the putative molecular weights assigned to the circumferin bands during this

study are not accurate. The true molecular weight of circumferin will not be known until the sequence is obtained.

Due to the changes in distribution of circumferin during the cell cycle, and due to the detection of four bands by SDS-PAGE, the phosphorylation state of circumferin was investigated. After treatment with lambda phosphatase, followed by 1D SDS-PAGE and immunoblotting, all four bands were detected by mAb P1, and at the same molecular weights as untreated samples. It cannot be stated conclusively, however, that circumferin is not phosphorylated. The small size of phosphate moieties means that the shift in mobility by SDS-PAGE caused by their removal is often imperceptible. Indeed, the observation that all known proteins modified by *O*-GlycNAc are also phosphorylated (Snow and Hart, 1998) suggests that circumferin is likely phosphorylated. This will need to be examined again once the sequence of circumferin has been obtained.

Glycosylation and phosphorylation are only two of the fourteen types of post-translational modifications known to occur on proteins, any of which might occur on circumferin. Among them, however, ubiquitination may be one of the more likely. The molecular mass of ubiquitin is 8 kDa, which is very similar to the difference of 9 kDa between the masses of circumferin Bands 1 and 4. Mono-ubiquitination, which growing evidence implicates in signalling responses (Hicke, 2001), may account for this difference.

4D. Efficiency of magnetic cell separation

Phosphatidyl serine expression on the outer plasma membrane is one of the

earliest hallmarks of apoptosis. Using Annexin V to detect phosphatidyl serine is a simple and effective way to distinguish between living and dead or dying cells.

Fluorescence microscopy revealed some cells that were labelled with Alexa 568-conjugated Annexin V but appeared morphologically normal. The other methods used in this study for detecting dead cells, Trypan Blue staining and flow cytometry, tend to be less sensitive. Cells which stain with Trypan Blue have lost selectivity of their plasma membranes. Cells recognized as dead by flow cytometry exhibited less DNA and a high degree of granularity. These events all occur later in the apoptotic pathway than phosphatidyl serine expression. It is, therefore, possible that cells which bound the Annexin V-conjugated beads may still exclude Trypan Blue and may not yet exhibit degradation of their DNA. This suggests that the proportion of living cells in the Living fraction, as reported by flow cytometry and Trypan Blue counts in Figure 2 may be artificially high. Since circumferin has been shown to be detected by immunoblotting at a greater intensity in dead cells than in an equivalent number of living cells, quantification of the changes in circumferin with stimulation may be affected by dead cells in the samples. Specifically, since there is a greater proportion of dead cells in the sample at 48 h p.s. compared to 24 h p.s., all quantifications of circumferin in Day 2L samples may be somewhat inflated above the true values.

Separation of the living and dead cell populations was always less effective on Day 2. A single-cell suspension is important for achieving a high quality magnetic separation. The tendency of stimulated lymphocytes to clump together was one factor contributing to this problem. Various methods were tried to improve the suspension,

such as passing the suspension through needles of different gauges, and vortexing during incubation with the magnetic beads. Passing the suspension through a needle was found to damage the cells, but brief, gentle vortexing early in the incubation with the magnetic beads gave a suspension with fewer clumps of cells, without harming the cells. Still, some clumps remained, and these likely reduced the efficacy of the cell separation, particularly on Day 2 when the clumps and the cells themselves were larger.

The quality of the separation on Day 2 may also have been affected by the degree of death in these samples. Cells in the later stages of apoptosis may have been so degraded as to no longer be able to bind the Annexin V-conjugated beads. These cells would then pass through the column into the Living fraction, thereby decreasing the purity of this fraction. Immunofluorescence microscopy and immunoblots both showed that circumferin was still detectable in apoptotic cells. It is therefore possible that even highly degraded cells possessed circumferin that could be detected by mAb P1, although they would likely not contribute much to total protein in the samples. This would have the effect of inflating the quantification of changes in circumferin relative to total protein.

4E. Conclusions

Circumferin is a protein with several distinctive characteristics. The cell cycle-dependent distribution of the protein suggests a potential role as a chromatin-binding protein of the nuclear envelope. The retention of circumferin until the removal of chromatin during nuclear fractionation, as seen by immunoblotting, supports previous results (Chaly *et al.*, 1985) and corroborates this hypothetical role. That circumferin is

detected in apoptotic cells, apparently without degradation, after significant degradation in total cellular protein, suggests that circumferin may also play a role in the packaging of degraded chromatin during apoptosis. The glycosylation state of circumferin is particularly interesting, as circumferin appears to be neither secreted, nor a membrane protein, yet exhibits both N- and O-linked glycosylation. The presence of the four bands detected by SDS-PAGE has not been fully explained by glycosylation or phosphorylation, and remains to be elucidated. The ability to detect circumferin by SDS-PAGE in cell types other than primary cultures of murine lymphocytes, too, remains a challenge to be resolved. Known to be present in virtually every tested species and cell type, circumferin is worthy of much further study.

The difficulties encountered in using mAb P1 severely limit future research on circumferin at this time. Therefore, obtaining the cDNA sequence must now be a priority. This will allow the production of circumferin protein in *in vitro* translation systems, which can then be used in the production of a more amenable antibody. It will then be possible to identify explicitly the role of circumferin in nuclear structure and function.

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