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CHROMAFFIN CELL SCINDERIN, A NOVEL CALCIUM DEPENDENT ACTIN SEVERING PROTEIN

Manjula Jeyapragasan

Thesis submitted to the Department of Biochemistry
in partial fulfillment of the requirements for the degree of
Master of Science

University of Ottawa
Ottawa, Ontario, Canada
May 1990

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UNIVERSITY OF OTTAWA

ABSTRACT

Several actin modulator proteins have been purified from a variety of sources and characterized in recent years. The present study describes the purification and characterization of a novel Ca^{2+} activated actin severing protein, scinderin present in the chromaffin cell of the adrenal medulla.

Scinderin has been purified to homogeneity using a combination of chromatographic procedures. The protein has an apparent molecular weight of 79,600±450 daltons, three isoforms (pIs: 6.0, 6.1 and 6.2) and two calcium binding sites; a high affinity site, (K_d= 5.85 X 10^{-7}M, B_{max}=0.81 mol Ca^{2+}/mol protein), and a low affinity site (K_d=2.85 x 10^{-6}M, B_{max}=1.87 mol Ca^{2+}/mol protein). Scinderin interacts with F-actin in the presence of Ca^{2+} and produces a decrease in the viscosity of actin gels as a result of F-actin filament severing as demonstrated by electron microscopy. Study of the physiochemical properties indicate that scinderin is a structurally and immunologically different protein from chromaffin cell gelsolin, another actin severing protein described earlier. Scinderin and gelsolin have different molecular weights, isoelectric points, and yield different peptides after limited proteolytic digestion by either staphylococcal V8 protease or chymotrypsin. Moreover, scinderin antibodies do not cross-react with gelsolin and gelsolin antibodies fail to recognize scinderin. Immunofluorescence with anti-scinderin demonstrated that this protein is mainly located in the subplasmalemma region of the chromaffin

cell. Immunoblotting procedures with anti-scinderin revealed the presence of scinderin in other tissues notably brain , anterior and posterior pituitary gland and kidney. In all these tissues scinderin was found to co-exist with gelsolin. Unlike gelsolin it was not present in liver, blood, skeletal or heart muscle. Presence of scinderin and gelsolin, two Ca^{2+} dependent actin severing proteins in the same cell suggests the possibility of synergistic functions by the two proteins in the control of cellular actin filament networks. Alternatively, the actin severing activity of the two proteins might be under the control of different transduction and modulating influences.

The findings on the 80 kDa protein, scinderin present in tissues with secretory activity, are discussed in relation to its possible role as a calcium dependent modulator of actin in cytoskeletal reorganization which occurs in response to agonist stimulation.

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ABBREVIATIONS USED IN THIS THESIS

APUD	amine precursor uptake and decarboxylation
ATPase	adenosine 5′- triphosphatase
BSA	bovine serum albumin
cAMP	adenosine 3′,5′- monophosphate, cyclic
DβH	dopamine β hydroxylase
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino-ethyl ester) N,N′-tetraacetic acid
GTP	guanosine 5′-triphosphate
kDa	kilodalton
MAP	microtubule associated protein
NaN_3	sodium azide
PBS	phosphate buffered saline
PMSF	phenyl methyl sulfonyl fluoride
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
TPBS	Tween phosphate buffered saline

CHAPTER I

INTRODUCTION

A. THE ADRENAL CHROMAFFIN CELL AND THE PARANEURON CONCEPT

The adrenal medulla occupies a unique position in the metazoan organism. The parenchymal cell of the adrenal medulla, the adrenal chromaffin cell is a modified postganglionic sympathetic ganglion cell which synthesises and secretes adrenergic neurotransmitters into the blood stream. The chromaffin cell thus occupies an unusual and interesting position in the bipartite neuroendocrine system and has served as one of the most readily available model systems for the study of the mechanisms of neuro-transmitter release.

The nervous system and the endocrine system have always been considered as two distinct entities comprising of unique cells with different activities. However, the advances in electron microscopy, cytochemistry and electrophysiology showed features which were thought to be unique to neurons were actually common to several non-neuronal cells. Pearse (1969) noted that cells capable of amine precursor uptake and decarboxylation have many common cytochemical features such as common neuroectodermal origin, and common mechanisms of hormone synthesis and secretion. He proposed the acronym APUD for these cells. Endocrine tissues, such as chromaffin cells and thyroid parafollicular cells, as well as some neuronal cells belonged to this "APUD" series. However, later studies by Le Douarin and Teillet (1973) and Andrew (1974) indicated that some members of this APUD series were not derived from the neural crest. It was also found that many

endocrine cells and neurons did not possess the APUD ability and thus it was felt that these criteria were insufficient to classify neuron-related cells. In contrast to Pearse's cytochemical view point, Fujita (1977) categorised the neuron-like cells on the basis of their structure, function, metabolism and origin. These cells called the paraneurons, are traditionally not considered as neurons but are related to them. The paraneurons are characterized as follows:

1) Production of substances identical or related to neurotransmitters or neurohormones
2) Presence of neurosecretory-like and/or synaptic-vesicle-like granules
3) Release of secretions in response to adequate stimuli acting upon a cell membrane receptor.
4) The cell should be of neuro-ectodermal origin.

Some members of this family include gastroenteric endocrine cells, adrenal chromaffin cells, mast cells, melanocytes, pancreatic islet cells, pinealocytes, adenohypophysial cells, parafollicular and Merkel cells (Fujita, 1980). Adrenal chromaffin cells are typical paraneurons (Kobayashi and Coupland, 1977) and are the largest homogenous population of paraneurons. It is now recognized that neurons and paraneurons share much in common in terms of structure and function. As a result of this observation, many parallels have been drawn between them. As well as sharing such features as exocytosis, these tissues also seem to possess similar mechanisms for the synthesis, storage and

Fig.1: Schematic representation of three different portions of the cell membranes (ie. receptive, conductile and secretory) in a neuron (α-motoneuron), a neurosecretory cell and a paraneuron. (Taken from "The Paraneuron", Fujita, T., Kanno, T. and Kobayashi, S., 1988)

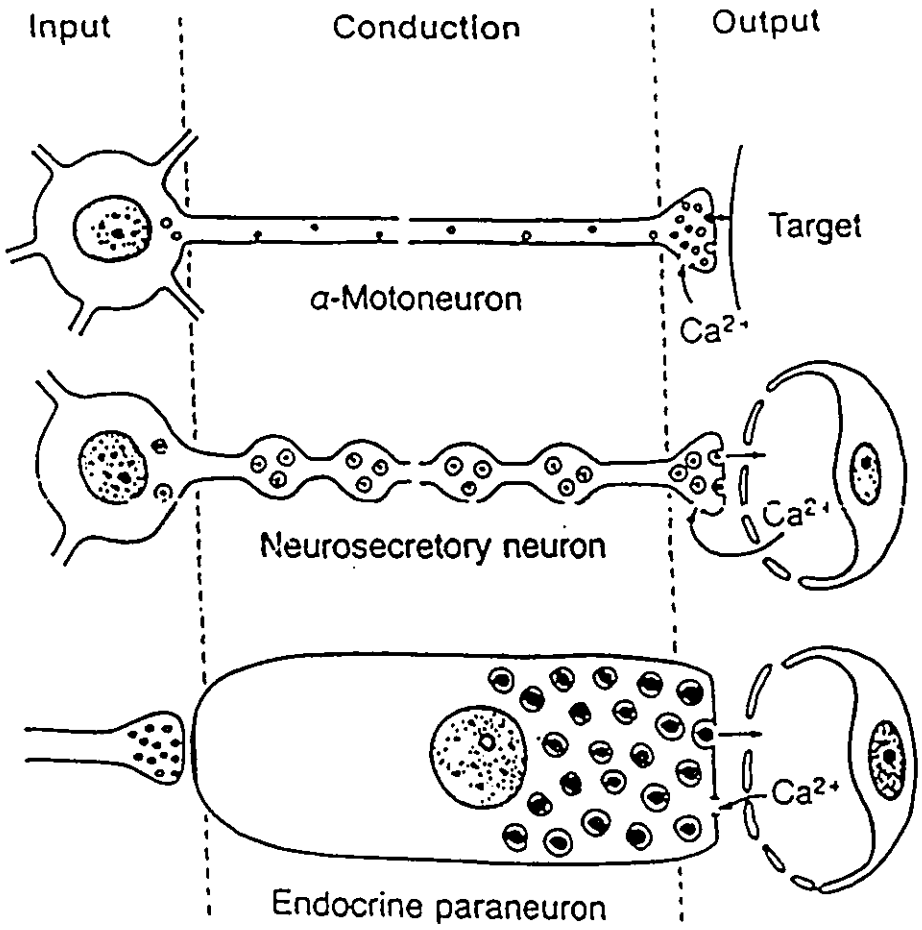


Fig. 1. Schematic representation of three different portions of the cell membrane.

release of their secretory products. Much of the interest generated by the adrenal medulla is due to its relationship to the nervous system. The adrenal chromaffin cell is the main cell type used in the present study. This is a typical endocrine paraneuron which is capable of secreting both peptides and biogenic amines. As pointed out by Kobayashi (1977) the study of the cell biology of the adrenal chromaffin cell may advance our understanding of the nature and function of paraneurons in general. Figure 1 demonstrates the relationship between neurons (alpha-motorneurons), the neurosecretory cell and endocrine paraneurones and shows the similarities in input, conduction and output.

B. THE ANATOMY AND MORPHOLOGY OF THE ADRENAL CHROMAFFIN CELL

As mentioned earlier, the adrenal medullary chromaffin cells provide an excellent system to study the molecular mechanisms underlying the transport and release of secretory products (Livett et al., 1983; Livett, 1984; Trifaró, 1982). It presents a large population of similar cells with many nerve-like or paraneuronal properties. Thus, the morphology, biochemistry, physiology, pharmocology and the pathology of these neural elements can be studied with relative ease. Many important biological concepts such as amine and nucleotide uptake, trans-synaptic induction, transmitter secretion and release mechanisms, and stimulus-secretion coupling have been developed from studies of the adrenal medulla

(Carmichael, 1979). Very little was known about these cells until 1953 when a major breakthrough in the understanding of transmitter storage occurred. Blaschko and Welch (1953) and Hillarp et al. (1953) simultaneously demonstrated that centrifugation of adrenal medulla homogenates sedimented the major secretory product of this gland, the catecholamines, thus setting the stage for the isolation of chromaffin granules. In 1955, Lever published the first electron micrographs showing the presence of secretory granules in chromaffin cells. This discovery was shortly followed by the observation of De Robertis and Vaz Ferreira (1957) demonstrating the release of granules by exocytosis. Since these initial studies, research on chromaffin cells and chromaffin granules has yielded a wealth of information on secretory mechanisms.

The adrenal medulla originates from the neural crest, sharing the same precursor as the sympathetic ganglia (Coupland, 1965; Le Douarin, 1982). Adrenal chromaffin cells are directly innervated by preganglionic sympathetic neurons arising in the intermediolateral horn of the spinal cord at segments T1 to L1 (Keese et al. 1988). These preganglionic neurons appear to be surrounded by fibers containing enkephalin, serotonin, substance P and somatostatin (Holets and Elde, 1982). These peptides may modulate the action of nervous stimulation on chromaffin cells. The adrenal medulla also receives adrenergic innervations (Prentice and Wood, 1975) from sympathetic postganglionic neurons. This rich innervation is not shared by the adrenal cortex.

The adrenal medulla consists of three cell types which are distinct from other neural, vascular and supportive cells;

Adrenaline storing (A), nor-adrenaline storing (NA) and small granule chromaffin (SGC) cells (Hillarp and Hokfelt, 1953; Unsicker, 1976). These cells differ in terms of the morphology of their granules and their staining. The "A" cells possess rounded secretory granules, whereas the granules of "NA" cells are irregular in shape. The granules of both cell types also show an electron dense core. The "SGC" cells appear to contain two vesicle types, a dense core vesicle and a smaller "synaptic" type vesicle (Kobayashi and Coupland, 1977). The content of the granules of SGC cells is unknown. The ultrastructure of the adrenal chromaffin cell appears to be relatively well preserved among the various mammalian species (Grynszpan-Winograd, 1975). It has been demonstrated that in humans, the "A" cells constitute approximately 95% of the chromaffin cell population (Benchimol and Cantin, 1977).

C. SECRETORY PROCESS IN THE ADRENAL CHROMAFFIN CELL

It is clear that the major activity of the adrenal medulla is to secrete primary amines in response to the appropriate stimuli. When a nerve impulse causes neurons innervating the adrenal medulla to fire, the released acetylcholine interacts with nicotinic receptors in the chromaffin cell membrane, setting in motion a process in which the calcium channels open and allow the intracellular calcium ion concentration to rise. In addition to calcium, magnesium, ATP, other ions and

second messengers, protein kinase C, calmodulin and calmodulin binding proteins, guanine nucleotide binding proteins, and yet unknown other factors have been shown to be essential to mediate the complex mechanisms underlying secretion and release of secretory granules (Knight and Baker, 1987). This complex process is comprehensively termed stimulus-secretion coupling. A single chromaffin granule contains, in addition to the catecholamines adrenaline and noradrenaline, a diverse mixture of proteins that may have widespread effects on the nervous system and on other organs. Chromogranin A, B and C, enkephalins and neuropeptides are some of the proteins stored and co-secreted with catecholamines. The contents of the chromaffin granule are widely thought to be released to the exterior by the process of exocytosis. The weight of evidence supporting the idea that exocytosis is the mechanism by which secretion occurs is overwhelming. On the biochemical front there is the observation that all the soluble components of the secretory vesicles are released together into the extracellular fluid (Viveros et al., 1968) and that there is correlation between the incorporation of vesicle membrane antigens into the plasma membrane and secretion (Lingg et al., 1983; Phillips et al., 1983). Electron microscopy and image-enhanced microscopy have captured the various stages of the fusion event and have shown the rapid disappearance of the secretory products from the vesicles (Ornberg and Reese, 1981). These findings are further substantiated by data from studies

using false transmitters, whereby chemically modified transmitter substances fail to be secreted if they are not accumulated within secretory vesicles (Smith and Winkler, 1972) and from electrophysiological studies which demonstrated unit sizes of miniature end plate potentials (Katz and Miledi, 1969). More recently electrical capacitance measurements of patches of membranes under conditions favouring secretion have proven that vesicle membranes do fuse to plasma membrane prior to the release of the vesicular contents (Fernandez et al., 1984). Extensive research in secretory mechanisms has now established exocytosis as the final phase of stimulus-secretion coupling in secretory cells. Exocytosis is widely accepted as the primary release mechanism for all neurotransmitters and hormones, except for the steroids secreted by the adrenal cortical cells and the gonads (Trifaró, 1977; Trifaró and Cubbedu, 1979; Trifaró and Poisner, 1982; Knight and Baker, 1987).

The main considerations thus far indicate that a rise in intracellular free calcium facilitates catecholamine secretion. The coupling between increased calcium levels and secretion is controversial, but several investigators maintain that calcium activates contractile proteins in the chromaffin cell and this leads to secretion. In recent years contractile proteins of the chromaffin cell cytoskeleton have been purified and characterised and the role of the chromaffin cell cytoskeleton and its regulatory proteins in mediating the release process

has been extensively studied (Trifaró et al., 1985a, 1985b; Trifaró et al., 1988; Trifaró et al., 1989). The present project also deals with a probable cytoskeleton modulating protein which is activated by elevation of intracellular calcium levels.

D. CHROMAFFIN CELL CYTOSKELETON AND ITS REGULATORY PROTEINS

D.1.Cytoskeleton

The cytoskeleton is described as a framework of cytoplasmic filaments which are retained together with the nucleus and display very good ultrastructural preservation when cells are extracted under controlled conditions by non-ionic detergents. It corresponds to the three-dimensional network formed by the nucleus, organelles, fibrous systems, and membranes with the assumption that these structures function co-ordinately. Several aspects related to the organization and functions of the cytoskeletal filament networks and their associated proteins are extensively reviewed (Stossel et al., 1985; Oliver et al., 1985). A series of specific proteins, some of them previously known from studies of muscle, form a cytoplasmic network in all nonmuscle cells. Immunocytochemical studies have shown the existence of at least three different filament systems, namely microfilaments, microtubules and intermediate filaments (neurofilaments in neuronal tissues) which are distinguished principally by their size and location. Cooke and Poisner (1979) pointed out that these fibrillar cytoplasmic organelles are strategically located where

they can participate in moving chromaffin vesicles to the cell surface during exocytosis. In addition, several specific proteins associated with these filamentous networks have been described.

D.2. Components of the cytoskeleton

D.2.i.Microfilaments

These submembranous structures are composed of G-actin subunits which are helically arranged in a double-stranded filament (F-actin) with a diameter of 4-7 nm. Two distinct systems of micro-filaments have been observed, the core microfilament system and the cross-bridging microfilament system (Cohen and Nemhauser, 1985; Fox, 1985). The microfilament network has often been associated with structural roles in maintaining the cytoarchitecture and cytoplasmic consistency. However, at present, more than forty micro-filament associated proteins have been isolated and characterized from various non-muscle cell systems as well as in muscles (Craig and Pollard, 1982; Cooke, 1985). These proteins have been shown to regulate assembly of microfilament structures, to mediate microfilament reorganization and to be involved in the interaction of microfilaments with cytoplasmic and subcellular organelle membranes. Consequently, the numerous microfilament-associated proteins were classified into three functional groups:

1) Proteins that promote microfilament formation - ie. actin gelling and crosslinking proteins, actinogelin, gelactin (I-IV), filamin, fimbrin and fascin.

2) Proteins which disrupt microfilament formation or existing

microfilament structures such as gelsolin, brevin, fragmin, villin, 60kD-capping protein, profilin, β-actinin and α-actinin. The protein scinderin referred to in the present study may be included in this category.

3) Anchorage proteins such as alpha-actinin, spectrin, globin, ankyrin, vinculin, 110 kDa-microvillus protein and talin.

The functional activity of some microfilament associated proteins has been found to be regulated by different factors such as Ca^{2+} concentrations, pH, ionic strength and temperature. The effects of Ca^{2+} is most notably seen in villin which produces opposite effects (promoting and disrupting) on microfilament systems. The two distinct actin-binding sites of villin are differentially regulated by Ca^{2+}, whereby at Ca^{2+} concentrations less than 10^{-6} M Ca^{2+}, villin promotes microfilament association but at concentrations greater than 10^{-6}M Ca^{2+}, villin inhibits microfilament formation (Glenney et al., 1981). Gelsolin which is functionally and structurally related to villin also expresses effects on actin-severing, nucleation and end-blocking which are principally regulated by intracellular calcium concentration (Yin ,1988). Therefore, intracellular elevation in calcium plays a pivotal role in restructuring the actin cytoskeleton in response to agonist stimulation.

D.2.ii. Microtubules

Microtubules appear as tubular structures of indefinite length with an approximate outer diameter of 25-30 nm when examined under the

electron microscope. These are highly ordered polymers of the globular protein tubulin (Mr 110,000 Daltons). This protein is composed of two similar but not identical subunits (alpha and beta) with molecular weights of about 53,000. Native single microtubules are composed of about thirteen protofilaments which are elongated tubulin (alpha,beta) heterodimers arranged end to end. In addition to monomer concentrations, several factors appear to regulate the assembly-disassembly process of micro-tubules. These include guanine nucleotides, divalent cations (Mg^{2+}, Ca^{2+}), pH, temperature and the presence of microtubule-associated proteins and also their phosphorylation. Microtubules appear to be central elements of cellular organization. They have been implicated in several cellular functions such as changes in cell shape, cell motility, axonal transport, subcellular organelle motility, DNA-synthesis, and cell membrane related events (Vale et al., 1985a, 1985b). They play an important role in the secretory granule transport and in the neurite outgrowth and maintenance in chromaffin cells (Trifaró et al., 1985a). The presence of high affinity binding sites for tubulin on the chromaffin granule membrane support the idea of a possible association between microtubules and chromaffin granules (Aunis et al., 1987). The best documented function of microtubules is their role in cell membrane-related events. These filamentous structures have been demonstrated to modulate the response of cells to receptor triggering at multiple sites. Microtubules are involved indirectly in the distribution and motility of cell surface receptors. Surface receptors may be linked through the

membrane with microfilament networks, the activity and distribution of which could in turn be coordinated by a microtubule-dependent mechanism. This type of interaction could take place through microfilament-associated proteins such as spectrin or calmodulin-dependent linker proteins (Sobue et al., 1985). Although molecular interactions of microtubules with other cellular structures are not completely understood, microtubules appear to transfer information through the cytoplasm in a bidirectional fashion. Inspite of their possible role in the transfer of chromaffin granules to the cell periphery, microtubules do not seem to be involved in the final stages of secretion (Trifaró et al., 1985a).

D.2.iii. Intermediate filaments (Neurofilaments)

The term "intermediate filament" is now applied to all 10nm filaments (7-11 nm) regardless of their location. Current biochemical and immunofluorescence methods have established the intermediate filaments as a distinct class of cytoplasmic proteins, that differ with respect to the physical properties of their subunits. In contrast to the major structural protein subunits of microfilaments and microtubules, the intermediate filament proteins do not appear to be highly conserved and exhibit a relatively high degree of tissue specificity. The chromaffin cell expresses neurofilament proteins because of its derivation from the neural crest (Bader et al., 1984; Trifaró et al., 1985a).

The exact functional significance of the different

intermediate filament systems which are present in various cell types is still not fully understood. They seem to have a cytoskeletal function in many cell types (Weber and Osborn, 1982), and appear to interact extensively with membranes through desmosomes (Jones and Goldmann, 1985). They may also be involved in nuclear anchorage and act in muscle cells as "mechanical integrators of cellular space" (Zackroff et al., 1981). In addition, many intermediate filaments seem to exist in a state of interdependence with microtubule expression (McIntosh, 1982) and interact with microfilaments through specific proteins such as spectrin, ankyrin and syndeins (Goodman and Zagon, 1986).

D.3. Contractile proteins of the cytoskeleton

D.3.i. Actin

The unequivocal demonstration of actin in the chromaffin cell can be found in the studies of Lee et al., (1979) on the characterisation of this protein. It possesses an apparent molecular weight of 42 kDa and contains two isomeric forms namely β-actin and -actin. Immunohistochemical studies using actin antibodies show that actin is widely distributed in the cells and is associated with many subcellular structures. The granular distribution pattern observed for actin in these experiments was similar to that observed when chromaffin cells were stained with antibody against dopamine β hydroxylase (Trifaró et al., 1985a) This similarity as well as other biochemical studies suggested that actin could be in association with chromaffin

granules. Under certain experimental conditions actin interacts with chromaffin granules as well as other chromaffin cell organelles (Burridge and Phillips, 1975; Fowler and Pollard, 1982; Trifaró et al., 1982; Perrin and Aunis, 1985). The binding of actin to chromaffin granules is affected by the calcium concentration of the medium as well as the ionic strength. Membranes from granules isolated at high ionic strength bind more actin than those isolated at low ionic strength, a condition that removes α-actinin from the membranes (Burridge and Phillips, 1975). So α-actinin may play a facilitatory role in the chromaffin granule - actin interaction. The presence of calcium decreases but does not abolish the binding of actin to granule membranes, indicating that part of the binding is calcium independent (Aunis and Perrin, 1984). Furthermore in vitro experiments show that chromaffin granule membranes will induce polymerisation and gel formation, effects which are blocked by raising calcium concentration in the medium (Fowler and Pollard, 1982). The association of actin filaments with chromaffin granules has been confirmed by electron microscopy (Burridge and Phillips, 1975; Wilkins and Lin, 1981). Friedman et al., (1980) demonstrated that catecholamine secretion from isolated chromaffin cells can be increased by the addition of an enzyme DNAse 1 that depolymerises actin filaments. So it may be concluded that rise in intracellular calcium levels may activate certain proteins which may have a local disruptive effect on the

preformed actin filaments and thus bring about the release of secretory vesicles. Gelsolin is one such protein and its presence in the chromaffin cells have been demonstrated (Trifaró et al., 1985a; Bader et al., 1986).

D.3.ii. Chromaffin cell myosin

The presence of myosin in chromaffin cells was first suggested by the presence of actomyosin-like protein in these cells (Trifaró and Ulpian, 1975; Ulpian, 1977). This protein had ATPase activity that was activated by Ca^{2+} and Mg^{2+} and decreased by the presence of high concentrations of K^{+}, which is characteristic of muscle actomyosin. The chromaffin cell myosin was purified by classical salting out procedure followed by gel-filtration chromatography (Trifaró and Ulpian, 1976). There was a close similarity in the aminoacid composition among chromaffin cell myosin, rabbit skeletal muscle myosin and the myosin from Plasmodium physarum (Trifaró and Ulpian, 1976). The adrenal myosin molecule, which is 160 nm long and consisted of two globular heads joined by a flexible tail, is morphologically similar to muscle myosin.

By performing immunofluorescence studies using myosin antibodies (Trifaró et al., 1978) determined that myosin in the adrenal medulla is primarily associated with the plasma membranes. This observation was substantiated by Hesketh et al., (1978) who studied the distribution of K-ATPase activity in

subcellular fractions. Highly purified myosin is poorly activated by actin and the interaction of myosin with actin requires another cofactor, which in the tissues has not been identified. This cofactor probably contains myosin light chain kinase and calmodulin. In other tissues these two factors are known to mediate phosphorylation of the myosin light chain which is required for myosin activation (Adelstein and Eisenberg, 1980).

D.3.iii. Chromaffin cell α-actinin.

The presence of α-actnin in the chromaffin cells has been demonstrated by indirect immunofluorescence using either bovine skeletal muscle α-actinin (Aunis et al., 1980) or chicken skeletal muscle α-actinin (Trifaró et al., 1984). The staining pattern showed α-actinin to be distributed over the entire cell body. A punctate staining pattern was observed in the cytoplasm and along the neurites (Trifaró et al., 1984) and a very strong fluoresence was observed at the neurite endings where granules accumulate. This staining pattern appeared to correlate with those of DβH and chromogranin A. Thus it was suggested that α-actinin may be present in chromaffin granules (Burridge and Phillips, 1975) and was confirmed by later studies by Bader and Aunis (1983).

α-actinin is a 97 kDa protein which appears to be localized on the external surface of the granule membrane (Bader and Aunis, 1983). The results obtained from pronase digestion of intact

and broken granules sugested a localization on the cytoplasmic surface of the granule for both α-actinin and actin. α-Actinin molecules are suggested to act as either nuclei which promote actin polymerisation and the formation of membrane bound actin filaments or they may stabilise the actin nuclei probably by anchoring them to the granule membrane (Trifaró et al., 1985a).

D.3.iv. Chromaffin cell tropomyosin

Tropomyosin is present in both muscle and nonmuscle systems as a major contractile protein and as an important component of the microfilaments maintaining the cytoarchitecture (Côté, 1983; Payne and Rudnik, 1985). In the adrenal medulla three major heat stable peptides were isolated (38, 35.5 and 32 kDa). They have similar biochemical properties as other muscle and nonmuscle tropomyosins (Côté et al., 1984). The functional implications of tropomyosin in the chromaffin cell have not been established yet.

D.4. Other regulatory proteins of the cytoskeleton

D.4.i. Chromafin cell Spectrin (Fodrin)

Spectrin is an important constituent of the erythrocyte cytoskeleton. This binds to other cytoskeletal proteins including actin microfilaments, ankyrin and band 4.1 protein (Branton et al., 1981). A spectrin like protein (α-fodrin) has been demonstrated in the chromaffin granule membranes and it appears to be localised on the cytoplasmic surface of the granule. It consists

of two subunits (α and β) of molecular weights 240 kDa and 235 kDa respectively. Fodrin, like α-actinin is believed to play a role in anchoring the actin nuclei to the granule membrane. Aunis and Perrin (1984) found that chromaffin vesicle membranes cause a large increase in viscosity of F-actin solutions, probably due to the presence of α-fodrin and suggested that the interaction of F-actin with the fodrin on the chromaffin vesicle membrane is important in controlling the movement of chromaffin vesicles.

D.4.ii. Chromaffin cell calmodulin

Kuo and Coffee (1976) isolated a troponin-C-like protein from the adrenal medulla. They demonstrated this protein was similar to calmodulin based on its physico-chemical properties. The presence of calcium dependent calmodulin binding sites of high affinity has been demonstrated in chromaffin granule membrane (Hikita et al., 1984; Burgoyne and Geisow, 1981). Two calmodulin binding membrane proteins of 65 kDa and 53 kDa have been identified in the chromaffin granule membrane (Bader et al., 1985). Many of the calcium dependent events in the eukaryotic cell are mediated by the intracellular calcium binding protein calmodulin (Cheung, 1980). The process of secretion is a Ca^{2+} mediated event and by the use of calmodulin antibodies in intact chromaffin cells, evidence of the inhibitory effects of these compounds has been established (Kenigsberg and Trifaró, 1985).

Moreover the subcellular distribution of calmodulin depends on the presence or the absence of calcium, suggesting that the changes in the cellular levels of Ca^{2+} might control the subcellular distribution of calmodulin in the chromaffin cell (Hikita et al., 1984). How calmodulin may be involved in secretion is yet to be determined. However the presence of calmodulin binding proteins in the chromaffin granules may suggest that calmodulin may be involved either with the transport of granules to the releasing sites or with the process of interaction between granule and plasma membrane during exocytosis. Calmodulin has been shown to regulate the phosphorylation of myosin light chain which in turn is required for the activation of myosin ATPase by actin (Adelstein and Eisenberg, 1980). Alternatively calmodulin might be involved with the process of retrieval of granule membrane after exocytosis.

D.4.iii. Chromaffin cell gelsolin

Gelsolin is a globular protein first isolated from macrophages, that regulates the network structure of actin filaments (Yin and Stossel, 1979). A gelsolin-like protein has been isolated from chromaffin cells and was found to cross-react with an antibody raised against macrophage gelsolin (Trifaró et al., 1985a). Later Bader et al., (1986) compared its properties with that of macrophage gelsolin and demonstrated that, structurally and immunologically, both proteins are identical.

In the presence of micromolar concentrations of calcium, gelsolin binds to calcium and to actin filaments and prevents gelation of these filaments by actin binding proteins (Yin and Stossel, 1980; Yin et al., 1981). The net effect of gelsolin on actin filaments is to promote the number of short actin filaments which are capped at the barbed end; thus, the addition of monomeric actin to this end to produce elongation is effectively inhibited (Yin, 1988). Thus gelsolin is proposed to act as a calcium dependent modulator of the cytoskeleton, mediating the transition from gel state to the sol state following an agonist stimulation of the secretory cell. This transition precedes exocytosis and may be required to allow the secretory vesicles to reach releasing sites in the plasma membrane .

D.5. Contractile proteins and the secretory process

The presence of contractile proteins and cytoskeletal components as well as the regulatory protein calmodulin in chromaffin cells, and in particular, their interaction with chromaffin granules have led several investigators to postulate that these components may be involved in the secretory process of chromaffin cells. Actin is widely distributed and forms a meshwork underneath the plasma membrane (Lee and Trifaró, 1981). Myosin is located in the plasma membrane while the α-actinin and fodrin which are granule membrane proteins, interact with subplasmalemmal actin. Fodrin is located in the plasma membrane too. So actin

microfilaments not only interact with the inner surface of the plasma membrane but also with the cytoplasmic surface of the chromaffin granules, probably through their binding to α-actinin and fodrin, two actin anchorage proteins.

Two molecular mechanisms of action for the cytoskeletal proteins possibly involved in the secretory process have been put forward (Trifaró et al., 1985a, 1989). The leading hypothesis was that exocytosis is a true contractile event and therefore, the cytoskeletal components and their regulatory proteins might be involved in the mechanism of transport of secretory granules to the plasma membrane, in the fusion process or in the extrusion phenomenon itself. This hypothesis was based on the similarities between stimulus-secretion coupling and excitation-contraction coupling in the muscle. The two mechanisms of action proposed for the intervention of contractile proteins are described briefly.

1) The first mechanism of action involves the contractile proteins actin, myosin, and α-actinin as well as the regulatory protein calmodulin.

Similar to muscles, a sliding filament system would be operative in chromaffin cells. Therefore, myosin should be arranged in bipolar filaments and actin-binding sites shoud be present on both granule and plasma membranes. In this case, α-actinin present in granule membranes would provide binding sites for actin while an anchorage protein (?) would play a similar role in plasma membrane. As cell depolarization induces Ca^{2+} entry into the cell this results in a transient increase in

intracellular Ca^{2+}. These events, in turn, would activate the sliding filament mechanism via a calmodulin-regulated activity. This implies the activation of myosin ATP-ase by actin via the phosphorylation of the 20kDa-myosin light chain which is regulated by calmodulin.

2) The second mechanism of action is based on the viscosity properites of actin and does not necessarily involve myosin (see Fig.2B). In this case under resting conditions actin controls cytosol viscosity through the formation of a mesh of microfilaments which are cross-linked and stabilized by fodrin and alpha-actinin. These events appear to be regulated by Ca^{2+} as demonstrated in recent studies. Therefore, under resting conditions, (10^{-8} M free Ca^{2+}) the cytosolic actin network would oppose the movement of secretory granules towards the release sites at the level of the plasma membrane (Fig.2A). Upon cell stimulation, Ca^{2+} enters the cell and produces,

(i) a dissociation of actin from fodrin

(ii) patching of fodrin along the plane of plama membrane, and

(iii) activation of gelsolin with a consequent capping and shortening of the actin microfilaments.

Consequently, the cytosol viscosity decreases, allowing the movement of granules towards the plasma membrane releasing sites. The regulatory protein calmodulin could be involved in the modulation of these events or alternatively may be involved in the granule-plasma membrane fusion process itself. The two mechanisms of action described above are substantiated by numerous studies conducted in chromaffin cells. However, they do not exclude the possibility that other cytoskeletal and

Fig.2: A schematic representation of a possible mechanism in which cyto skeleton proteins may play a role in chromaffin cell (CC) secretion. (A) under resting conditions actin (——) filaments which are cross-linked and stabilised by (i) fodrin (■) and by (ii) α-actinin (▲). The secretory granule membranes also contain a calmodulin (CM)-binding protein (●). A similar CM-binding protein is also present in the plasma membranes. At the Ca^{2+} concentration found in the resting cells, CM (⋈) and gelsolin (⧫) are not activated and there is a large percentage of non-filamentous, non-phosphorylated myosin (⊱). (B) When chromaffin cell is stimulated , Ca^{2+} enters the cell and produces

(i) a dissociation of actin from fodrin, (ii) patching of fodrin along the plane of plasma membrane (▬▬) and (iii) activation of gelsolin with the consequent capping and shortening of the actin micro-filaments. Calcium does not affect the binding of actin to granule membrane α-actinin. As a result of both (i) and (iii), the cytosol viscosity decreases, allowing the movement of granules towards the plasma membrane releasing sites. Whether an actin-myosin interaction (sliding mechanism) is also involved in granule movement remains to be determined. The intracellular Ca^{2+} concentration reached during stimulation is sufficient to activate calmodulin dependent processes, including the binding of CM to granule membranes and plasma membranes (fusion) and the phosphorylation of myosin-like chains, a condition required for myosin activation and bipolar filament formation (⋘).

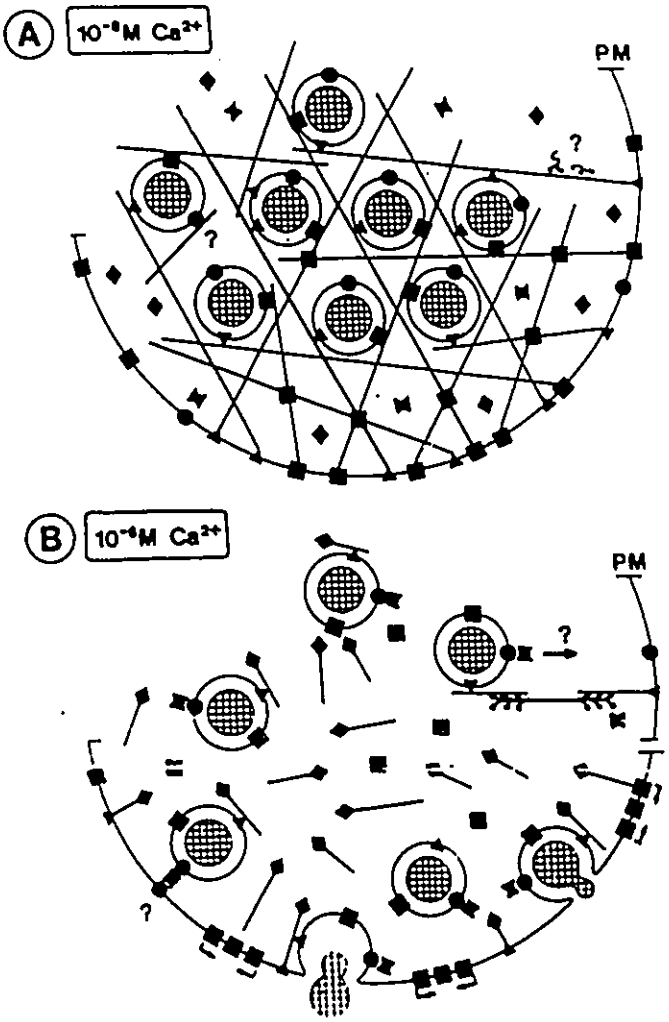


Fig. 2. A schematic representation of a possible mechanism in which cytoskeleton proteins may play a role in chromaffin cell secretion.

regulatory components might be involved in some steps of the secretory process. For instance, an ankyrin-like protein could be involved in the anchorage of actin microfilaments at the level of the plasma membrane, or the contractile protein tropomyosin might interact with the microfilament network and in some way modulate the association of some cytoskeletal components with this filamentous system.In addition, the phosphorylation of some cytoskeletal components or structures may provide a regulatory mechanism for their interaction with other cellular components or structures. Furthermore, other regulatory proteins such as caldesmon, which has been recently detected in chromaffin cells, might provide an altenative regulatory mechanism ("flip-flop" mechanism) for the interaction of calmodulin-binding proteins with the microfilaments or the actin-myosin interaction (Sobue et al., 1983, 1985).

E.ACTIN AND CALCIUM DEPENDENT ACTIN SEVERING PROTEINS

E.1.Actin

Actin is one of the most abundant protein in eukaryotic cells. Actins isolated from different sources including skeletal muscles, smooth muscles and nonmuscle cells have similar physicochemical properties. Actin has a subunit molecular weight of 42,000 and can exist in two structural states, namely G-actin, the globular monomer, and F-actin, a filamentous polymer. In skeletal muscles, F-actin is the double helical polymeric structure which makes up the thin filaments . These actin filaments have a diameter of 4-6 nm and possess a definite polarity as indicated by heavy meromyosin arrowhead formation.

Central role of actin is its self - association from a monomeric G-actin to filamentous F-actin at physiological ionic strengths. This knowledge is made use of in the isolation and purification of actin described in the methods section. The polymerisation process has been studied in detail (Korn, 1982). The rate of filament assembly is controlled by the formation of nuclei and their production is favoured in the presence of Mg^{2+} ions. Filaments are lengthened by the addition of actin monomers to both ends of the growing filaments. Pollard and Mooseker (1981) estimated the rates of assembly and disassembly at each end and have shown that preferred assembly takes place at the barbed end while preferred disassembly occurs at the pointed end (based on the structure of actin decorated by myosin sub fragment-1, these ends have been termed barbed and pointed by analogy with the flights on an arrow). One consequence of a difference in assembly rates at the two ends is that the filament stability is altered if one end is blocked. Capping the preferred assembly end will cause monomer dissociation from the other end to establish a new monomer-polymer equilibrium (Kirschner, 1980). This is the mode of action of actin severing and capping proteins and is discussed below. In nonmuscle cells the major microfilament protein actin can undergo substantial structural and functional changes, depending on the physiological state of the cell as described above.

There exist four muscle actins (alpha family) consisting of two

sarcomeric actins, one specific for skeletal muscle and one for cardiac muscle, and two smooth muscle actins, one found mainly in visceral smooth muscle and the other related to vascular smooth muscle. In the case of nonmuscle cells, they contain two generally occurring cytoplasmic actins termed β and -actin (Vandekerckhove and Weber, 1979). β-actin and -actin are very similar to each other (only 4 amino acid substitutions and closely related to the actin found in lower eukaryotes (Vandekerckhove and Weber, 1980). In skeletal muscles, the thin actin filaments are attached to the sarcomeric Z-line structure via the anchorage protein α-actinin. In smooth muscles, the actin filaments are inserted into α-actinin containing dense bodies which are associated with the plasma membrane. In nonmuscle cells, it has been shown recently that actin filaments are associated to the plasma and subcellular organelle membranes via the interaction of α-actinin (Burridge and Feramisco, 1982) and probably via fodrin (Aunis and Perrin , 1984). Although the functional significance of actin in nonmuscle cells is not fully understood, several passive and active roles have been ascribed to this protein. The passive roles include a cytoskeletal role which is involved with structural support for cell attachment and a role in the maintenance of cytoplasmic viscosity (Byers et al., 1984; Tucker et al., 1985). The active roles are more diversified and include axonal transport (Lasek, 1982), chromosome segregation involved in cell division (McIntosh, 1982; Nagata and Ichikawa, 1984), changes in cell shape (Albertini and Herman, 1984), regulation of the topographical distribution of membrane proteins

(Oliver and Berlin, 1983), endocytosis (Buckley, 1983) and exocytosis (Poisner et al., 1967; Fox, 1985; Trifaró et al., 1985a, 1985b).

E.2. Actin binding proteins

Many actin binding proteins have been isolated and characterised. They have been classified as actin cross-linking proteins, actin filament severing, capping and depolymerising proteins and anchorage proteins. Profilins which are present in high concentrations (> 30 μM) in the cytoplasm bind only to monomeric actin and stabilize the monomer pool.When filaments are formed the cross-linking proteins stabilise them in three dimensional isotropic networks or tight bundles of actin filaments, in a calcium sensitive or insensitive manner. The three dimensional network further associates with membranes and cellular organelles via the anchorage proteins. The proteins which produce a solation effect upon increase in the intracellular calcium are described below.

E.2.i. Actin filament severing proteins

A large number of actin-binding proteins has now been described, which on account of their properties in vitro and in some instances their cytoplasmic locations, are thought to determine the behaviour of actin and thus have a crucial role in the regulation of cell motility as well as in maintaining the dynamic state of cytoskeletal assembly and disassembly. The calcium dependent actin filament severing and capping proteins are

believed to exert their effects through the regulation of the consistency of the cytoplasm of the cell (Yin and Stossel, 1979). Proteins which fragment actin filaments in a calcium dependent manner have been identified in many organisms: gelsolin from macrophages (Yin and Stossel, 1979), villin from intestinal brush border (Glenny et al., 1981), gelsolin like proteins from a variety of tissues, in platelets (Wang and Bryan, 1981), brain (Petrucci et al., 1983), serum brevin (Harris and Schhwarz 1981),adrenal medulla (Trifaró et al., 1985a; Bader et al., 1986), skeletal muscle (Yin et al., 1981; Carron et al.,1986) and smooth muscle (Ebisawa et al., 1985), fragmin from Physarum plasmodium (Hasegawa et al., 1980) and severin from Dicotyostelium amoeba (Yamamoto et al., 1982) and a 45 kDa protein from sea urchin egg (Wang and Spudich, 1984). There is a group of heterodimers with subunits of 30-35 kDa, which are not sensitive to calcium, and cap and nucleate but do not sever actin filaments (Pollard and Cooper, 1986).

The above mentioned proteins can be grouped into distinct families.

a) Gelsolin and villin family

Gelsolin (90 kDa) and villin (95 kDa) are monomers which are sometimes isolated as 1:1 complexes with actin and are present in different mammalian tissues. Gelsolin resides in the cortical cytoplasm and during cell activity it is concentrated in the area of cytoplasm actively engaged in movement (Yin et al., 1981). Villin on the other hand is the major actin associated protein

in the core bundles of microfilaments from microvilli of intestinal epithelial cells. Sequence data reveal a limited area of homology between gelsolin and villin, which may have a common function. Brevin found in plasma, is nearly identical to gelsolin, found in cytoplasm, except that brevin has 25 extra residues at the N-terminus, based on limited aminoacid sequence data and peptide maps. Therefore, brevin is sometimes called plasma gelsolin. Brevin is synthesised by muscular tissues and liver to be secreted into the blood. The gelsolin like proteins described in a variety of tissues have similar molecular weights of 88 - 93 kDa, and some of them show immunological cross-reactivity. Functionally, the proteins in this group cap, nucleate, and sever in a calcium dependent manner. Binding to calcium induces a conformational change in gelsolin which is then able to bind actin. The expression of the different effects on actin filaments depends on the prevailing calcium concentration.

Cytoplasmic gelsolin requires calcium for activity, and binds calcium with a dissociation constant of 1 μM and stoichiometry of 1.7 calcium per gelsolin. Plasma gelsolin binds more than one calcium with a Kd of 20 μM. Gelsolin has three non homologous actin binding sites, two at the NH_2 terminal half of the molecule which appear to be largely calcium insensitive, and one at the COOH-terminal half of the molecule which requires calcium for binding to actin (Bryan, 1988). Despite the presence of two calcium insensitive sites, the intact gelsolin molecule is inactive in the absence

of calcium. In gelsolin severing activity has the most stringent requirement for calcium and does not occur at submicromolar Ca^{2+} concentration. Although μM $Ca^{2+}$is required for gelsolin to bind actin, once it binds two actin monomers in the presence of calcium, only one of the actins can subsequently be dissociated by chelating calcium with EGTA. Unlike free gelsolin this complex does not fragment actin filaments, but blocks them with high affinity even at sub micromolar levels of calcium. Therefore once gelsolin blocks the barbed end of a filament in vitro it can no longer be dissociated from actin by removal of calcium. Data from further studies (Yin, 1988) suggest that cells possess a mechanism, not directly involving Ca^{2+}, for dissociating actin-gelsolin EGTA-resistant complexes subsequent to agonist stimulation.

Villin is unique among capping proteins in also possessing bundling activity.Nucleation and filament shortening by villin require calcium, but bundling does not. Villin has three binding sites for calcium and undergoes a conformational change upon binding calcium. One binding site has high affinity with little or no exchange, and the other two have K_ds of about 5 μM. The change in conformation may not, however, be related to the change in function since limited proteolysis eliminates the conformational change but not the calcium binding and calcium sensitivity of the functions. The dependence of the capping activity on calcium concentration shows that one of the 5 μM sites may be involved, but the severing activity has a different calcium

dependence that is not explained by the calcium-binding data. Villin has two non homologous actin binding sites present in the same polypeptide chain, which can be separated and analyzed after limited proteolytic digestion of the whole molecule. One site is inhibited by micromolar concentrations of calcium. Therefore, at less than 10^{-6}M Ca^{2+}, villin will only cross-link actin, forming microfilament bundles. At this calcium concentration gelsolin is inactive. At greater than 10^{-6}M calcium, villin binds to filamentous actin but cannot cross-link as only one actin binding site is functional (Glenney et al., 1981), hence shortens the actin filaments and caps the barbed end. Unlike gelsolin villin readily dissociates from actin on addition of EGTA.

b) Fragmin and severin family

The fragmin and severin family consists of polypeptides of about 45 kDa and these proteins may be universal in their distribution since they have been isolated from protozoa and vertebrates. These too cap, nucleate and sever actin filaments in a calcium dependent manner and are isolated as 1:1 complexes with actin. Fragmin, like gelsolin can exist as 1:1 complex with G-actin but this has no severing activity. Tropomyosin or heavy meromyosin binding protect against fragmentation of filaments. In Physarum, Cap 42a and Cap 42b subunits have extensive homology with actin and fragmin, respectively, but in Acanthamoeba no homology is found by comparing the available sequences of profilin or gelsolin with

that of actin (Pollard and Cooper, 1986). The calcium dependence of cap 42(a+b) from Physarum varies with phosphorylation of the protein by a specific kinase. This protein does not sever filaments unlike fragmin which is also from Physarum.

E.2.ii. Mode of action

E.2.ii.a. Effects on Actin Polymerization

A protein that binds to the end of an actin filament can interfere with either the association of monomers, the dissociation of the terminal subunits, or both. Gelsolin, villin, fragmin, severin and other capping proteins block one end of actin filaments and prevent the reannealing of actin filament fragments. They also inhibit the addition of actin monomers to growing barbed ends. One possible outcome of such capping is an increased monomer concentration as a result of G-actin dissociation from the free pointed ends. In the presence of profilins , proteins which bind specifically to G-actin and prevent polymerisation, complete depolymerisation may occur. These lead to inhibition of the rate of actin polymerisation seeded by actin filament nuclei.

E.2.ii.b. Nucleation of actin

When added to G-actin solution in assembly conditions, they promote the formation of actin nuclei by binding to more than one monomer and thereby facilitate polymerisation. This effect could be demonstrated by assaying the actin

polymerisation by high shear viscometry. These proteins eliminate the lag phase of the viscosity increase. As the rate of viscosity increase is limited by the rate of nucleus formation capping proteins must increase the rate of nucleus formation. The mechanism by which nucleation is enhanced has not been studied in detail, but could result from stabilisation of actin dimers or trimers (Craig and Pollard, 1982). Gelsolin, fragmin and Cytochalasin D have been shown to form stable complexes with mononeric or dimeric actin (Pollard and Cooper, 1986).

So accelerated nuclei formation and decreased rates of elongation occur simultaneously resulting in an increased number of short filaments capped at one end (Weeds, 1982).

E.2.ii.c. Actin filament severing activity

The proteins categorised above actively break the actin filaments, a process usually called severing. Here the protein molecule binds to the side of an actin filament and intercalates between two adjacent actin subunits and breaks the non covalent bond between actin-actin monomers. Two new filament ends are created-the new pointed end is free and the new barbed end has the protein bound to it i.e. the barbed end is capped. Large effects are observed with relatively lower molar ratios of (1:1000) regulatory protein to actin. The impetus for this theory is that capping proteins shorten actin filaments very rapidly. There exist two theories to explain the rapid reduction in viscosity of F-actin solution or for

the appearance of short actin filaments on electron micrograph. One theory is that the proteins involved shorten actin filaments by a direct action as described above. The second theory suggests these proteins may shorten the performed actin filaments by blocking the ends of spontaneously or mechanically broken filaments. In many cases, the mixing process fragments filaments so that capping proteins may simply rapidly cap the newly created barbed ends. However, in some experiments fragmentation is minimized, and shortening is faster than expected from depolymerization. These experiments have been performed by mixing a capping protein either with actin filaments in solution, with filaments attached to Limulus bundles, or with actin filaments attached to erythrocyte membranes and the data support the first postulate.While the apparent depolymerization rates are very rapid, and severing seems to be the most likely explanation, there is some reservation about this conclusion simply because of uncertainties about actin polymerization, especially the rate constants for elongation, fragmentation, and annealing.

E.2.iii. Possible physiological role of calcium dependent actin filament severing proteins

Although all these proteins share similar activity on both monomeric and polymeric actin, (inhibit subunit exchange at ends of actin filaments), they vary in their ability to sever filaments, promote nucleation, bind to actin monomers, and bundle actin filaments. Various regulatory factors sometimes distinguish various activities. For

example, with villin and gelsolin the capping and severing activities depend on different concentrations of calcium. Gelsolin-actin interactions in addition to calcium, are regulated by polyphosphoinositides (Janmey et al., 1987). Comparing the functional activities of the various proteins has been complicated because different techniques and conditions are employed in different laboratories and many assays do not have a unique quantitative interpretation.

However some generalised roles may be postulated. Their in vitro properties suggest that these proteins may have several roles in cells: binding of capping proteins to filament ends would stop subunit addition and loss, stabilize filament length, and stop treadmilling (Weeds, 1982). Since capping proteins make actin filaments short, they may control filament length and thereby cytoplasmic viscosity. Local variations in viscosity may control the flow of cytoplasm and the movement of particles in the interior of cells. The notion is that intracellular organelles can flow into an area of a sol, but not a gel (Livett, 1984). Interestingly, gelsolin was localized by immunofluorescence microscopy to an area of cytoplasm under a particle being phagocytized, where breaking apart a filament network may be important (Yin, et al., 1981) . One theory of cell movement holds that solation of the cytoplasm (that might be accomplished by severing actin filaments) is necessary for myosin-mediated contraction to occur (Tayler and Condeelis, 1979).

F. EFFECT OF CALCIUM ON THE CYTOSKELETON

It is clear that Ca^{2+} ions regulate the formation of of actin bundles or gel networks in two distinct ways. Cross-links formed by α-actinin are broken when the calcium ion concentrations rise above μM levels but readily reform when calcium is removed. Actin gels are disrupted by gelsolin in the presence of calcium but in this case recovery is probably achieved by gelsolin-actin dissociation induced by polyphosphoinositides (Janmey et al., 1987). In addition Ca^{2+} ions independently regulate myosin filament formation and acto-myosin interaction via a calmodulin dependent myosin light chain kinase. Calcium transients therefore cause both contraction and solation. Calcium ions also regulate the interactions of gelsolins and other capping proteins with both monomeric actins and with the ends of preformed actin filaments.

Although it is possible to propose theories for control of actin polymerisation, gelation, and cytoskeletal assembly-disassembly including the control by calcium, there is no direct evidence that these proteins play their assigned roles inside the cell. At most immunofluorescence has shown that particular proteins are in the right place at the right time. The next step is to determine by intracellular manipulations whether these promising candidates actually regulate actin structure and therefore cell motility and cell shape. In the meantime actin binding proteins will find important use at tools for isolation and

analysis of individual steps in actin polymerisation and as selective probes for analysing the relationship between cytoskeletal structure and function in living cells.

G. STATEMENT OF THE PROBLEM

As discussed in the introduction the chromaffin cell cytoskeleton and its regulatory proteins play an important role in bringing about the release of chromaffin granules. The subplasmalemmal meshwork of actin filaments is disrupted following an increase in intracellular calcium and the granules trapped in this meshwork under resting conditions are now released to the release sites of plasma membrane. The exact mechanism as to how this is carried out is not known yet. One of the proteins which could regulate the dynamics of cytoskeletol actin in the presence of calcium is gelsolin. Extensive work on gelsolin has unequivocally proven that gelsolin is a promising candidate to mediate such interactions.

In the earlier work from this laboratory on the characterisation of chromaffin cell actin, it was observed another protein was consistently eluted together with chromaffin cell actin from DNAse I- Sepharose affinity columns (Lee et al., 1979). Subsequent work determined that this protein was gelsolin (Trifaró et al., 1985a; Bader et al.,1986). The association of gelsolin was further studied and characterised in our laboratory. In these studies it was observed that in addition to gelsolin another protein of unknown function was retained by the DNAse I-Sepharose 4B affinity columns and eluted by EGTA-containing buffers. This second protein was earlier referred to as the 85

KDa protein (Bader et al., 1986).

In addition to these findings different investigators have independently identified gelsolin-like proteins in adrenal medulla (Grumet and Lin, 1981; Ashino et al., 1987). However attempts were not made to carry out a comparative study of the properties of these proteins with that of macrophage gelsolin to determine their true identity.

Therefore the present project was undertaken for the following purposes.

a) To purify the 85 kDa (now 80 kDa) protein to homogeneity to study its physico-chemical properties.

b) To establish the identity of the protein and to verify whether it was a breakdown product of gelsolin.

c) To compare the properties of this protein with chromaffin cell gelsolin under similar experimental conditions.

d) To determine its interaction with calcium and actin to obtain an insight of its possible role in the cell.

e) To study its tissue distribution and intracellular localisation to further characterise its role.

The physiological role of actin-binding proteins and of actin itself is under extensive investigation. A new actin-binding protein which could play a role in the reorganisation of the cytoskeleton following an agonist stimulation is described in the present studies describe the isolation, characterisation and

functional properties of a novel calcium dependent actin filament severing protein. We have named this protein, SCINDERIN (derived from the Latin word "scindere" meaning "to cut") because of its actin filament severing properties. It is hoped the data presented here will contribute to the the ever expanding knowledge of molecular mechanisms underlying the secretory process and exocytosis of secretory vesicles.

CHAPTER II

EXPERIMENTAL PROCEDURES

IIa. MATERIALS

The CNBr activated Sepharose 4B, DEAE-Sepharose CL-6B and Sephadex G-100 were purchased from Pharmacia Fine Chemicals (Dorval, Que.). All electrophoresis chemicals and alkaline phosphatase conjugated IgG antibodies and their respective staining kits were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Ont.).The Waters DEAE-5PW column was purchased from Millipore (Mississauga, Ont.) The nitrocellulose was obtained from Technical Marketing Ltd. (Ottawa, Ont.).Carbon coated chromium steel balls were purchased from Microbalt Co. (Peterborough, NH.). Staphylococcal V8 protease was purchased from Boehringer Manheim (Dorval, Que.). ^{45}Ca and Aquasol Scintillation Fluid were purchased from New England Nuclear (Montreal, Que.).All other chemicals were of reagent grade and purchased from either Sigma Chemical Co. (St. Louis, MO),BDH Laboratories (Ottawa, Ont.) or Fisher Scientific.

IIb. METHODS

A. PURIFICATION OF ACTIN

A.1. Preparation of acetone powder

Acetone powder was prepared from rabbit skeletal muscle using the technique described by A.Szent-Gyorgi (1951).

A.2. Preparation of F-actin

F-actin was prepared from the acetone powder according to the method of Pardee and Spudich (1982). The procedure is outlined in Fig.3. The acetone powder was extracted at 0-0.5°C for 30 min by stirring with extraction buffer (20 ml/g acetone powder) containing 2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$ and 0.005% sodium azide (pH 8.0). The extract was separated from the hydrated acetone powder by squeezing through several layers of cheese cloth. The residue was re-extracted by stirring for 10 min in the same volume of extraction buffer. Extracts were combined and centrifuged at 20,000 x g for 1 hr at 4°C. The KCl concentration of the supernatant was brought to 50 mM, Mg^{2+} to 2 mM and ATP to 1 mM. After 2 hr of gentle stirring at 4°C solid KCl was slowly added to a final concentration of 0.6 M and the solution was stirred gently for 0.5 hr. This high salt wash removes tropomyosin. The polymerised actin is centrifuged at 80,000 g

Fig .3: Flow diagram of the method followed for the purification of actin from rabbit skeletal muscle. Reproduced from Pardee and Spudich (1982). Buffer A is the extraction buffer containing 2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM 2-mercapto ethanol, 0.2 mM $Cacl_2$ and 0.005% sodium azide.

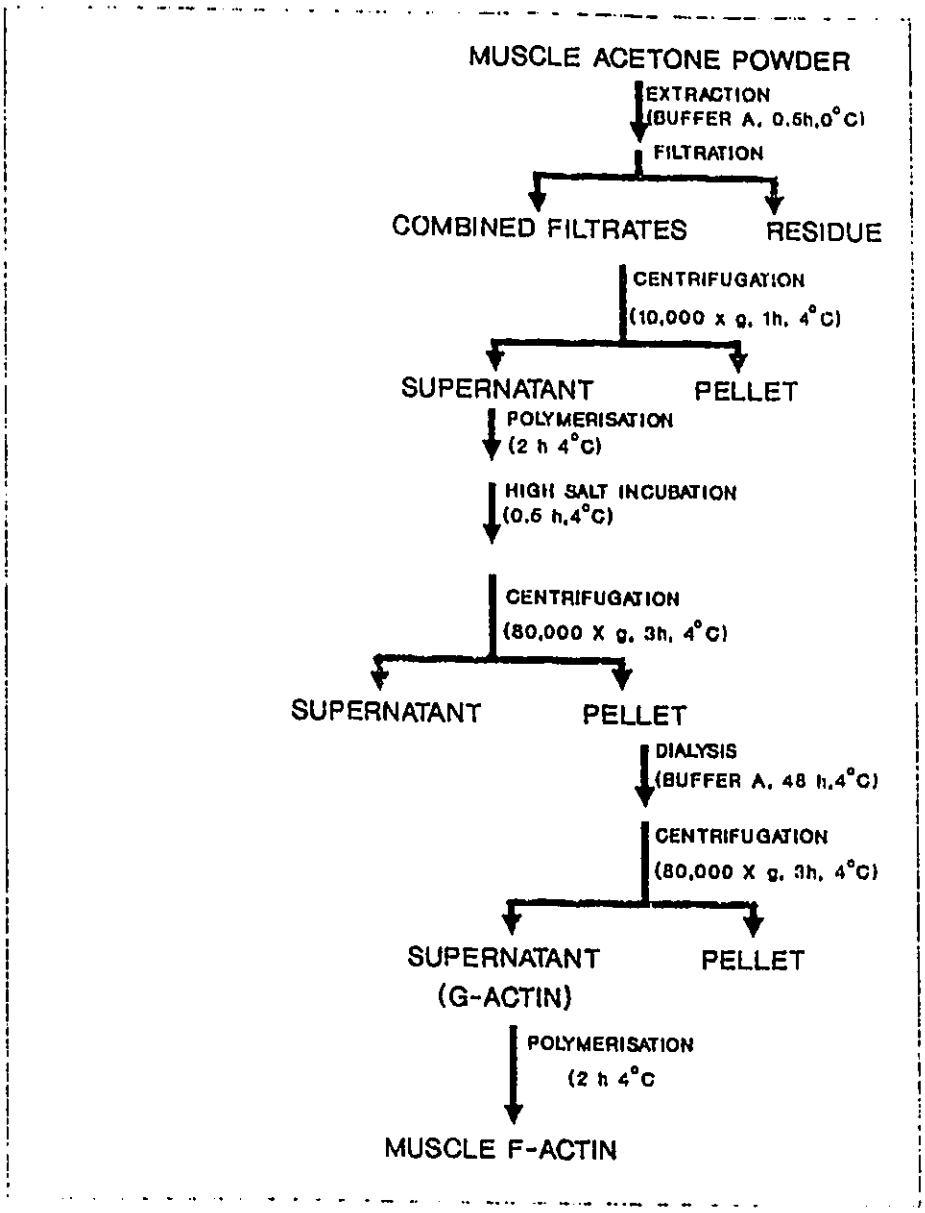


Fig.3. Flow diagram of the method followed for the purification
----- of actin.

for three hours at 4°C. To remove the contaminants trapped in the liquid phase of the sediment, sedimented F-actin was resuspended in 150 ml of fresh extraction buffer containing 0.6 M KCl, 2 mM $MgCl_2$, 1 mM ATP and resedimented as described earlier.

A.3. Conversion of F-actin to G-actin.

The sediments containing F-actin were resuspended by gentle homogenisation in 3 ml of cold extraction buffer/g of acetone powder originally extracted. Dialysis of resuspended pellet at 4°C against 1 L of ice cold extraction buffer with three buffer changes at 12hr intervals was carried out to bring about depolymerisation of F-actin. Then, the dialysed actin was centrifuged at 80,000 x g for 3hr to separate soluble G-actin from filamentous F-actin. The supernatant containing the G-actin fraction was submitted to another cycle of polymerisation and depolymerisation to increase the purity of the final product. The purity of the product was determined by SDS-PAGE (Fig.4). G-actin thus obtained was immediately used or polymerised and stored as F-actin in the presence of 0.02% NaN_3 at 4°C.

A.4. Preparation of actin-DNAse I affinity column

Purified muscle G-actin obtained by the above mentioned procedure was used in the preparation of actin-DNAse I affinity column. DNAse I-Sepharose 4B was prepared by a modification of the

Fig. 4.: Purity of the skeletal muscle G-actin. A sample of the G-actin (40μg) purified from the rabbit skeletal muscle was run in parallel with 5μg of G-actin form Sigma Chemical Co. Lane A shows the electrophoretic pattern of commercial G-actin and lane B shows that of G-actin purified from rabbit skeletal muscle. The position of actin is indicated by the white arrow.

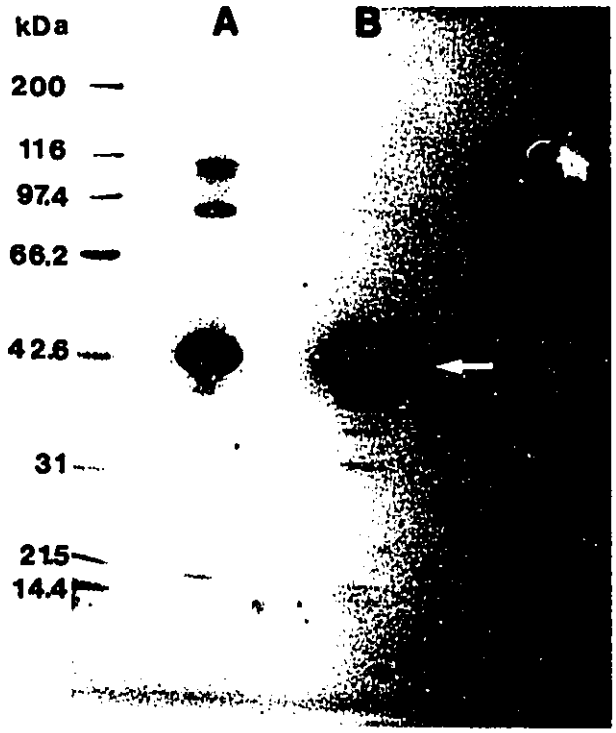


Fig.4. Purity of the skeletal muscle G-actin.

procedure described by Bader et al., (1986). Briefly, 5 g of CN-Br activated Sepharose 4B was swollen and washed in 500 ml of 1 mM HCl on a sintered glass funnel. Then the resin was washed with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl at pH 8.3) and immediately mixed with 5 mg of DNAse I in coupling buffer. The slurry was rotated slowly overnight and then the gel was extensively washed with coupling buffer. Residual reactive groups were blocked by incubation for 24 hr in 100 ml coupling buffer containing 0.2 M glycine. The gel was subsequently washed 3 times with 500 ml coupling buffer and 500 ml acetate buffer (0.1 M Na-acetate, 0.5 M NaCl, pH = 4). Finally, the gel was washed with 500 ml of 2 mM Tris-HCl buffer (pH 7.5) and poured into the column. The DNAse I column was equilibrated with 20 mM Tris-HCl buffer (pH 7.5), 0.1 mM DTT, 2 mM $CaCl_2$, 1 mM ATP and 0.25 mM phenylmethyl-sulfonyl-fluoride (PMSF). G-actin in 2mM Tris-HCl buffer (pH = 8.0), 0.2 mM ATP, 0.5 mM 2- mercaptoethanol, 0.2 mM $CaCl_2$ and 0.005% NaN_3, was extensively dialyzed against 20 mM Tris-HCl buffer (pH 7.5), 0.1 mM DTT; 2 mM $CaCl_2$; 1 mM ATP and 0.25 mM PMSF overnight. Then, 10 mg G-actin (0.5 mg/ml) were loaded onto the DNAse I sepharose column. All steps were carried out at 4^oC. Coupling procedures were monitored at 280 nm to assess the extent of the coupling, which was between 80-90% and 45-50% for DNAse I and G-actin respectively.

B. PARTIAL PURIFICATION OF SCINDERIN AND GELSOLIN

In order to test the tissue distribution pattern of scinderin

and gelsolin, the partial purification procedure described by M.F. Bader et al.(1986), was used. 25g each of the following bovine tissues, adrenal medulla, brain, heart muscle, skeletal muscle, kidney, liver, anterior and posterior pituitary gland and 100 ml of heparinised whole blood were tested. Except blood the rest of the tissues were washed in ice cold Locke's solution (154 mM NaCl, 2.6 mM KCl, 0.85 mM KH_2PO_4, 2.15 mM K_2HPO_4, 1.2 mM $MgCl_2$, 2.2 mM $CaCl_2$ and Dextrose 10.0 mM) to remove the blood and homogenised in the same homogenisation buffer used for scinderin purification with the omission of 300mM KCl (1 g tissue in 4 ml solution) using a Sorval omni-mixer. The homogenates were centrifuged at 1000 x g for 10 minutes. The resulting supernatants and the whole blood were then centrifuged at 100,000 x g for 60 minutes and dialysed against 20 mM imidazole(pH 7.5), 1 mM dithiothreitol(DTT), 0.25 mM PMSF, and 1 mM $CaCl_2$. After the dialysis step, $CaCl_2$ and ATP were added to obtain a final concentration of 2mM and 0.5 mM, respectively. The solutions were then passed through the DNAse 1-Sepharose 4B columns (4 cm x 0.9 cm) prepared as described earlier but without loading G-actin . These columns had been pre-equilibrated with 20 mM imidazole(pH 7.5), 1mM DTT, 0.25 mM PMSF, 1mM $CaCl_2$ and 0.5 mM ATP. The columns were then washed with the same buffer containing 0.6M NaCl. The calcium activated proteins were eluted with 20 mM imidazole (pH 7.5), 0.25 mM PMSF, 10 mM EGTA, 1 mM DTT and 0.5 mM ATP. When optical density at 280 nm has returned to the basal

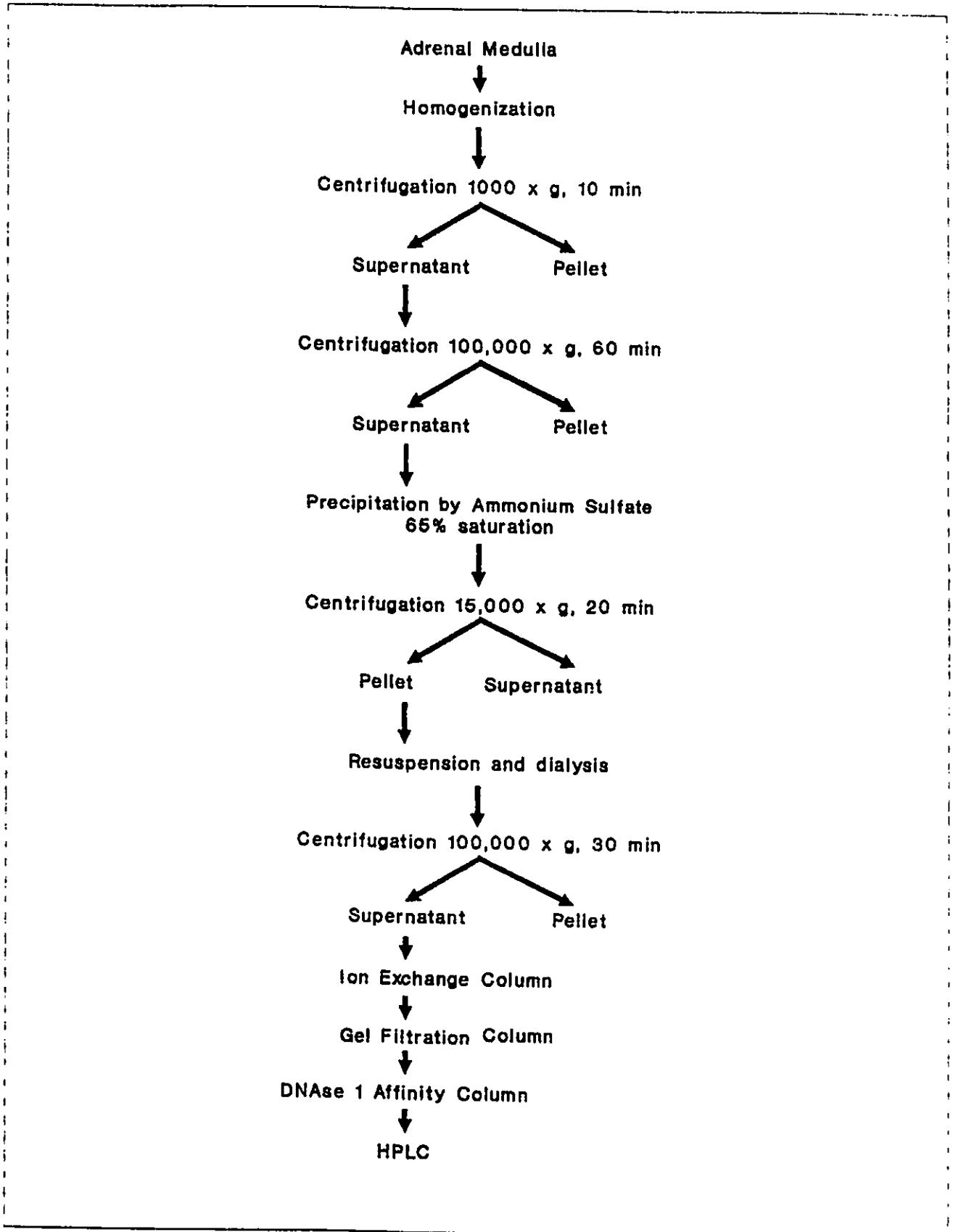
value, the columns were washed with 3 M Guanidine-HCl, 0.5 M Na-acetate, 30% glycerol, 1 mM $CaCl_2$, 0.5 mM ATP(pH 6.5). Finally the columns were regenerated by washing with the equilibration buffer mentioned above . Proteins eluted with EGTA were dialysed against 20 mM imidazole (pH 7.5), 0.25 mM PMSF, 1 mM EGTA, 0.75 mM NaN_3, 0.5 mM DTT and concentrated under high pressure . The EGTA eluate of each tissue was run in triplicate on 10% SDS-PAGE gel and transferred to nitrocellulose. Staining with Amido-Black 10B , immunoblotting with scinderin antibody (1:125 dilution) and immunoblotting with gelsolin antibody (1:1000 dilution) were carried out on the three strips of membrane bearing the transferred proteins from each EGTA eluate.

C. PURIFICATION OF SCINDERIN

The purification procedure for scinderin is briefly outlined in fig.5. Bovine adrenal glands obtained from a local slaughterhouse were kept on ice and transported to our laboratory. The adrenal glands were extensively perfused with ice-cold Locke's solution to remove traces of blood and the medullae were dissected from the cortices. Between 70-100 g of medullae from 25-30 glands were usually obtained. Adrenal medullae were homogenized in 0.3 M sucrose, 20 mM Imidazole (pH 7.5), 5 mM DTT, 1 mM PMSF, 0.75 mM NaN_3, 0.1 mM EGTA, 1 mM ATP, 300 mM KCl, 10 μg/ml soybean trypsin inhibitor, 1μg/ml of Leupeptin, and 0.1 mM Di-isopropylfluorophosphate (DFP).(1 g of medulla in 4 ml of solution)

Fig. 5: Flow diagram of the method followed for the purification of scinderin

Fig.5. Flow diagram of the method followed for the purification of scinderin



using a motor-driven Potter Elvehjem homogenizer. The homogenate was centrifuged at 1000 x g for 10 min and the resulting supernatant was centrifuged again at 100,000 x g for 60 min. To the supernatant thus obtained (cytosol), enough ammonium sulfate was added to reach 65% saturation. This mixture was stirred for 20 min and then centrifuged (15,000 x g for 20 min). The sediment was collected and dissolved in 80 ml of buffer A (20 mM Tris HCl, pH 7.5; 0.1 mM DTT and 0.1 mM EGTA) containing 20 mM KCl and dialysed against the same buffer for at least 12 hr. The dialysed material was clarified by centrifugation at 100,000 x g for 30 min. The sample was then loaded onto a DEAE-Sepharose CL-6B column (2.5 x 35 cm) which had been pre-equilibrated with the same buffer. The column was eluted at 20 ml /hr with a linear KCl gradient of 0.02 - 1 M KCl (540 ml total volume) and 6 ml fractions were collected. Fractions were analysed on SDS-PAGE and were tested by immunoblotting with an antibody raised against scinderin (80 kDa protein; see below). Sixteen immunoreactive fractions were concentrated under high pressure. The concentrated material was separated by gel filtration on a Sephadex G-100 column (2.5 x 90 cm) preequilibrated with buffer A containing 100 mM KCl. The column was perfused at a flow rate of 20 ml/hr and fractions with the same volume as above were collected. Immunoreactive fractions were combined again and $CaCl_2$ was added to obtain a final concentration of 2 mM. Combined fractions from the previous eluates were loaded onto an actin-DNAse I Sepharose 4B affinity column (0.9 x 10 cm) that had been preequilibrated with buffer A containing 100 mM KCl and 2 mM $CaCl_2$. The column was then

washed extensively with buffer A containing 0.5 M KCl. Finally, actin-binding proteins were eluted with buffer A containing 20 mM KCl and 10 mM EGTA. The EGTA eluate was concentrated as above and analysed by SDS- PAGE. Finally, further purification was carried out in an ion-exchange (Waters DEAE-5PW) column (0.75 x 7.5 cm) using a Waters HPLC system. The column was washed for 10 min at 0.8 ml/min with buffer A containing 20 mM KCl then with linear gradient ranging from 0 to 10% buffer B (1 M KCl in buffer A) over 5 min, from 10 to 50% B for 50 min and finally from 50 to 100% B for 10 min. The eluate was collected in 0.8 ml fractions. These were also analysed by SDS- PAGE to check the purity of the final product and the identity of the protein was confirmed by immunoblotting.

D.PURIFICATION OF GELSOLIN

As the physico-chemical properties of scinderin were to be compared with another similar adrenal medullary actin binding protein gelsolin the purification of this protein from the adrenal medulla was undertaken. The starting material for the purification of gelsolin was 80% ammonium sulfate precipitate of adrenal medullary cytosol. As the adrenal medulla was rich in gelsolin the concentration of ammonium sulfate required to precipitate gelsolin was determined by serial precipitation of adrenal medullary homogenate by increasing ammonium sulfate concentrations, separating the proteins in the 15,000 x g sediments by SDS-PAGE and immunoblotting with gelsolin

Fig. 6: Serial precipitation of adrenal medullary cytosol by ammonium sulfate. 50 ml of adrenal medullary cytosol prepared as described in methods, was stirred gently at 4°C while solid ammonium sulfate was slowly added to it to obtain the indicated concentrations. After 1 hr of equilibration, the solution was centrifuged at 20,000xg for 20 min. The sediments thus obtained were resuspended in 20 mM Tris-HCl (pH 7.5), 0.1 mM DTT and 0.1 mM EGTA and was dialysed against the same buffer overnight. The dialysed samples were analysed by SDS-PAGE. (A) shows the Coomassie Brilliant Blue staining of the proteins precipitated by corresponding % saturations of ammonium sulfate shown above each line.

(B) Immunoblot. A similar nitrocellulose sheet containing the transferred proteins was cut at the molecular weight level corresponding to the 70-95 KDa region. The strip was immunoblotted with anti-gelsolin (1:1000 dilution). No immuno reactivity can be noticed after precipitation at 80% saturation.

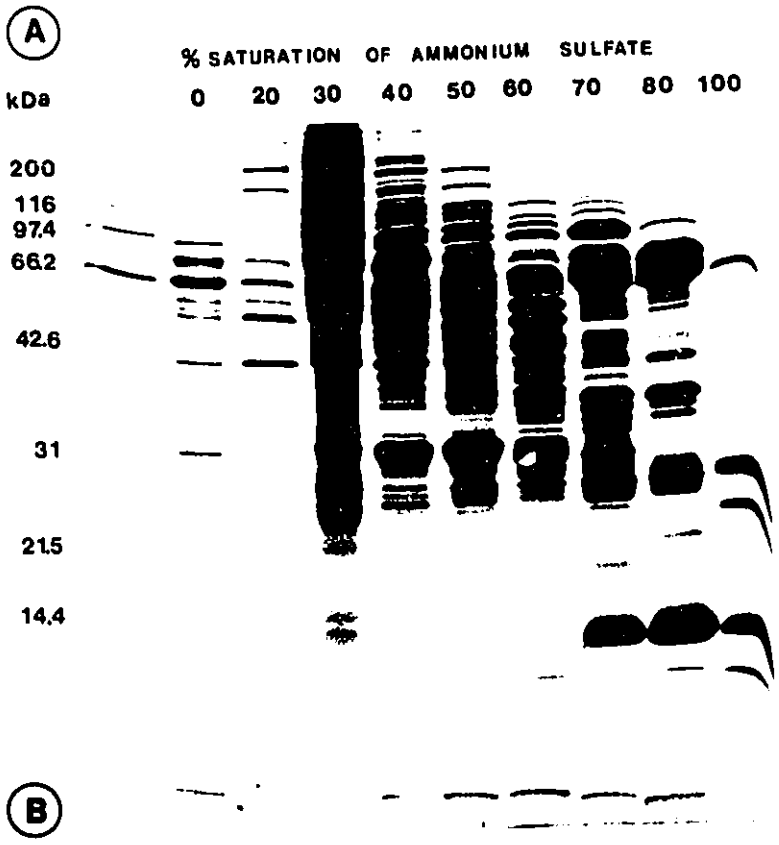


Fig.6. Serial precipitation of adrenal medullary cytosol by ammonium sulfate.

antibody (Fig.6). Fig. 6A shows the electrophoretic pattern of the proteins precipitated by gradually increasing concentrations of ammonium sulfate and 6B shows the immunoblot of the region containing the 70-95 KDa proteins. From the immunoblot it could be seen that almost all the gelsolin was precipitated at 80% saturation .The protocol followed in the purification of gelsolin was similar (4 chromatography steps) to that used in the purification of scinderin, except that in this case, fractions 40-55 were collected from the eluate from a DEAE-Sepharose Cl 6B column. These fractions were positive for gelsolin in immunoblot tests. Further purification was made using the gel filtration chromatography, actin affinity chromatography and high performance liquid chromatography in the order mentioned. Immunoblot tests were used in the monitoring of all these subsequent chromatography eluates. The final preparation of adrenal medullary gelsolin was analysed on SDS-PAGE to check for purity and was confirmed by immunoblotting. Samples were concentrated with Amicon filters with 30 KDa cut off point .

E.INTERACTION OF SCINDERIN WITH ACTIN

Purified G-actin obtained from rabbit skeletal muscle was subjected to polymerisation and the F-actin thus obtained was used in these experiments.The effect of scinderin on F-actin was tested by viscometry and electron microscopy.

E.1. Measurement of viscosity

Apparent viscosity was measured by the method of MacLean-Fletcher and Pollard (1980) using a low-shear falling ball viscometer. G-actin was polymerized in 100 mM KCl, 1 mM $MgCl_2$, 1 mM ATP for 2 hrs at 4°C and sedimented by centrifugation (100,000 x g; 3 hrs). The F-actin was homogenized in assay buffer (40 mM Pipes, 100 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 0.05% NaN_3, pH 6.8, containing 5 mM EGTA and $CaCl_2$ to give the indicated final free Ca^{2+} concentration (Caldwell, 1970). Samples were drawn into 100 µl capillary tubes, sealed with plasticine at one end, and incubated at 25°C for 120 min. Viscosity was measured at an angle of 45° with 0.8 mm carbon chromium steel balls (material 440C). Viscosity curves were obtained using a)different free calcium concentrations while keeping the actin : scinderin ratio constant and b) using different concentrations of scinderin while keeping the actin and free calcium ion concentrations constant. The values used to draw the curves were the averages of three separate experiments.

E.2. Electron microscopy

F-actin (0.2 mg/ml) in assay buffer (40 mM Pipes, 0.1 M KCl(pH 6.8), 2 mM $MgCl_2$, 2 mM ATP, 0.05% NaN_3), containing either 5 mM EGTA or EGTA and $CaCl_2$ to give final free calcium concentration 10^{-5} M, was incubated with scinderin for 120 min at 25°C. The final concentration of the actin-binding protein was 18.6 µg/ml, corresponding to a molar ratio of scinderin to actin 1:400. The samples were mounted

on carbon-coated Formvar grids, negatively stained with 1% aqueous uranyl acetate and examined at x 33,000 magnification with a Phillips EM 420 electron microscope. The length of the
F-actin filaments treated with scinderin (80 kDa protein) was calculated from twenty randomly choosen fields from prints prepared at a final magnification of x 82,500.

F. AMINO ACID ANALYSIS

Hydrolysis of the purified samples of scinderin and gelsolin was carried out at 108°C for 24 hrs in 6 M HCl, 0.1% mercaptoethanol (6 μg protein in 20 μl). Amino acid analysis of acid digests was performed by HPLC on a Pico-Tag column (0.3 x 25 cm; Waters) after the derivatization of amino acids with phenylisothiocyanate as described previously (Bidlingmeyer et al., 1984).

G. PREPARATION OF ANTIBODIES

Polyclonal antibodies against gelsolin (90 kDa protein) and scinderin (80 kDa protein) were prepared and characterized as previously described (Bader et al., 1986). In this earlier publication from our laboratory, scinderin was referred to as a 85 kDa actin-binding protein of unknown function. For the preparation of antibodies, a sample of adrenal medullary cytosol was applied directly to a DNAse I Sepharose affinity column in the presence of calcium. The mixture of actin-

binding proteins (90 kDa, 80 kDa etc.) obtained upon elution was subsequently separated by SDS-PAGE. Gels were briefly stained with Coomassie Blue, scanned at 633 nm and protein bands representing gelsolin and scinderin were cut off the gels. Individual polyacrylamide peptide bands were homogenized in complete Freund's adjuvant and injected subcutaneously into rabbits. A similar injection was repeated 2 weeks later except that the bands were homogenised in incomplete Freund adjuvant . Rabbits were bled from the ear vein 40 days after the first injection . More recently antibodies to scinderin were raised in two rabbits (antibodies 6 and 7) injected with scinderin purified as described in this thesis.

H. ELECTROPHORETIC TECHNIQUES

H.1. One dimensional electrophoresis :

Sodium dodecyl sulfate polyacrylamide (10 %) gel electrophoresis (SDS-PAGE) was carried out as described by Doucet and Trifaró (1988). The separating gel (12.5 cm long x 14 cm x 1.5 mm) consisted of 10% acrylamide, 0.1 % bis-acrylamide, 0.4% SDS, 5% glycerol, 200 mM Tris-HCl and 100 mM glycine (final pH = 9.0). Polymerisation was achieved with 0.1% ammonium persulfate and 0.05% TEMED. The stacking gel consisted of 4% acrylamide, 0.04% bis-acrylamide, 0.4% SDS, 4 mM EDTA, 5% glycerol and 70 mM Tris-HCl (pH = 6.7). Polymerisation was achieved in the

same manner as described above. The upper tank buffer contained 0.1% SDS, 100 mM Tris-HCl and 150 mM glycine (pH = 8.45). The lower tank buffer was a 1:1 dilution of the upper tank buffer. Samples for the gel were solubilized in a mixture containing 8M urea, 3% SDS, 100 mM dithiotreitol, 70 mM Tris-HCl (pH = 6.7) and 0.005% bromophenol blue. Electrophoretic molecular weight standards were of the following composition; Myosin (200kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.6 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysosome (14.4 kDa). Electrophoresis was performed at 60 V overnight in a BioRad Protean I apparatus.

H.2. Two Dimensional Gel Electrophoresis

Isoelectric focussing was performed according to the technique of O'Farrel et al.,(1977) with modifications described by Bravo (1984) and Doucet and Trifaró (1988). Briefly, protein samples were solubilised in a mixture containing 9.8 M urea, 2% (w/v) NP-40, 2% ampholines (pH = 7 - 9) and 100 mM dithiothreitol. These proteins were separated in the first dimension by electrofocusing on prerun gel rods (2.7 mm diameter x 11 cm long) containing 9.16 M urea, 3.31 % acrylamide, 0.19% bis- acrylamide, 2% NP-40, 6% ampholine (pH = 5 - 9) and 1.2% ampholines (pH = 3 -10). The samples were overlaid with buffer containing 8M urea, 1% ampholines (pH = 7 - 9), 5% NP-40 and 5% β-mercaptoethanol.

Gels were then run 18 hr at 400 V in a BioRad Protean 1 apparatus. Upper and lower tank buffers contained 20 mM NaOH and 10 mM $H_3 PO_4$ respectively. After the first dimensional electrophoresis, each gel rod was equilibrated 30 min in the sample mixture for regular SDS- PAGE, and then loaded on the gel for the second dimension. The gel for the second dimension was performed according to Laemmli (1970) using linear 7.5 - 15% acrylamide slab gels (3.0 mM thick), containing 1% SDS, 7.5% glycerol, 375 mM Tris-HCl (pH = 8.8). The stacking gel consists of 3% acrylamide, 62.5 mM Tris (pH =6.8) and 1% SDS. Electrophoresis was performed overnight at 80 V in a BioRad Protean 1 apparatus. The upper tank buffer consists of 25 mM Tris (pH = 8.3), 0.5% SDS and 200 mM glycine. The composition of the lower tank buffer was 50 mM Tris (pH = 8.3).

H.3. Gel staining procedure

Gels were first fixed for 45 min in 25% 2-propanol and 10% acetic acid. This was followed by a 20 - 30 min staining with 0.1% Coomassie brilliant blue (R- 250) in 25% 2-propanol and 10% acetic acid. The gels were destained with several changes of a solution containing 25% methanol and 10% acetic acid.For the second dimension of the two-dimensional gels, a different protocol was followed. Gels were fixed for 1 hr in 30% methanol, 10% trichloroacetic acid and 3.5% sulfosalicylic acid. This was followed by a 2 hr wash in several volumes of 30%

methanol and 10% trichloroacetic acid and a 30 min wash with 10% ethanol and 5% acetic acid. The gels were then stained for 1 hr in 0.4% Coomassie brilliant blue (R-250) in 50% trichloroacetic acid. Destaining was achieved with several changes of a solution containing 7% acetic acid and 25% ethanol.

I. IMMUNOBLOTTING TECHNIQUE AND I^{125}-PROTEIN "A" OVERLAY PROCEDURE

The protocol for immunoblotting was based on the technique of Towbin et al. (1979). Protein samples (20 μg) were subjected to electrophoresis on 10% polyacrylamide gels (Doucet and Trifaró, 1988) and transferred to nitrocellulose membranes for 1.5 hrs in a LKB Transphor unit (setting 100%, with water cooling and current increased from 0.6 to 1.6 Amp) containing transfer buffer of same composition as mentioned earlier. The membranes were incubated for 1 hr in 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS: 130 mM NaCl, 7.6 mM Na_2HPO_4 and 2.3 mM $NaH_2PO_4.H_2O$ (pH = 7.4). The blots were then washed three times with PBS containing 0.05% Tween 20 (TPBS) at 10 min intervals and then incubated in TPBS for 1 hr with their respective antibodies at the following dilutions: anti-scinderin 1:125 and anti-gelsolin 1:1000. Following 3 washes with TPBS at 10 min intervals, the blots were incubated with alkaline phosphatase conjugated anti-rabbit IgG (1:3000 dilution) for 1 hr. After 3 washes with TPBS, the blots were then stained with nitro-blue tetrazolium 5-bromo-4-chloro-3-

indolyl-phosphate to visualize protein bands.The procedure for ^{125}I-Protein A immunoblotting was performed essentially as described by Burnette (1981). Briefly, nitrocellulose membranes were incubated first with antibody against scinderin (80 kDa protein) for 60 min. After 3 washes with TPBS, the nitrocellulose membranes were incubated with ^{125}I-Protein A (250,000 CPM/ml, Amersham) for 45 min. After several washes with TPBS, the nitrocellulose membranes were dried and placed in a Cronex cassette equipped with a Dupont Lighting plus screen against Trimax XM film for 18 hrs, at -80°C. Autoradiograms were scanned using a transmission densitometer connected to a Beckman 10-inch pen recorder to measure the relative intensities of the labeled proteins. This information was used to calculate total and specific activities of different protein fractions containing scinderin.

J. IMMUNOHISTOCHEMISTRY

J.1. Chromaffin cell cultures

Chromaffin cells were isolated from bovine adrenal glands and cultured as described by Trifaró and Lee (1980). For immunohistochemistry chromaffin cells were grown on collagen coated coverslips. 8 days old cells were used for the study.

J.2. Indirect immunofluoresence of cell cultures

Chromaffin cells grown on coverslips were processed for immunofluorescence as described by Lee and Trifaró (1981). The

cells were washed 3 times with Locke's solution and were fixed with 3.7% formaldehyde in Locke's solution for 20 min. They were then washed 6 times with phosphate buffered saline (PBS, pH 7.2). Cells were permeabilised by treating with 50% acetone for 5 min followed by 100% acetone for 5 min and finally again treating with 50% acetone for 5 min. After 6 washes with PBS they were incubated with PBS (control), anti scinderin (1:20) or anti brevin (1:100) for 1 hour at 37°C. After this step cells were again washed with PBS and were incubated with fluorescein isothiocyanate (FITC, 1:35) conjugated to anti rabbit IgG for 1 hour at 37°C. After washing 10 times with PBS cells were mounted with 50% glycerol in PBS.

K. PEPTIDE MAPPING

Scinderin and gelsolin, both purified separately, were subjected to limited proteolytic digestion with either α-chymotrypsin or staphylococcal V8 protease. Twenty μg of each protein in 20 mM Tris-HCl (pH 7.5), 200 mM KCl, 0.1 mM EGTA and 0.1 mM DTT was incubated at 25°C with either 50 ng of α-chymotrypsin (Kwiatkowoski et al., 1985) or 50 ng of staphylococcal V8 protease (Bader et al., 1986). After 15 min incubation, the digestion was stopped by adding enough ice-cold PMSF (100 mM) and di-iso propyl fluorophosphate (DFP, 100 mM) to reach final concentrations of 1 mM and 0.5 mM respectively. Digested proteins were subjected to two dimensional electrophoresis according to the method

of Doucet and Trifaró (1988) but with the omission of dithiotreitol from the medium. The molecular weights and the isoelectric points of the fragments obtained following proteolysis were determined by using standard methods.

L. CALCIUM BINDING STUDIES

L.1. ^{45}Ca overlay procedure

Purified protein (20 μg) was subjected to SDS-PAGE on 10% polyacrylamide gel (Doucet and Trifaró, 1988). After overnight electrophoresis, transfer to nitrocellulose was performed at room temperature for 1½ hr at 1.6 Amp. Transfer buffer contained 25 mM Tris-HCl (pH 8.3), 150mM glycine, 20% (v/v) methanol. For ^{45}Ca overlay, the procedure described by Hincke (1988) was followed. After transfer, the nitrocellulose membrane was washed four times at 30 min intervals with overlay buffer (60 mM KCl; 5 mM $MgCl_2$; 10 mM imidazole (pH 6.8), 0.5 mM DTT, 0.02% NaN_3). Membranes were either incubated in overlay buffer containing 10^{-6} M ^{45}Ca (specific activity = 17 μCi/nmole) or in overlay buffer containing 10^{-6} M ^{45}Ca and $2x10^{-3}$ M $CaCl_2$. Non-radioactive calcium was used to ascertain the specificity of binding. After incubation for 20 min, the membranes were washed twice (at 2 min intervals) in 5% (v/v) ethanol and blotted to dryness between two sheets of filter paper. The membranes were then subjected to autoradiography by placing them in a Cronex cassette equipped with a Dupont lighting plus screen against Trimax XM film for

72 hrs, at - 80°C.

L.2. Calcium-binding measurements

Ca^{2+} binding to scinderin was determined by equilibrium dialysis. The protein was dialyzed overnight against 0.1 M KCl, 0.1 mM EGTA and 10 mM -imidazole-HCl (pH 7.1), to remove Ca^{2+} bound to the protein. For equilibrium dialysis the microdialysis procedure of Overall (1987) was used. One ml sample of protein (200 μg/ml) in a microfuge tube was dialysed with shaking at 4°C against 300 ml of above solution containing 16 μCi of $^{45}CaCl_2$ for 24 hrs. In order to achieve a desired free Ca^{2+} concentration, $^{40}CaCl_2$ was added to the dialysis buffer in the amount required according to the method of Caldwell (1970). The pH of the buffers was maintained at 7.1. Following equilibration, 100 μl samples taken from the solutions inside and outside the dialysis bags were added to 10 ml vials containing Aquasol scintillation fluid and the radioactivity was measured in a scintillation spectrometer . Ca^{2+}-binding data was first subjected to Marguard non-linear least squares procedure SSQMIN (Potter et al., 1983) and then analyzed with a curve-fitting computer program EBDA to determine K_d and B_{max} values (McPherson, 1983) by the method of Scatchard (1949).

M. PROTEIN DETERMINATION

Protein assays in the range of 1-20 μg were performed according to Bradford (1976) using a commercially available product (Bio-Rad protein

assay). Bovine serum albumin was used as a standard for the establishment of calibration curves.

CHAPTER III

RESULTS

CALCIUM DEPENDENT ACTIN BINDING PROTEINS IN ADRENAL MEDULLA

A.1. Isolation and purification of scinderin

In order tc purify scinderin in a larger scale chromaffin cell cytosol was initially treated with ammonium sulfate (65% saturation). This step was used to precipitate scinderin out from the adrenal medullary homogenate. The sediment obtained by centrifugation was dialyzed overnight. After dialysis the sample was clarified by centrifugation at 100,000 x g for 30 min . The preparation was sequentially subjected to four chromatographic procedures, DEAE-Sepharose Cl-6B, Sephadex G-100, actin-DNAse I-Sepharose 4B and DEAE-5PW. Fig.7 shows typical elution profiles. The elution profile of the first column (DEAE Sepharose Cl-6B) is shown in A. Fractions 22-38 (shaded area 1) which tested positive for scinderin in immunoblots were pooled, concentrated and filtered through Sephadex G-100. (B) shows the elution profile of the gel filtration column. Scinderin positive fractions (24 -30; shaded area 2) were combined and applied to an actin-DNAse I affinity column (C). The actin-DNAse I affinity column step was used on the basis of our previous observations (Bader et al., 1986) on the retention by this column of scinderin together with gelsolin and their subsequent elution by EGTA containing buffers. The fractions collected during elution with EGTA-containing buffer (shaded area 3) were pooled, concentrated and subjected to HPLC (D) using a DEAE-5PW column. The shaded area 4 under the peak corresponds to fractions

Fig.7: Purification of scinderin. Adrenal medullae were homogenized, centrifuged and a 35% ammonium sulfate fraction was prepared, dialysed and clarified by centrifugation as described in methods. The preparation was then subjected to four subsequent chromatographic procedures (A-D). The elution profile of the first column (DEAE-sephadex CL6B) is shown in A. Fractions 22-38 (shaded area 1) which tested positive for scinderin in immunoblots were pooled, concentrated and filtered through sephadex G-100 (B). Scinderin positive fractions (24-30; shaded area 2) were combined and applied to an actin-DNAse I affinity column (C). The fractions collected during elution with EGTA-containing buffer (shaded area 3) were pooled, concentrated and subjected to HPLC (D) using a DEAE-5PW column. The shaded area 4 under the peak corresponds to a fractions containing 98-99% pure scinderin. For further details on chromatography, see Materials and methods. At the bottom of the figure, electrophoretic patterns and immunoblots of different fractions are shown. Protein aliquots (150 μg) were separated by electrophoresis (S,C,A,1-4) and subsequently electroblotted (C', A', 1'-4'). The lanes correspond to molecular weight standards (S), cytosol (C), ammonium sulfate sediment (A) and pooled fractions (shaded areas 1-4) from column effuents. Lanes C', A', 1', 2', 3'and 4' are immunoblots of the same fractions. Scinderin was too diluted to be detected by immunoblotting in cytosolic (C') and ammonium sulfate (A') fractions.

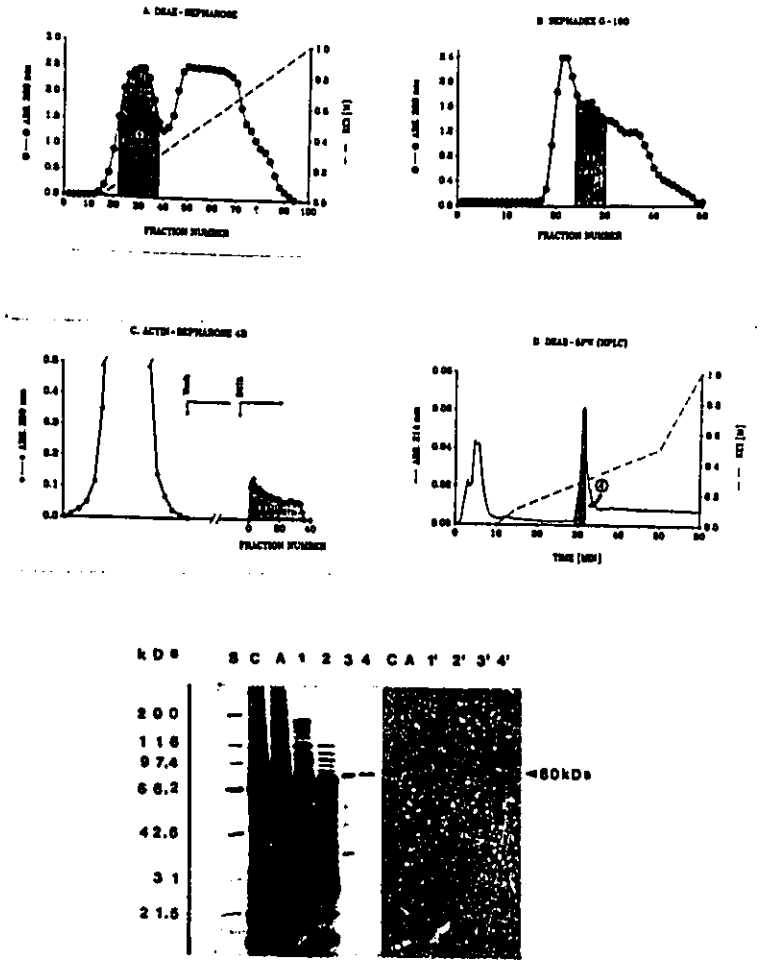


Fig. 7. Purification of Scinderin.

containing 98 - 99% pure scinderin. This purification protocol was always reproducible and 400 - 450μg of scinderin was obtained from 80- 100g of adrenal medullary tissue. It also allowed the early separation of as much as 90% of gelsolin (after ammonium sulfate precipitation and DEAE sepharose chromatography) from scinderin and at the same time provided evidence for the interaction of scinderin with actin rather than gelsolin, since the preparation loaded on the actin DNAse I affinity column was almost devoid of gelsolin. This was not the case in our earlier experiments (Bader et al., 1986). The electrophoretic pattern of the pooled fractions and the corresponding immunoblots are also shown in fig.7. Lane C represents Coomassie blue staining of adrenal medullary cytosol and lane A represents that of dialysed ammonium sulfate sediment after clarification by 100,000 x g centrifugation. Lane 1 represents the electrophoretic pattern of the proteins in the pooled fractions from the DEAE-Sepharose column and lane 2 represents the pattern after gel filtration chromatography. Lane 3 and lane 4 represent the electrophoretic pattern of the proteins in the eluates after actin DNAse-I affinity chromatography and after HPLC on a DEAE-5PW column respectively. The preparation of scinderin obtained was 98% pure and the ^{125}I protein A assay indicated that scinderin had been enriched 181 times when compared to the protein preparation eluted from the first column (Table 1). The final purification factor was obviously

TABLE 1 Purification of adrenal medullary scinderin

Purifaction Step	Total protein (mg)	Scinderin			Recovery (% total activity)	Purificati (times)
		*Total Activity	Protein (mg)	Specific Activity		
Cytosol	3440	ND	ND	-	-	-
Ammonium sulfate (0-65%)	686	ND	ND	-	-	-
DEAE-Sepharose CL-6B	290	5,136	1.60	17.7	100	1
Sephadex G-100	139	4,077	1.27	29.3	79	1.6
Actin-Sepharose 4B	0.94	1,926	0.60	2,049	37	116
DEAE-5PW	0.41	1,316	0.41	3,210	26	181

Cytosol was prepared from 25 bovine adrenal medullae.

*Scinderin total activity (relative densitometric units) was measured by the ^{125}I-Protein immunoblotting technique described in Materials and Methods.

ND: Scinderin was too diluted to be detected by immunoblotting in cytosolic and ammoni sulfate fractions.

Fig. 8: Apparent molecular weights of scinderin and gelsolin as determined by SDS-PAGE. Plot of the apparent molecular weights of molecular weight standards (BioRad) versus their relative mobilities. The markers used were myosin (200,000), β-galactosidase (116,250),phosphorylase B (97,400), bovine serum albumin (66,200), ovo-albumin (42,699), carbonic and anhydrase (31,000) and soybean trypsin inhibitor (21,500). The mean ± S.E. apparent molecular weight values of gelsolin (■) and scinderin (o) calculated from 4 and 11 different electrophoresis runs respectively are indicated in the figure.

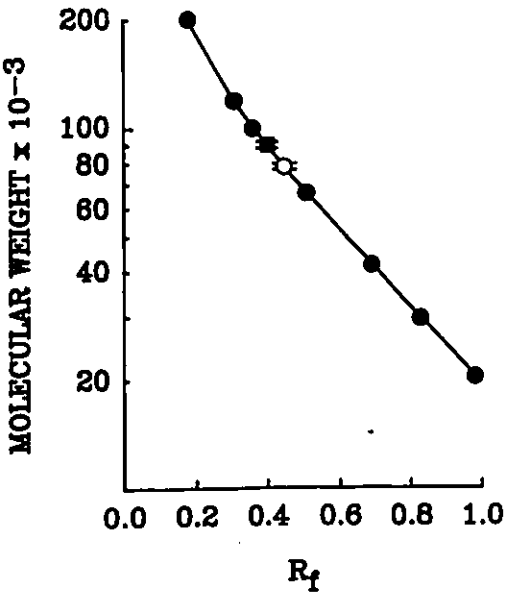


Fig.8. Apparent molecular weights of scinderin
----- and gelsolin as determined by SDS-PAGE.

greater than 181 because scinderin was too dilute in cytosol and ammonium sulfate fractions to be detected by immunoblotting followed by the ^{125}I protein A assay (Table 1). Therefore, the ammonium sulfate cut was determined by trial and error, and by measuring scinderin activity by immunoblotting only after the DEAE Sepharose chromatography.The property of decreasing actin gel viscosity observed in the cytosol, ammonium sulfate sediments as well as in the eluates from the DEAE Sepharose column were due in part, to the presence of gelsolin and consequently viscosity measurements were not used to monitor the fractions. The apparent molecular weight of scinderin, as determined by SDS PAGE (Fig. 8) and confirmed by immunoblotting was 79,600 ± 450 (n=11).

In ion exchange chromatography gelsolin was eluted later than scinderin enabling the separate purification of each protein. The molecular weight of gelsolin as determined by the SDS-PAGE (Fig.8) and confirmed by immunoblotting was 89,800 ±480 Daltons (n=4).

B. CHARACTERISATION OF SCINDERIN

B.1. Scinderin, a calcium binding protein

As the calcium dependent actin binding activity of scinderin was used in its purification, the calcium binding properties of scinderin were investigated next. Samples of scinderin were run on SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes containing scinderin were incubated with ^{45}Ca in

Fig. 9: Overlay test of ^{45}Ca binding by scinderin. Samples (20 μg protein) of purified scinderin were subjected to SDS-PAGE and subsequently transferred to nitrocellulose membranes as described in Materials and methods. The nitrocellulose membranes were incubated with 10^{-6} M ^{45}Ca (μCi/ml) in the absence (Lane 1) or in the presence (Lane 2) of $2x10^{-3}$ M $CaCl_2$. After several washes, the nitrocellulose membranes were dried and exposed for autoradiography as indicated in Methods. Autoradiographies are shown in lanes 1 and 2 and lanes 1' and 2' show the immunoblots of the same samples of scinderin performed with scinderin antibody.

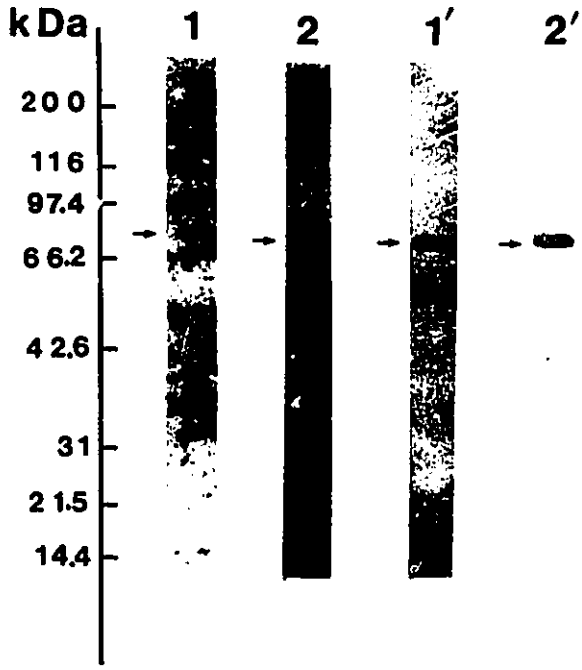


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Fig.9. Overlay test of Calcium binding by scinderin.

the absence or presence of an excess of non-radioactive calcium. Fig. 9 lane 1 shows the binding of ^{45}Ca by scinderin in the absence of cold calcium in the incubation medium and lane 2 shows the competition and displacement of radio-active calcium by non radioactive Ca^{2+} when present in excess. Lane 1' and 2' show the corresponding immunoblots which confirm the presence of scinderin in both lanes. These results demonstrate that binding of scinderin to radio-active calcium is specific and not due to a nonspecific interaction.

The interaction of scinderin with calcium was further determined by equilibrium dialysis. The relationship between calcium concentration and its binding to scinderin is shown in fig.10. This was drawn from the average values obtained from three different experiments. From the binding curve, a mean saturation value of 1.8 ± 0.08 mol Ca^{2+}/mol protein; n=3) was obtained at calcium concentrations of 10^{-4} - 10^{-3}M. The insert in fig. 10 shows the Scatchard plot obtained with the binding values. The data indicate the presence of two Ca^{2+} binding sites of different affinities, one site with a K_d of 5.85×10^{-7}M and a B_{max} fo 0.81 mol calcium/ mol protein and the second site with a k_d and B_{max} of 2.85×10^{-6} M and 1.87 mol calcium/ mol protein, respectively.

B.2.Effect of scinderin on F-actin solution viscosity and filament length:

Actin filament severing activity of gelsolin in a calcium

Fig.10 : Ca^{2+} binding to scinderin. Equilibrium dialysis was carried out at free Ca^{2+} concentrations between 10^{-8} and 10^{-3} M as described in Materials and methods. The Ca^{2+} saturation curve and the Scatchard plot (insert) were constructed from data obtained in duplicate from 3 different preparations of scinderin. Two Ca^{2+} binding sites were observed, a high affinity site with a K_d = 5.85×10^{-7} M and B_{max} = 0.81 M Ca^{2+}/mole protein and a low affinity site with a K_d = 2.85×10^{-6} M and B_{max} = 1.87 M Ca^{2+}/mole protein.

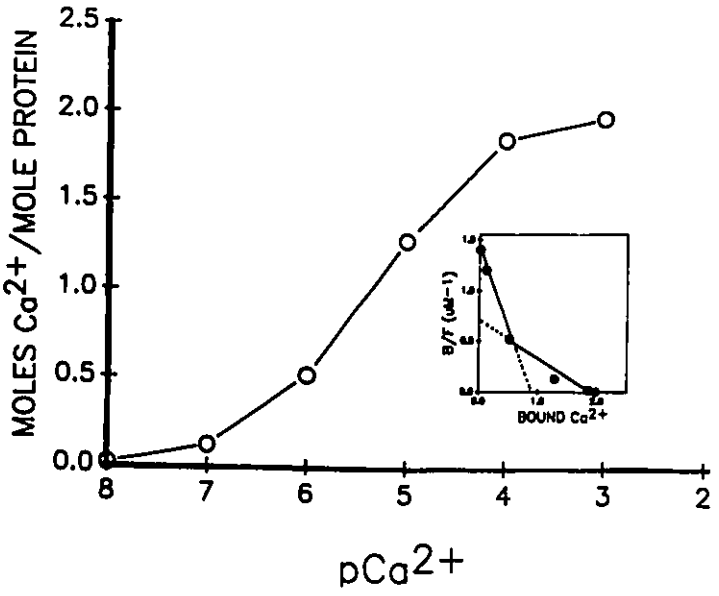


Fig.10. Ca^{2+} binding to scinderin- Equilibrium ------- dialysis studies

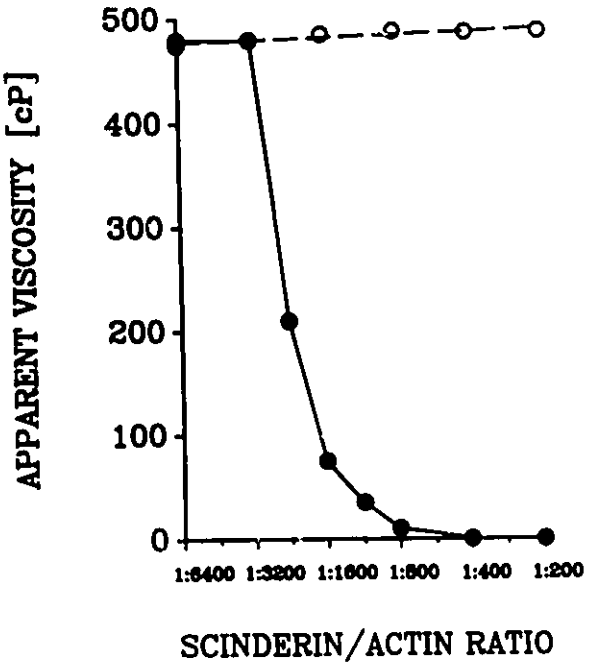
dependent manner has been well documented. Therefore the effect of scinderin on actin was tested in a similar manner to detect any such activity. This was tested by two methods; (a) falling ball viscometry and (b) electron microscopy. Mixtures of scinderin and F-actin at different molar ratios were prepared and tested in the absence (presence of 5 mM EGTA) or in the presence of 10^{-5} M free calcium. The addition of scinderin did not modify the viscosity of the actin solutions when calcium was absent. In the presence of calcium, a decrease in the viscosity of actin solutions was observed with molar ratios of scinderin to actin greater than 1:3200 (Fig.11 A). At the scinderin - actin molar ratio of 1:800, a decrease in viscosity from 480 cP to 10 cP was observed. The effect of calcium concentration on scinderin induced decrease in actin solution viscosity was also tested at a fixed molar ratio of 1:1600. Changes in the viscosity were observed between calcium concentrations of 10^{-8} and 10^{-6} M with the maximal fall in viscosity observed at 10^{-6} M Ca^{2+} (Fig.11 B). For this particular scinderin - actin molar ratio, a 50% reduction in the viscosity of the preparation was observed at 0.4×10^{-7}M Ca^{2+}. Since the actin preparation used was pure as judged by SDS - PAGE (Fig.4) and because the polymerised actin preparation did not change in its viscosity in the presence of calcium alone (Fig.11 B), it can be concluded that the changes in viscosity observed were due to scinderin.

The interaction of scinderin with actin was also investigated

Fig. 11A: Effect of various concentrations of scinderin on the apparent viscosity of F-actin. G-actin (10 mg/ml) in 2 mM Tris-HCl, pH 8, 0.2 mM ATP, 0.2 mM $CaCl_2$ and 0.005% NaN_3 was polymerized for 2 hr at 4°C by addition of 100 mM KCl, 1 mM $MgCl_2$ and 0.8 mM ATP.The F-actin thus obtained was sedimented by centrifugation at 80,000 X g for 3 hr and resuspended in incubation buffer (40 mM Pipes, pH 6.8, 100 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 5 mM EGTA and 0.05% NaN_3.Apparent viscosity was determined using a falling ball viscometer.F-actin (final concentration 1.91 mg/ml =44.37 µM) and scinderin (final concentration 17.6-0.56 µg/ml=0.22 - 0.007 µM) were mixed together in the presence of either $CaCl_2$ - EGTA buffer (10^{-5} M free Ca^{2+} or 5 mM EGTA). The sample (200 µl) was shaken at high speed for 10 sec and approximately 100 µl were drawn into a capillary tube. The tube was sealed with plasticine at one end and incubated in horizontal position for 120 min at 24°C.At the end of the incubation period,the tube was mounted at an angle of 45° and apparent viscosity was measured and calculated as described by MacLean-Fletcher and Pollard (1980).

Fig. 11B: Effect of free Ca^{2+} concentration on F-actin-severing activity of scinderin.Apparent viscosity was measured with a falling ball viscometer. F-actin (44.37 µM) was incubated in a capillary tube with 2.21 µg scinderin/ml (molar ratio to actin 1:1600) for 120 min at 25°C. The incubation buffer was of the following composition (mM): Pipes, 40 (pH 6.8),KCl,100; $MgCl_2$,2; ATP,2 and 0.05% NaN_3 containing 5 mM EGTA and different concentrations of $CaCl_2$ to obtain the free Ca^{2+} concentrations indicated in the abcissa.pCa^{2+} values were calculated using the binding constant for Ca-EGTA at pH 6.8 of 2.14×10^6 (Caldwell, 1970). Filled circles and solid lines represent incubation of F-actin and scinderin in the presence of 10^{-10} to 10^{-5} M free Ca^{2+} and open circles and broken lines represent incubation of F-actin alone in the presence of the same Ca^{2+} concentrations.

A.



B.

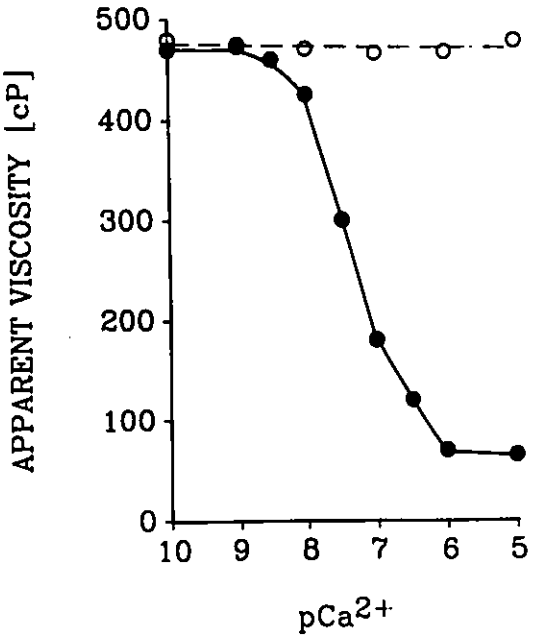


Fig. 11. A- Effects of various concentrations of scinderin on the apparent viscosity of F-actin.

B- Effect of free calcium concentration on F-actin severing activity of scinderin

Fig. 12: Electron micrographs of negatively stained mixture of F-actin filaments and scinderin (scinderin to actin molar ratio of 1:20) in the absence (A) or in the presence (B) of 10^{-5} M free Ca^{2+}. A mesh of interconnecting filaments can be observed in A. The bar indicates 0.1 μm. In B, as a result of the Ca^{2+} activated effect of scinderin on F-actin, short filaments are only observed. The bar represents 0.1 μm. A histogram with the distribution of the length of 183 short F-actin filaments is shown in C.

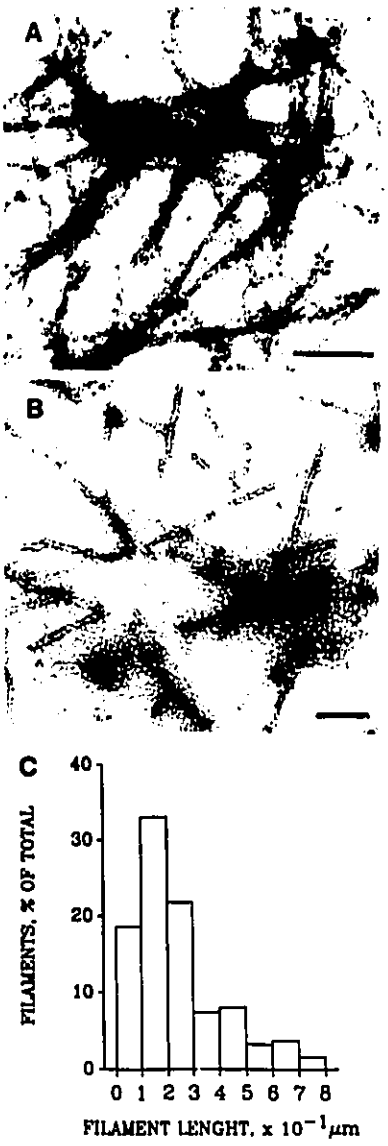


Fig.12. Electron micrographs of negatively stained mixture of F-actin filaments and scinderin in the absence (A) or in the presence (B) of 10^{-5}M free calcium.

by electron microscopy. Under conditions suitable for polymerisation, actin alone, either in the presence of 10^{-5} M Ca^{2+} and in the presence of 10^{-5} M Ca^{2+} or in the absence of $Ca^{2+}$and in the presence of EGTA and scinderin, formed a network of very long filaments (Fig.12 A). Short filaments were never observed under these conditions. In contrast only short filaments were observed when scinderin was added to actin networks in the presence of calcium (Fig.12 B). Under these conditions, it was possible to measure the length of actin filaments. The filaments observed were shorter than 0.8μm with over 50% of the filaments with lengths of 0.2μm or shorter (Fig.12 C). The average filament length observed under these conditions was 0.32 ± 0.04 μm (n=183). This length corresponds to filaments formed by approximately 58 actin monomers (Pollard and Cooper, 1986).

C. COMPARISON BETWEEN SCINDERIN AND GELSOLIN

C.1.Specificity of the antibodies

A partially purified preparation of adrenal medullary cytosol obtained as described in section B of experimental procedures, containing both scinderin and gelsolin in detectable amounts was subjected to SDS-PAGE. The proteins were then transferred to nitrocellulose membranes. Immunoblots prepared with scinderin antibody and gelsolin antibody indicated that the two proteins

Fig. 13: Specificity of the scinderin and gelsolin polyclonal antibodies. Partially purified preparation of adrenal medullary cytosol (ammonium sulfate sediment) containing both proteins was subjected to SDS-PAGE and subsequently electrotransferred to nitrocellulose membranes. Lane 1 shows proteins stained with amido black 10B, lane 2 after incubation with anti-scinderin and lane 3 after incubation with gelsolin antibody. Antibody dilutions used were 1:125 and 1:1000 for anti-scinderin and anti-gelsolin, respectively. The scinderin antibody specifically cross-reacts with a 80 kDa band and did not cross-react with either gelsolin (90 kDa) or actin (42 kDa). Similarly, gelsolin antibody only recognizes a 90 kD protein and shows no cross-reactivity with either actin or scinderin.

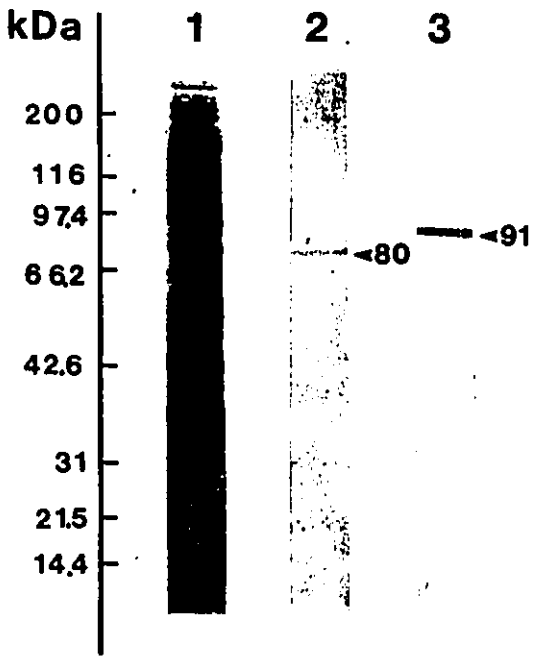


Fig.13. Specificity of the scinderin and gelsolin
------ polyclonal antibodies.

were immunologically different (Fig.13), since no cross-reactivity was observed with either of the two antibodies. Fig. 13 lane 1 shows the proteins transferred to nitrocellulose membrane and stained with Amido Black 10B. Lane 2 shows the interaction of scinderin antibody with only scinderin (80 KDa) and not with either gelsolin (90 KDa) or actin (42 KDa). Similarly gelsolin antibody recognises only gelsolin and shows no cross-reactivity with either scinderin or actin. This indicates that gelsolin and scinderin do not share antigenic sites and supports the view that scinderin is a different protein and not a breakdown product of gelsolin.

C.2. Comparison of isoelectric points and peptide maps

A mixture containing both purified scinderin and purified gelsolin was subjected to two dimensional electrophoresis. Fig. 13D shows the electrophoretic pattren of the mixture. Three isoforms for each of the two proteins, gelsolin(G): pI =5.8, 5.9 and 6.0 and scinderin(S): pI = 6.0, 6.1 and 6.2 were observed. These results indicate that scinderin has a more basic nature compared to gelsolin. the other three isoforms (Br.) seen slightly above gelsolin(90 KDa) may correspond to brevin (93 KDa) which is the secreted form of gelsolin found in blood plasma. THe similarities between adrenal medullary gelsolin and plasma brevin have been described previously in the work from our lab (Bader et al.' 1986). Fig.14 shows the two dimensional electrophoretic pattern

Fig. 14 : Chymotrypsin cleavage products of adrenal medullary gelsolin and scinderin. Gelsolin (G) and scinderin (S) were digested with chymotrypsin at a protein-enzyme ratio of 400:1 as indicated in Materials and methods. Peptide maps of gelsolin and scinderin are shown in A and B respectively. In C, a compositive diagram of superimposed peptide maps for both gelsolin and scinderin is shown; the arrows (solid spots) indicate cleavage products of scinderin, whereas open spots correspond to cleavage products of gelsolin. A mixture of gelsolin and scinderin prior to digestion is shown in D. Three isoforms for each of the two proteins (gelsolin, pI = 5.8, 5.9 and 6.0; scinderin, pI = 6.0, 6.1 and 6.2) were observed. The other 3 isoforms (Br.) may correspond to brevin as described previously (Bader et al., 1986).

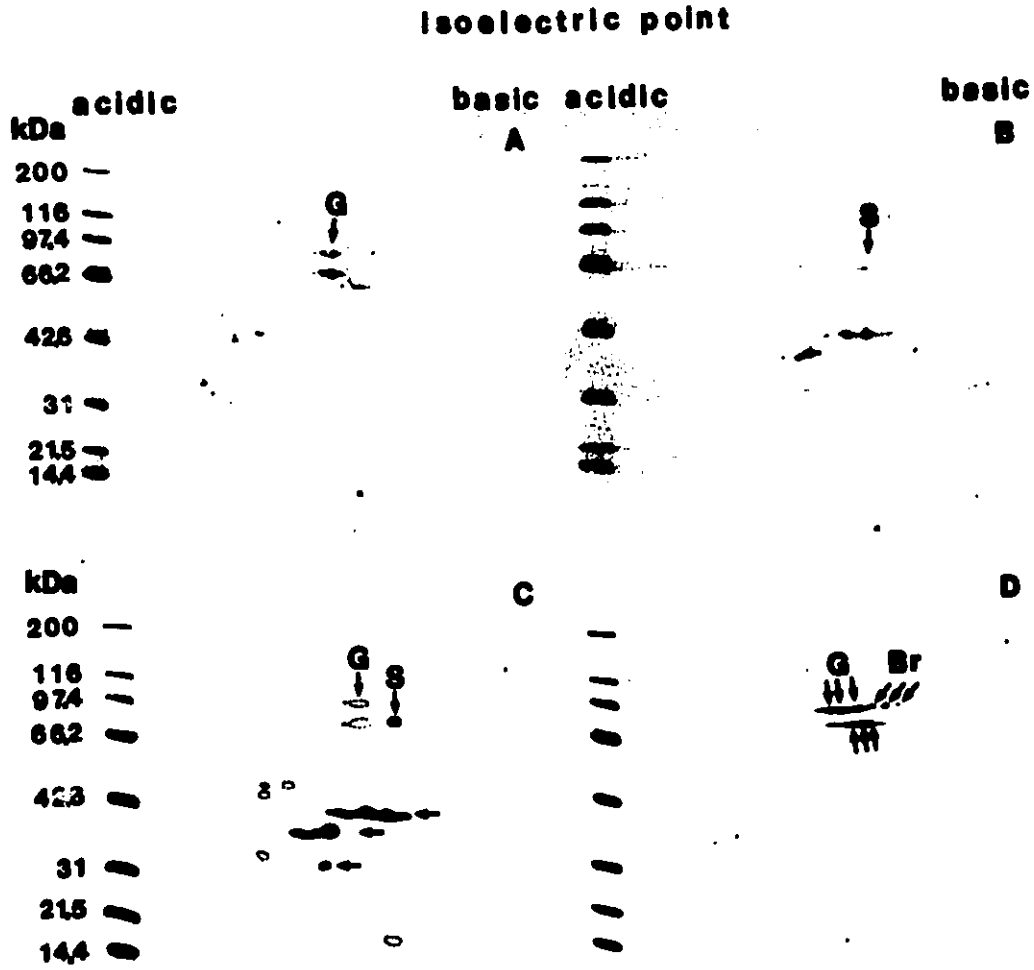


Fig.14. Chymotrypsin cleavage products of adrenal medullary scinderin ------ and gelsolin.

of chymotryptic fragments of gelsolin and scinderin. Peptide map of gelsolin is shown in fig. 14 A and that of scinderin is shown in fig. 14 B. In 14 C, a composite diagram of superimposed peptide maps for both gelsolin and scinderin is shown. The two dimensional peptide map obtained after limited proteolytic digestion with chymotrypsin showed marked differences in peptide composition (Fig. 14 C). The molecular weights of adrenal medullary gelsolin fragments obtained by chymotrypsin digestion were similar to those previously described for human plasma gelsolin (Kwiatkowski et al.' 1985). Upon incubation using a protein to enzyme ratio of 400:1, 5 fragments were obtained from gelsolin (Fig 14 A and C). Molecular weights and pI of these fragments were 80 KDa (pI 6.0), 50 KDa (2 isoforms of pI 5.5 and 5.7), 46.5 KDa (pI 5.5), 31 KDa (pI 5.5) and 16 KDa (pI 6.1). Chymotryptic digestion of scinderin under similar experimental conditions gave a quite different peptide pattern (Fig.14 B). The polypeptide pattern showed two main proteolytic fragments of molecular weights 40 KDa (2 isoforms of pI 6.0 and 6.1) and 34 KDa (2 isoforms of pI 5.7 and 5.9) respectively, and a small 32 KDa (pI = 5.8) fragment (Fig. 14 B and 14 C). The differences in the molecular weights of fragments as well as in the isoelectric points further support the view that gelsolin and scinderin are two different proteins.

C.3. Aminoacid analysis

The aminoacid composition of two different preparations of

Table 2: Comparison between the aminoacid composition of scinderin and gelsolin:[a]= Data from Yin and Stossel (1980) as modified by Petrucci et al., (1983). The data are expressed as number of residues per molecule of protein of molecular weight of 90,000. The molecular weights of preparations, assuming that their content in Phe was the same as that found in gelsolin.

TABLE 2: Comparison between the amino acid composition of scinderin and gelsolin

Amino acid	Scinderin		Gelsolin[a]
	Preparation 1	Preparation 2	
Asp	62	66	85
Glu	87	92	105
Ser	53	51	50
Gly	70	67	69
His	18	17	17
Arg	43	40	41
Thr	42	43	42
Ala	63	60	73
Pro	33	32	49
Val	61	57	58
Met	11	9	17
Ile	41	38	29
Leu	66	65	73
Phe	34	34	34
Lys	33	34	51
Tyr	32	31	24

purified scinderin were also determined. Table 2 shows these results together with those previously published for gelsolin (Yin and Stossel, 1980; Petrucci et al., 1983). The content of lysine is lower in scinderin compared to that of gelsolin. However, scinderin is a more basic protein than gelsolin (Fig.14 D), since the content of acidic residues (aspartic and glutamic) is much greater in the latter protein. Morever, the content of isoleucine and tyrosine residues is higher in scinderin than gelsolin, thus indicating that scinderin cannot be a breakdown product of gelsolin.

C.4. Tissue expression of scinderin

In order to test the tissue expression of scinderin the partial purification procedure described in the previous publication from our lab (Bader et al., 1986) was adopted ;(see experimental procedures, section B). The tissues were homogenised in the same homogenisation buffer with the omission of 3 M KCl. This was necessary because this form of purification depended on the presence of functional G-actin in the homogenate. As G-actin binds to DNAse-I with high affinity, the calcium dependent actin binding proteins will also be retained by the DNAse-I CNBr activated Sepharose affinity column. Elution with 10mM EGTA chelates the calcium and hence leads to the elution of calcium dependent actin binding proteins. A typical elution profile for adrenal medullary proteins is shown in fig.15. Lane A shows the

Fig. 15: Partial purification of scinderin and gelsolin from adrenal medullary cytosol. The cytosol fraction (100,000xg supernatant) was prepared from bovine adrenal medulla and dialysed as described in section B of experimental procedures. The dialysed fraction to which calcium and Na-ATP were added was passed through the DNAse I-Sepharose affinity column. The optical density of the eluted material was monitored at 280nm, and the peaks were collected. The major portion of the cytosol proteins were not retained by the column. Fig. 15 A shows the elution profiles with various buffers as indicated. Fig. 15 B shows the electrophoretic patterns of the corresponding eluates. Lane A is the electrophoretic profile of the proteins not retained by the column. The column was washed overnight with the equilibration buffer containing 0.6 M NaCl, and calcium dependent actin binding proteins were eluted with EGTA - containing buffer. Finally the column was washed with guanidine-HCl buffer. Lanes B and C are respectively the electrophoretic profiles of the proteins eluted with EGTA and with Guanidine - HCl. Scinderin (80 KDa) is indicated by the closed arrow and gelsolin (90 KDa) is indicated by the open arrow. Actin (42.6 Kda) is indicated by the arrow head.

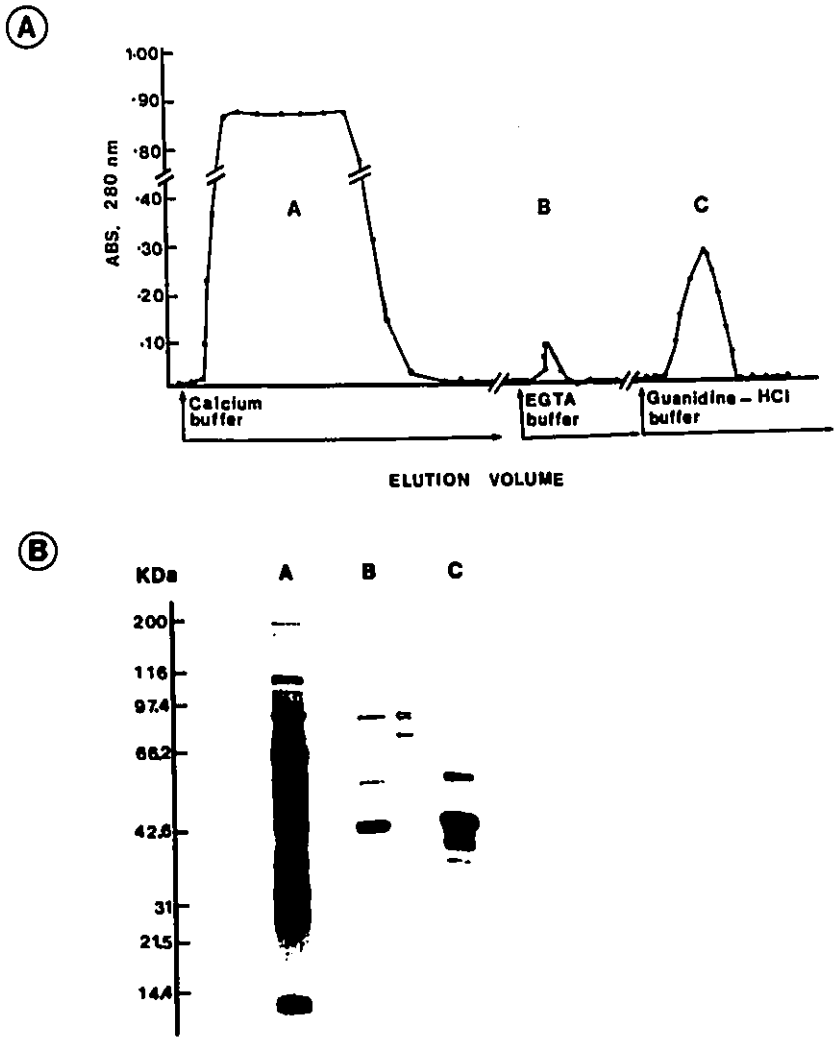


Fig.15. Partial purification of scinderin and gelsolin
------ from adrenal medullary cytosol.

electrophoretic pattern of the proteins not retained by the column. It shows that almost 90% of the proteins are not retained. Wash with 0.6 M NaCl removes the proteins that are bound nonspecifically. Lane B shows the electrophoretic pattern of the proteins eluted with EGTA and lane C represents the proteins eluted with 3M Guanidine-HCl. Elution with the latter buffer denatures actin, thereby resulting in the removal of bound actin from the column.

This same protocol was used to identify the presence of scinderin and gelsolin in other bovine tissues. Fig.16 shows the tissue expression of scinderin. Bovine tissues that were tested for the presence of protein were anterior and posterior pituitary gland, brain, kidney, skeletal muscle, heart muscle, liver and heparinised plasma. Fig. 16A shows the immunoblots of the tissues which displayed positive immunoreactivity with antiscinderin (1:500 dilution). Bovine adrenal medullary extract was used as control. The position of scinderin is indicated by a closed arrowhead. It is interesting to note that brain, pituitary and adrenal medulla also share the property of originating from neural tissue and that all three express scinderin . Fig. 16B shows the tissues which did not express the protein. Here adrenal medullary EGTA eluate (lane 1) as well as pure scinderin (lane 2, open arrow) were used as controls. In each group lane 1 represents the EGTA eluted proteins separated by SDS-PAGE, transferred to nitrocellulose and stained with amidoblack 10 B; lane 2 represents a similar membrane immunoblotted with scinderin

Fig. 16 A: Tissue expression of scinderin. Samples of the concentrated EGTA eluates obtained as described in methods were subjected to 10% SDS-PAGE and transferred to introcellulose membranes. Bovine adrenal medullary exract was used as control. Bovine anterior and posterior pituitary glands, brain and kidneys were tested and found to display positive immunoreactivity when tested against scinderin antibodies at antibody dilution of 1:500. The position of scinderin is indicated by arrowheads.

Fig. 16 B: Samples of EGTA eluates obtained under similar conditions to those described above were transferred to nitrocellulose membranes. A purified sample of chromaffin cell scinderin was used as control. Bovine skeletal muscle, heart muscle, liver and plasma were tested with anti scinderin (1:500 dilution). For each group lane 1 represents the transferred proteins after staining with Amido Black 10B. Lane 2 shows the immunoblot of the same proteins after incubating with anti-scinderin. The presence of gelsolin in the same preparation was tested with anti-gelsolin (1:1000 dilution).This is shown in lane 3. Closed arrowheads indicate the position of gelsolin, open arrowheads indicate that of scinderin and arrows indicate that of brevin.

A.

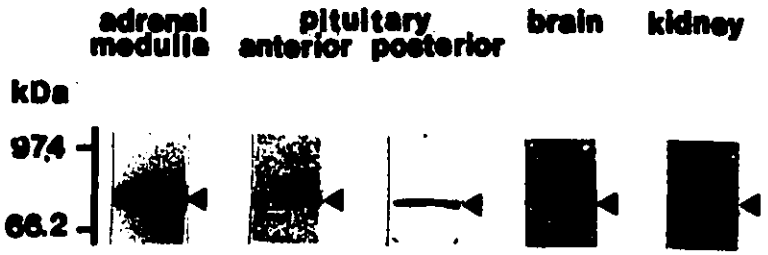


Fig. 16.A. Tissue expression of scinderin.

B.

Fig. 16.B. Different pattern of tissue distribution between scinderin and gelsolin.

antibody and lane 3 represents the immunoblot obtained with gelsolin antibody. As the gelsolin antibody was polyclonal it cross-reacts with both gelsolin (closed arrowhead) as well as with brevin which is a contaminant from blood. It can be seen that gelsolin is ubiquitous but scinderin does not share the same property. Four repeated experiments on different sets of bovine tissues showed that scinderin was notably absent from these tissues. The immunoreactivity of scinderin like proteins in different tissues were not the same; while adrenal medulla, anterior pituitary and posterior pituitary gave strong reactions brain and kidney gave weak positive reactions. This might be due to differences in the efficiency of the transfer among different lanes or may suggest variations in the amount of scinderin found in different tissues as the amount of starting material was the same for each tissue. This difference in distribution between scinderin and gelsolin indicates that scinderin is a novel protein by itself and may have a specific role in the tissues in which it is present. The presence of scinderin in all the secretory tissues tested suggests that scinderin may be expressed in secretory tissues and be involved in the secretory machinery.

C.5. Scinderin distribution in cultured chromaffin cells

Immunofluorescence studies with scinderin antibody on cultured chromaffin cells showed a weak and diffuse cytoplasmic

Fig. 17: Chromaffin cells as they appeared after staining by antibodies against scinderin (A and C) and gelsolin (D and F). Chromaffin cells cultured on collagen coated coverslips were fixed and stained with corresponding antibodies as previously described (Lee and Trifaró, 1981). The preparations were examined by indirect light fluorescence (epifluorescence). Strong and well defined cortical cytoplasmic fluorescence is observed with anti-scinderin (A and C, curved arrows) whereas anti-gelsolin staining shows a strong but more diffuse cortical cytoplasmic fluorescence, (D and F, straight arrows). B and E are phase contrast images of A and D, respectively. Magnifications were x 400.

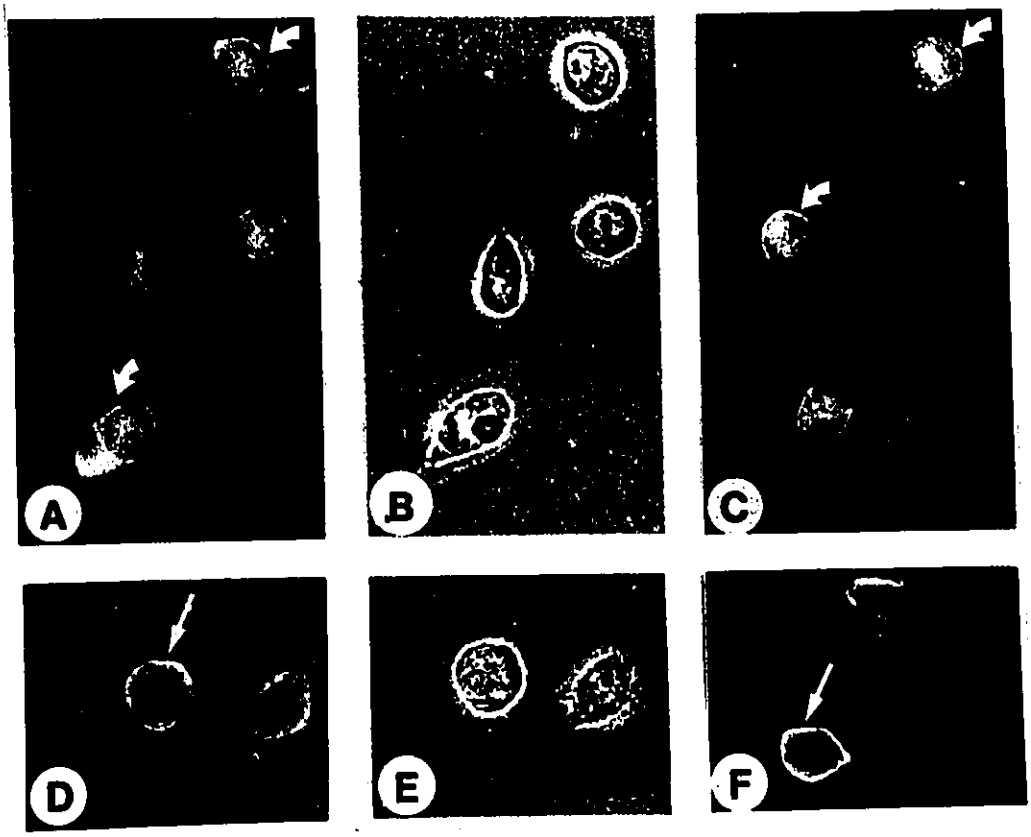


Fig.17.Cellular localisation of scinderin and gelsolin
------ demonstrated by immuno-fluorescent staining.

staining with a strong fluorescence ring pattern at the cortical cytoplasmic region, thus suggesting a preferential subplasmalemmal localisation for this actin severing protein (Fig. 17 A and C, curved arrows). Cortical surface distribution of gelsolin has been previously reported (Yin et al., 1981). In our experiments anti-gelsolin staining showed a strong but more diffuse cortical cytoplasmic fluoresence (D and F straight arrows). B and E are phase contrast images of A and D, respectively. The detection of scinderin in the chromaffin cells by scinderin antibody further confirms the presence of this protein in the adrenal chromaffin cell and excludes the possibility of contamination from another source in the adrenal medulla.

CHAPTER IV

DISCUSSION

Early work from our laboratory demonstrated the presence in the chromaffin cells, of gelsolin, a calcium-activated actin-severing protein (Trifaró et al., 1985a; Bader et al., 1986). In these studies chromaffin cell gelsolin was found to be structurally and immunologically identical to macrophage gelsolin on the basis of a) macrophage gelsolin and chromaffin gelsolin co-migrated in one and two-dimensional electrophoresis with the same apparent molecular weight and isoelectric points; b) the peptide maps generated after limited proteolytic digestion by staphylococcus V8 protease were similar; c) both macrophage and chromaffin cell gelsolins under similar conditions severed actin filaments; d) antibodies raised to macro-phage gelsolin cross-reacted with chromaffin cell gelsolin.

In addition to above described findings, two other laboratories have described the presence of gelsolin-like proteins in adrenal medulla (Grumet and Lin, 1981; Ashino et al ., 1987). Although the data from these studies suggested that experiments were performed with proteins which promote nucleation and severe actin, the true identity of these proteins was not determined. Thus, in contrast to observations by Bader et al., 1986 no comparisons between macrophage gelsolin and these adrenal medullary actin modulators was made, in these studies by Grumet and Lin, 1981 and by Ashino et al., 1987.

The present studies demonstrate that in addition to gelsolin, the chromaffin cells contain scinderin, another calcium

activated actin filament severing protein. As the presence of scinderin in adrenal medulla has been described in the earlier work from our laboratory it became interesting to purify the protein to homogeneity and study its properties in comparison to chromaffin cell gelsolin. This will enable the determination of the true identity of the protein as well as identify the relationship of scinderin to the gelsolin like proteins described in the adrenal medulla by other researchers.

A. ISOLATION OF CALCIUM DEPENDENT ACTIN BINDING PROTEINS

A.1. Purification of scinderin and gelsolin from adrenal medulla.

Actin-DNAse 1 affinity chromatography is a commonly used technique, which enables the isolation and purification of several actin-associated calcium dependent proteins from tissue extracts. This method was used as the main step in the isolation of scinderin and gelsolin from the adrenal medulla. As each protein was to be purified separately to homogeneity, additional steps (ammonium sulfate precipitation and ion exchange chromatography) were required to provide early seperation of these two proteins prior to the application on actin-DNAse 1 affinity column as this step cannot separate the two proteins. The final HPLC step was required to remove some minor contaminants from the EGTA eluate. This purification protocol offered the maximum yield of pure scinderin and gelsolin among

the various methods tried.

The purity of the final product of scinderin used for physico -chemical studies described below was established by the following findings, a) scinderin migrated as a single major band during electrophoresis in polyacrylamide gels heavily loaded with the protein sample. Identity of the protein was confirmed by immunoblotting; b) the purified protein always showed a constant specific activity of calcium dependent severing of F-actin.

A.2. Determination of molecular weight.

The subunit molecular weight of scinderin was estimated directly by SDS-PAGE. Scinderin migrated as a single band, with a Mr of 79,600 ± 450 (n = 11) both in the presence as well as in the absence of dithiotreitol (DTT). This indicates that scinderin is composed of a single polypeptide and it does not contain subunits linked by interchain disulfide bonds. The value obtained for chromaffin cell gelsolin was in agreement with the figure published previously (Yin and Stossel, 1980).

A.3. Partial purification of scinderin from other tissues.

As complete purification was not required to study the tissue expression of scinderin attempts were not made to separate scinderin and gelsolin before the affinity chromatography step. Based on the previous observations from our laboratory the 100,000 x g supernatants of various bovine tissue extracts

were loaded directly on the CNBr-DNAse 1 affinity column in the presence of calcium. As G-actin was ubiquitously present this was used as the ligand to trap the actin binding proteins present in various tissues. The actin-binding proteins which bind to G-actin in a calcium dependent manner were trapped along with the G-actin by the DNAse 1 in the affinity column and were eluted by a buffer containing 10 mM EGTA. To analyse the eluates from different columns a new SDS-PAGE system developed recently in our laboratory (Doucet and Trifaró, 1988) was used. This offered the advantage of resolving high molecular weight proteins quite well. Three major proteins namely brevin gelsolin , scinderin and some minor proteins were observed when the different EGTA eluates were analysed in this manner. The presence or absence of each protein was confirmed by immunoblotting with the corresponding antibody. Scinderin while being present in brain, pituitary and kidney was notably absent from liver, muscle and blood. As gelsolin and brevin were detected in the same EGTA eluates that were used for testing the presence of scinderin it could be safely concluded that the absence of scinderin in the EGTA eluates of liver and blood was due to the absence of this protein in the tissues concerned and not due to an experimental error.

B. INTERACTION OF SCINDERIN WITH CALCIUM

Equilibrium dialysis studies indicated scinderin posseses two Ca^{2+} binding sites of different affinities, one high affinity site and one low affinity site. Calcium binding by scinderin was saturable as shown by the binding curve. No cooperative effect was observed between these two binding sites when the data were analysed by the method of Scatchard(1949). Similarly published studies for gelsolin also showed the presence of two calcium binding sites for this protein but the presence of only one class of high afffinity binding (Yin et al., 1980). The differences in the calicum binding nature of scinderin and gelsolin suggests that the two proteins may undergo different types of regulation. Calcium was shown to be essential for the actin binding activity of both scinderin and gelsolin. However the significance of the calcium - scinderin interactions can be fully understood only by studying the calcium induced conformational changes of scinderin and by assessing the precise influence of Ca^{2+} on the actin recognition sites of scinderin.

C. SCINDERIN - ACTIN INTERACTIONS

As described in the purification procedures, scinderin bound to a G-actin coupled DNAse 1 Sepharose column in a calcium

dependent manner. Low shear viscometry study and electron microscopy revealed the calcium dependent actin severing nature of scinderin. Under the present assay conditions F-actin was used at a relatively high concentration to allow the quantitative determination of the decreasing viscosity of actin filaments. In the presence of 10^{-6} M Ca^{2+}, a dramatic decrease in the viscosity of the actin solution was observed with molar ratios of scinderin to actin greater than 1:1600. As shown in the negatively stained electron micrograph scinderin randomly severed actin filaments in the presence of calcium while no bundling or depolymerisation of the actin filaments was observed. These data clearly demonstrate that scinderin breaks the non-covalent bond between actin-actin monomers within a filament in the presence of calcium resulting in the fragmentation of the F-actin filament. This results in a decrease in the viscosity of F-actin solution as observed by low shear viscometry. So scinderin too can be included in the class of calcium dependent actin modulators occurring very widely from lower eukaryotes to mammals.

The interaction of gelsolin with actin are multiple and complex (Yin, 1988). In addition to actin severing activity it has an end blocking effect on actin filament, inhibiting monomer exchange from that end. It also nucleates actin. The combined effect of these interactions is to promote the

formation of a large number of short actin filaments which are capped at their barbed ends. The expression of these three effects of gelsolin is dependent on the calcium concentration of the reaction medium (Bryan and Kurth, 1984 ; Janmey et al., 1985; Weeds et al., 1986). Such understanding of the gelsolin-actin interactions led to the postulation of the role of gelsolin in secretory and non secretory cells (Yin, 1988). Simlar activities for scinderin are currently being investigated. A complete understanding of scinderin-calcium-actin interactions will enable the postulation of the functional role of scinderin in the chromaffin cell as well as in other tissues.

D. COMPARISON BETWEEN SCINDERIN AND GELSOLIN

The data obtained from the study of the physico-chemical properties of scinderin indicate that scinderin is a distinct protein by itself and is not a breakdown product of gelsolin. This conclusion was made on the basis of the following findings.

a) During various steps of purification, the two proteins showed different chromatographic behaviour. This enabled the separate purification of each protein.

b) Scinderin and gelsolin have different apparent molecular weights, aminoacid compositions and isoelectric points.

c) Scinderin has two calcium binding sites with different

affinities whereas gelsolin has two calcium binding sites with the same affinity.

d) Limited proteolytic cleavage with either staphylococcal V8 protease or chymotrypsin clearly yielded different peptide maps for these two proteins with the fragments demonstrating different isoelectric points as well.

e) The two proteins are immunologically different as no cross-reactivity was observed with either of the two antibodies.

f) The tissue expression of scinderin and gelsolin is not the same. In the tissues tested positive , both scinderin and gelsolin were coexistant.

g) The immunofluorescence study of the chromaffin cell demonstrated different cellular localisations for scinderin and gelsolin.

Scinderin although structurally different from gelsolin shares all the gelsolin functions so far explored. Both are cytosolic proteins which bind actin and sever actin filaments in a calcium dependent manner, producing short F-actin filaments with a consequent solation and decrease in the viscosity of actin solutions. Preliminary immunocytochemical experiments also confirm the presence of both proteins in chromaffin cells. Although both are located in the sub plasmalemma region they appear to be distributed differently. So in contrast to macrophage and chromaffin cell gelsolins which were shown to be identical both structurally and immunologically, scinderin significantly differs

in these aspects from chromaffin cell gelsolin.

The gelsolin like protein described by Grumet et al., (1981) was a 90 kDa polypeptide which inhibited actin polymerization in the presence of 0.4 mM $MgCl_2$ and reduced the steady state viscosity of the F-actin solution. The properties described for this protein appear to be similar to that of macrophage gelsolin (Yin et al., 1980) and is unlikely to be related to scinderin. They did not report the presence of an additional protein of 80 - 85 kDa protein in their purification procedures. Ashino et al., (1987) described the purification of an 80 kDa calcium dependent actin modulating protein from the adrenal medulla. This may be related to scinderin as both of them have similar molecular weights and share the calcium dependent actin severing activity. The severing activity of the 80 kDa protein described by Ashino et al.,(1987) was slightly protected by the saturable amounts of tropomyosin present (0.14 mg/ ml) in the solution. This effect was not tested for in the studies described here. Ashino et al (1987) suggest that the 80 kDa protein belongs to the gelsolin family and they did observe some functional differences in the actin binding activity of the 80 kDa protein when they compared their data to the already published properties of a gelsolin-like protein in the brain. However a comparative study of the properties of the two proteins under similar exprimental conditions as has been carried out in the present study would have established the

identity of the 80 kDa protein they purified from the adrenal medulla.

E.TISSUE EXPRESSION OF SCINDERIN

The data presented suggest the presence of scinderin like proteins in other tissues. As expected gelsolin and brevin (except in the brain) were present in all the tissues tested in agreement with the data published previously (Yin et al., 1981; Carron et al., 1986). The absence of scinderin in the liver and blood on repeated experiments suggest that unlike gelsolin which has a secreted variant brevin, that is synthesised by the striated and smooth muscle tissues and liver to be secreted into the blood stream, the scinderin in the cytoplasm does not appear to have a secreted variant.

It is of interest to note that an 85 kDa calcium sensitive gelation factor that co-purified with a gelsolin like 90 kDa protein has been described in acanthamoeba extracts (Pollard et al., 1980).The gelation activity of this 85 kDa protein could only be detected once complete purification was achieved. Chromaffin cell scinderin however never exhibited gelation activity, but had a solation effect on the actin gels in the presence of calcium, suggesting that the 85 KDa acanthamoeba protein is not similar to scinderin. An 85 kDa actin binding protein was also purified from platelets (Lin et al.,

1982) and brain extracts, although the 85 kDa protein was isolated with a presumed gelsolin like protein (Petrucci et al.,1983). In the latter issue the authors assumed it to be a breakdown product of gelsolin but their assumption was not verified further as the functional properties of the 85 kDa protein was not studied. In the data presented in this study the brain also contained scinderin based on the finding on immunoblotting. Platelet preparations were not tested for the presence of scinderin.

Two individual research groups have described the isolation and characterization of calcium dependent actin severing proteins of 80-84 kDa in thyroid gland and muscular tissue respectively. Koboyashi et al., (1983) described the purification of the 80 kDa actin modulator protein from the thyroid gland. This protein also was purified using the DNase-I-Actin-Sepharose chromatography as the main purification step. It was found to be a single polypeptide with the isoelectric point in the basic range (6.35 - 7.0). Its aminoacid composition was different from those of villin, gelsolin and leukocyte actin polymerisation factor.It was shown to be an efficient Ca^{2+} dependent nucleator for actin assembly and it reduced the viscosity of F-actin solution in a calcium dependent manner. This 80 kDa protein was co-purified with another 40 kDa protein which rapidly inhibits the polymerisation of actin in the presence of physiological concentrations of calcium and magnesium. Future studies are planned to check for the

presence of scinderin in other secretory tissues including the thyroid gland by immunoblotting and if present to compare and contrast its properties with the data mentioned above. Kobayashi et al., (1983) also reported the presence of a 91 kDa protein (pI = 6.3) in the thyroid gland and suggested that it may be similar to macrophage gelsolin. However no comparative study was carried out to test this hypothesis. Ebisawa et al., (1985) identified the existence of an 84 kDa protein which severs actin filaments in a calcium dependent manner in several smooth muscle cells including chicken gizzard, bovine stomach and aorta . This protein had a pI of 6.1 (scinderin = 6.0-6.2) and had an amino acid composition similar to macrophage gelsolin, except that the content of basic residues was higher in this protein as opposed to the content of acidic residues which was higher in gelsolin. This feature was observed for scinderin too. The 80 - 84 kDa proteins described in the thyroid gland and smooth muscle may be related to scinderin and all three of them may belong to a novel subgroup of actin severing proteins in the large family of gelsolin-like proteins which disrupt microfilament formation. In the present study the presence of scinderin in the smooth muscle was not tested for but its absence in the cardiac and skeletal muscle was demonstrated by immunoblotting. The presence of scinderin like proteins in kidney,adrenal medulla and pituitary indicate that all organs with a secretory function may express scinderin. Like villin being

expressed by specialised tissues with primarily an absorptive function (eg. intestinal microvilli) the expression of scinderin by the tissues with a predominant secretory function may shed some light into its physiological role. However this has to be further verified future studies.

F.GENERAL DISCUSSION

The data presented here raise the obvious question as to why there are two actin severing proteins present in the same cell. It is possible that gelsolin and scinderin regulate actin filament network at two different cellular sites. This is an entirely possible situation, since it has been demonstrated that gelsolin and villin, another actin severing protein, are present in the same brush border of chicken intestinal epithelial cells, but with different cellular distributions. Villin is exclusively found in the microvilli (Brestcher and Weber, 1979, 1980) but not in the terminal web of intestinal epithelial cells. On the other hand, gelsolin antibody fluoresence was only detected in the terminal web of the same cells (Yin et al., 1981). Preliminary immunomcytochemical studies carried out in our lab revealed that distribution of scinderin and gelsolin is not the same as well. These different distribution patterns may be related to the functional role of each protein.

Difference in the calcium affinities of these two proteins may also account for different types of regulation. Furthermore gelsolin activity is also modulated by products of the phosphatidyl inositol pathway (Yin et al., 1988) and its not known whether scinderin is similarly regulated or whether other transducing mechanisms (eg. protein kinase C, calmodulin, cyclic AMP, G-proteins etc.) are also involved in the differential regulation

of these two proteins.The presence of scinderin like proteins in the cells and tissues in which gelsolin is also present could indicate a functional relationship between these two proteins regarding the regulation of actin filaments during cell activity. They could be acting synergistically in bringing about the change in cytoplasmic viscosity prior to secretion. The role of secreted form of gelsolin in the blood has not been well defined. One possibility is that it is involved in the clearance of actin filaments from the circulation, into which actin may be released from damaged tissue, (Yin, H.L. 1988). The absence of scinderin like protein in the blood was observed in the present study. The significance of this is not clear. Interestingly besides gelsolin another relatively abundant plasma protein, Vitamin D binding protein can also shorten actin filaments by sequestering actin monomers. The coexistence and complementary functions of these proteins suggest the presence of an efficient system in the blood for the removal of actin and the presence of an additional protein like scinderin to carry out the same function may not be required.

F.1. Chromaffin cell scinderin and regulation of the cytoskeleton

The chromaffin cells of the adrenal medulla store the secretory products in membrane bound granules (chromaffin granules). Upon chromaffin cell stimulation, the granule contents are released to the cell exterior by exocytosis. Due to

similarities between stimulus - secretion - coupling and excitation - contraction coupling in muscle, Poisner and Trifaró in 1967 proposed that the secretory process in the chromaffin cell might be mediated by contractile elements (cytoskeleton) either associated with the chromaffin granules or present elsewhere in the cell. During the past few years extensive work has been carried out on the chromaffin cell cytoskeleton and its regulatory proteins. Several such proteins , for eg. actin isoforms (Lee et al., 1979), myosin (Trifaró and Ulpian, 1976), α-actinin (Bader and Aunis, 1983), tropomyosin isoforms (Côté et al., 1984), neurofilament subunits (Trifaró et al., 1982), calmodulin (Kuo and Coffee, 1976) and calmomdulin binding proteins (Sobue et al., 1983; Bader et al., 1985) and gelsolin (Trifaró et al., 1985a; Bader et al., 1986) have been isolated and characterised. From work carried out more recently it has become clear that the viscosity of the cytosol is very important in the control of release reaction of secretion (Trifaró et al., 1985a ; 1985b ; Trifaró et al., 1988; Trifaró et al., 1989). Work from our as well as other laboratories seem to suggest that a network of actin, which is placed below the plasma membranes, is very important in controlling these events. The early work of Fowler and Pollard (1982) showed that when chromaffin granule membranes are mixed with F -actin, an increse in viscosity of the solution is observed and gel formation occurs: this effect is blocked when the calcium concentration of the medium is increased. Also

electron microscopy studies have shown chromaffin granules as well as neurohypophysial granules, to be embedded within a filamentous network in intact cells (Kondo et al., 1982, 1983). Recent experiments on intact chromaffin cells has demonstrated that stimiulation causes a 50% decrease in cytosolic, filamentous actin (Cheek and Burgoyne, 1986; Trifaró et al., 1989). This decrease is accompanied by a disappearance of the cortical (subplasmalemmal region) network of actin in these cells. If the cells are allowed to recover, the cortical network returns and F-actin values reach normal levels. These observations are consistent with a model in which cytoskeleton, by controlling the cell viscosity is able to regulate granule movement. Under resting conditions (10^{-8} M Ca^{2+}) actin filaments form a network which seems to oppose the movement of secretory granules towards the releasing sites ion the plasma membrane. When stimulation occurs and intracellular calcium rises (10^{-6} - 10^{-5} M Ca^{2+}), these actin filaments are disrupted, the cell viscosity decreases and granules are then free to move to the periphery (Trifaró et al.,1985a,1985b ; Trifaró et al., 1988). In this regard, the cytosolic proteins gelsolin (Trifaró et al., 1985a; Bader et al., 1986) and scinderin upon activation by Ca^{2+} cause a shortening of in the actin filament networks in vitro. Immunohistochemistry studies also revealed that both proteins are principally located in the subplasmalemma region close to the F-actin meshwork and this may enable them to effectively sever the

filaments of F-actin thereby releasing the trapped granules following a rise in the intracellular calcium concentration. The two proteins are activated by calcium concentrations in the order of 10^{-7} - 10^{-6} M. These are the calcium concentrations reached in the cells during cell stimulation. Also consistent with this hypothesis is the observation that agents which destabilise F-actin, such as cytochalasin D and DNAse 1 promote calcium stimulated release in permeabilised chromaffin cells and F-actin stabilisers such as phalloidin produce the opposite effect (Lelkes et al., 1986 ; Sontag et al., 1988). This is of course, an over-simplified picture of what might be the fine regulation of exocytosis in which intervention of other messengers and modulators such as calmodulin (Kenigsberg and Trifaro' 1985), cyclic AMP (Cheek and Burgoyne ,1987), G-protein induced actin ribosylation (Matter et al., 1989) etc. might take place.

F.2. Scinderin: its possible role in secretion:

Research work on scinderin is still at its infancy and no definite conclusions can be drawn regarding the functional role of the protein in the cell. However the presence of the protein in the different types of secretory tissues tested as well as the fact that it is not ubiquitous like gelsolin suggest that scinderin may be involved in the secretory function in the cell. The participation of scinderin in stimulus-secretion coupling could be determined by direct procedures such as introducing scinderin specific antibodies into the chromaffin

cell and determining the effect on secretory function following agonist stimulation or indirectly by immunohistochemistry by checking for reorganisation in the immunofluoresence of scinderin following cell stimulation.

Catecholamines are released from the chromaffin cell by exocytosis, a mechanism that follows the movement of secretory vesicles to the plasma membrane and involves the fusion of secretory granule membranes with cell plasma membrane and the subsequent extrusion of the soluable granule content to the exterior (Trifaró et al., 1975). The possible mechanisms underlying such events have already been addressed to in the introduction. As referred to in the introduction exocytosis has now been widely accepted as the mechanism for release of secretory granule contents. If scinderin is proven to be involved in the secretory response by any of the different methods, then most probably it will be functioning synergistically with gelsolin.

G. CONCLUDING REMARKS

The exact mechanism underlying the secretory process in cells is not yet clearly known. However the morphological and biochemical data available from the study of chromaffin cells suggest that actin based cytoskeleton in nonmuscle cells may undergo dynamic assembly and disassembly as the physiologic state of the cell changes. Recent studies have described proteins that interact with actin in vitro and could regulate the gel-sol state of the cytoplasm either by cross-linking, bundling, capping or severing actin filaments (Pollard and Cooper ,1986). The activity of several of these proteins is calcium dependent. The rise in intracellular calcium levels preceding exocytosis (Livett, 1984, Trifaró, 1977) may regulate the interactions of the actin filaments with actin binding proteins. From the data presented it could be proposed that scinderin too may play a role in regulating the dynamic state of the cytoskeleton in a calcium dependent manner. However it is too early to propose a functional role for scinderin as a wider knowledge of its structure-function relationship as well as its mode of regulation is required to understand its function in the cells.

The results clearly demonstrate that scinderin and gelsolin are two distinct proteins found to co-exist in the same cell in a variety of tissues. They may be regulated differently while

their actions may be synergistic. Scinderin and gelsolin may participate in the calcium dependent interactions between chromaffin granule membrane and actin filaments. However more research work is needed to demonstrate their involvement in the secretory process. Easily the most exciting approach to studying the role of scinderin in stimulus-secretion coupling will be the development of methods for selectively permeabilising the chromaffin cell plasma membrane and hence gaining direct access to the cytosol. Knight and Baker (1983) developed a method of high voltage discharge in which the cell membrane is permeated by small pores that allow the passage of small molecules , but not macromolecules and used this system efficiently to study the mechanisms of exocytosis. Since the leaky cell preparation provides an excellent in vitro assay for investigating the mechanisms of stimulus-secretion coupling the role of scinderin also could be determined by testing in the presence and absence of scinderin antibodies. This would also enable the determination of other factors which modulate the activity of scinderin. Alternatively techniques such as the micro-injection of proteins into intact cells (Trifaró and Kenigsberg, 1983) may prove useful for the introduction of antibodies specific to scinderin, and monitoring the catecholamine release might establish the participation of scinderin in the secretory process. Also recent advances in genetic engineering enable one to generate, by site directed mutagenesis different mutants of the same protein as desired, and this may provide valuable insight into the possible functional roles of

this novel actin binding protein in the secretory cell.

SUMMARY OF CONTRIBUTIONS TO THE ORIGINAL KNOWLEDGE

1. A variety of chromatographic procedures when used in combination with actin affinity chromatography enable the separate purification of both scinderin and gelsolin. In the earlier study from our laboratory the proteins were eluted together from the actin affinity column.

2. The identity of scinderin was clearly established. Although scinderin and gelsolin are calcium dependent actin binding proteins they are structurally and immunologically different.

3. Scinderin is a single polypeptide with an apparent molecular weight of 80,000 daltons.

4. The aminoacid composition of scinderin though similar to gelsolin, is not identical. The basic residues are relatively higher in scinderin when compaired to gelsolin.This dissimilarity is reflected in the isoelectric points of both proteins.

5. Scinderin interacts with two calcium ions with different affinities. The binding of calcium to scinderin is saturable.

6. Scinderin like gelsolin exhibits calcium dependent severing of

actin filaments. The relatively low levels of scinderin required to produce this effect indicates that scinderin can act as a potent modulator of actin.

7. The tissue distribution pattern suggests that there is no secretory variant of scinderin as has been observed with gelsolin. In the other tissues both scinderin and gelsolin may be co-existent. Tissues tested in addition to adrenal medulla were anterior and posterior pituitary,brain,kidney, heart and skeletal muscle, liver and blood plasma. Scinderin was present in pituitary, brain and kidney and was absent in skeletal and heart muscle, liver and plasma.

8. The localisation of scinderin in the chromaffin cell is in the subplasmalemmal region with a strong and well defined cortical fluorescence.

CHAPTER V

REFERENCES

Adelstein, R.S. and Eisenberg, E. (1980) - Regulation and kinetics of actin- myosin-ATP interaction. Ann. Rev. Biochem. 49, 921-956.

Albertini, D.F. and Herman, B. (1984) - Cell shape and membrane receptor dynamics: modulation by the cytoskeleton. Cell Muscle Motil. 5, 235-253.

Andrew, A.(1974) Further evidence that chromaffin cells are not derived from the neural crest. J. Embryol. Exp. Morphol. 31, 589-598.

Ashino, N., Sobue, K., Seino, Y. and Yabuuchi, H. (1987) Purification of an 80 kDa Ca^{2+}-dependent actin modulating protein, which severs actin filaments, from bovine adrenal medulla. J. Biochem. 101, 609-617.

Aunis, D., Guerold, B., Bader, M.F. and Cieselski-Treska, J. (1980) Immunocytochemical and biochemical demonstration of contractile proteins in chromaffin cells in culture. Neuroscience 5, 2261-2277.

Aunis, D. and Perrin, D. (1984) Chromaffin granule membrane-F-actin interactions and spectrin-like proteins in the subcellular organelles: A possible relationship. J. Neurochem. 42, 1558-1569.

Aunis, D., Bader, M.F., Langley, O.K. and Perrin, D. (1987) - Tubulin and actin binding proteins in the chromaffin cells. In Cellular and molecular biology of hormone and neurotransmitter containing secretory vesicles (Johnson,R.G. ed.), 493, 435-445.

Bader, M.F. and Aunis, D. (1983) - The 97 KDa α-actinin like protein in chromaffin granule membranes from adrenal medulla: evidence for localisation on the cytoplasmic surface and for, binding to actin filaments. Neuroscience 8, 165-181.

Bader, M.F., Georges, E., Mushynski, W.E. and Trifaró, J.M. (1984) - Neurofilament proteins in cultured chromaffin cells. J.Neurochem. 43, 1180-1193

Bader, M.F., Hikita, T. and Trifaró, J.M. (1985) - Calcium dependent calmodulin binding to chromaffin granule membranes: Presence of a 65 Kda calmodulin binding protein. J. Neurochem. 44, 526-539.

Bader, F.M., Trifaró, J.M., Langley, O.K., Thiersé, D. and Aunis, D. (1986) Secretory cell actin-binding proteins:Identification of gelsolin - like protein in chromaffin cell. J. Cell Biol. 102, 636-646.

Benchimol, S., and Cantin, M. (1977) - Ultrastructural cytochemistry of the human adrenal medulla. Histochem. 54, 9-26.

Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984) Rapid analysis of amino - acids using pre-column derivatization J. Chromatogr. 236, 93-104.

Blaschko, H. and Welch, A.D. (1953) Localisation of adrenaline in cytoplasmic particles of the bovine adrenal medulla. Naunyn-Schmiedebergs, Arch. Exp. Pathol. Pharmacol. 219, 17-22.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein - dye binding. Anal. Biochem., 72, 248-254.

Branton, D., Cohen, C.M. and Tylor, J. (1981) - Interactions of cytoskeletal proteins on the human erythrocyte membrane. Cell 24, 24-32.

Bravo, R. (1984) - Two-dimensional gel electrophoresis: In Two-Dimensional Gel Electrophoresis of proteins. Methods and Applications, (Celis J.E. and Bravo, R., eds) Academic Press Inc., New York, NY. pp. 3-36.

Bretscher, A. and Weber, K. (1979) - Villin: The major microfilament associated protein of the intestinal microvillus. Proc. Natl. Acad. Sci. USA. 76, 2321-2325.

Bretscher, A. and Weber, K. (1980) Villin is a major protein of microvillus cytoskeleton which binds to both G - actin and F - actin in a calcium dependent manner.Cell, 20, 839-847.

Bryan, J. (1988) - Gelsolin has three actin binding sites. J.Cell Biol. 106, 1553-1562.

Bryan, J.,and Kurth, M. (1984) Actin - Gelsolin interactions: evidence for two actin binding sites. J.Biol.Chem., 259, 7480 - 7487.

Buckley, I.K. (1983) - Movements in cultured chromaffin cells. In Actin Structure and Function in Muscle and Non-Muscle Cells, (dos Remedios C.G. Barden, J.A., eds) Academic Press, New York, NY. pp 211-226.

Burgoyne, R.D. and Geisow, M.J., (1981) - Specific binding of ^{125}I-calmodulin to and protein phosphorylation in adrenal chromaffin granule membranes. FEBS Lett. 131, 127-131.

Burnette, W.N. (1981) " Western blotting " Electrophoretic transfer of proteins from sodium Dodecyl Sulfate - polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Anal. Biochem.,112, 195-203.

Burridge, K. and Feramisco, J.R. (1982) - α-Actinin and vinculin from nonmuscle cells: calcium sensitive interactions with actin. Cold Spring

Harbor Symp. Quant. Biol. 46, 587-597.

Burridge, K. and Phillips, J.M. (1975) Association of actin and myosin with secretory granule membrane. Nature (London), 254, 526-529.

Byers, H.R., White, G.E. and Fugiwara K. (1984) - Organisation and function of stress fibers in cells in vitro and in situ. Cell Muscle Motil. 1, 289-333.

Caldwell, P.C. (1970) - Calcium chelation and buffers. In Calcium and Cellular Function, (Cuthbert, A.W. ed.), Macmillan and Co. Ltd., London, pp. 10-16.

Carmichael, S.W. (1979) - Secretory mechanisms. In The adrenal medulla, (Horrobin, D.F. ed.), Vol.1, 23-80.

Carron, C.P., Hwo, S., Dingus, J., Benson, D.M., Meza, I. and Bryan, J. (1986) - Re-evaluation of cytoplasmic gelsolin localisation. J. Cell Biol. 102, 237-245.

Cheek, T.R. and Burgoyne, R.D. (1986) Nicotine evoked disassembly of cortical actin filaments in adrenal chromaffin cells. FEBS Lett., 207, 110-114.

Cheek, T.R. and Burgoyne, R.D. (1987) Cyclic AMP inhibits both Nicotene - induced actin disassembly and catecholamine secretion from bovine adrenal chromaffin cell. J. Biol. Chem., 263, 11663-11666.

Cheung, W.Y. (1980) - Calmodulin plays a pivotal role in cellular regulation. Science 207, 19-27.

Cohen, W.D. and Nemhauser, I. (1985) - Marginal bands and the cytoskeleton in blood cells in marine invertebrates. In Blood cells of marine invertebrates : Experimental systems in Cell biology and comparative physiology. (Cohen, W.D., ed.) Alan R. Liss, Inc., Newyork, NY. pp 3-49.

Cooke, P. (1985) - A periodic cytoskeletal lattice in striated muscle. Cell Muscle Motil. 6, 287-313.

Cooke, P.H. and Poisner, A.M. (1979) - Role of cytoskeleton in adrenal medullary secretion . review BK # 06022, Univ. of Connecticut, ctr.HLTH. Dept.of Physiology. Farmington, CT 06032

Côté, G.P. (1983) - Structural and functional properties of non-muscle tropomyosins. Mol. Cell Biochem. 57, 127-146.

Côté, A., Doucet, J.P. and Trifaró, J.M. (1984) - Adrenal medullary tropomyosin. Int. Symp. Mol. Biol. Periph. Catechol. Stor. Tiss., 100.

Coupland, R.E. (1965) - The adrenal medulla. In: The cells in medical sciene. Vol.3, Cellular specialisation (Beck, F., and Lloyds, J.B., eds). Academic press, London.

Craig, S.W. and Pollard, T.D. (1982) - Actin-binding proteins. Trends Biochem. Sci. 7, 88-92.

De Robertis, E. and Vaz Ferreira, A. (1957) - Electron microscopic study of the excretion of catechol-containing droplets in the adrenal medulla. Exp.Cell Res. 12, 568-574.

Doucet, J.P. and Trifaró, J.-M. (1988) A discontinuous and highly porous sodium dodecyl sulfate polyacrylamide slab gel system of high resolution. Anal. Biochem., 168, 265-271.

Ebisawa, K., Maruyama, K. and Nonomura, Y. (1985) Ca^{2+} regulation fo vertebrate smooth muscle thin filaments mediated by an 84 k Mr actin-binding protein:purification and characterisation of the protein. Biomed. Research 6, 161-173.

Fernandez, J.M.., Neher, E., and Gomperts, B.D. (1984) - Capacitance measurements reveal stepwise fusion events in degranulating mast cell. Nature,(London), 312, 453.

Fowler, V.M. and Pollard, H.B. (1982) - Chromaffin granule membrane-F actin interactions are calcium sensitive . Nature 295, 336-339.

Fox, J.E.B. (1985) - The organisation of platelet contractile proteins. In Platelet Membrane Glycoproteins (George J.N., Nurden, A.T. and Phillips D.R., eds.). Plenum Publishing Corp., Newyork, N.Y. pp. 273-298.

Friedman, J.E., Lelkes, P.I., Rosenheck, K. and Oplatka,A. (1980) - The possible implication of membrane-associated actin in stimulus-secretion coupling in adrenal chromaffin cells. Biochem. Biophys. Res.Comm., 96, 1717-1723.

Fujita, T. (1977) - Concept of paraneurons. Arch. Histol. Jpn. 40(suppl), 1-12.

Fujita, T. (1980) - Paraneuron, its current implications. Biomed. Res.1 (suppl.) 3-9.

Fujita, T., Kanno, T. and Kobayashi, S. (1988) - "The Paraneuron", Springer-Verlag, Tokyo.

Glenney, J.R., Geisler, N., Kaulfus, P. and Weber, K. (1981) -

Demonstration of at least two actin binding sites in villin, a calcium-regulated modulator of F-actin organisation. J. Biol. Chem. 256, 8156-8161.

Goodman, S.R. and Zagon, I.S. (1986) - The neural cell spectrin skeleton - A review. Am. J. Physiol. 250(3), c347-c360.

Grumet, M. and Lin, S. (1981) - Purification and characterisation of an inhibitor protein with cytochalasin like activity from adrenal medulla. Biochim. Biophys. Acta 678, 381-387.

Grynszpan-Winograd, O. (1975) - Ultrastructure of the adrenal chromaffin cell. Handbook of Physiol. Sect.7, Vol. VI, 295-307.

Harris, D.A. and Schwartz, J.H. (1981) - Characterisation of brevin, a serum protein which shortens the actin filaments. Proc. Natl. Acad. Sci. USA. 78, 6798-6802.

Hasegawa, T., Takahashi, S., Hayashi, H. and Hatano, S. (1980) - Fragmin: A calcium sensitive regulatory factor on the formation of actin filaments. Biochemistry 19, 2677-2683.

Hesketh, J.E., Aunis, D. Mandel, P. and Devilliers, G. (1978) - Biochemical and morphological studies of bovine adrenal medullary myosin. Biol. Cell. 33, 199-208.

Hikita, T., Bader, M.F. and Trifaró, J.M. (1984) - Adrenal chromaffin cell calmodulin: Its subcellular distribution and binding to chromaffin granule membrane proteins. J. Neurochem. 43, 1087-1097.

Hillarp, N.A. and Hokfelt, B.(1953) - Evidence of adrenaline and noradrenaline in separate adrenal medullary cells. Acta Physiol. Scand. 30, 55-68.

Hillarp, N.A., Lagerstedt, S. and Nilson, R. (1953) - Isolation of granular fraction from suprarenal medulla, containing sympathomimetic catecholamines. Acta Physiol. Scand. 30, 55-68.

Hincke, M.T. (1988) - Conditions for improved absorption of calmodulin to nitrocellulose: Detection by ^{45}Ca binding. Electrophoresis 9, 303-306.

Holets, V. and Elde, R. (1982) - The differential distribution and relationship of serotoninergic and peptidergic fibers to sympatho adrenal neurons in the intermediolateral cell column of the rat : A combined retrograde axonal transport and immunofluorescence study. Neuroscience 7, 1155-1174.

Janmey, P.A., Chaponnier, C., Lind., S.E., Zaner,K.S., Stossel, T.P. and

Yin, H.L. (1985) - Interactions of gelsolin and gelsolin-actin complexes with actin. Effects of calcium on actin nucleation, filament severing and end-blocking. Biochemistry 24, 3714-3723.

Janmey, P.A. and Stossel, T.P. (1987) - Modulation of gelsolin function by phosphatidyl inositol-4,5-bis-phosphate. Nature, 325, 362-364.

Jones, J.C.R. and Goldman, R.D. (1985) - Intermediate filaments and the initiation of desmosome assembly. J. Cell. Biol. 101, 506-517.

Katz, B. and Miledi, R. (1969) - Tetrodotoxin resistant electric activity in presynaptic terminals. J. Physiol.(London), 203, 459-487.

Keese, W.K., Parker, T.L. and Coupland, R.E. (1988) - The innervation of the adrenal gland. I. The source of the pre and post ganglionic nerve fibers to the rat adrenal gland. J.Anatomy 157, 33-41.

Kenigsberg, R.L. and Trifaró, J.M. (1985) - Microinjection of calmodulin antibodies into cultured chromaffin cells, blocks catecholamine release in response to stimulation. Neuroscience, 14, 335-347.

Kirschner, M.W. (1980) - Implications of treadmilling for thestability and polarity of actin and tubulin polymers in vivo. J.Cell Biol. 86, 330-334.

Knight, D.E., and Baker, P.F. (1983) - The phorbol ester TPA increases the affinity of exocytosis for calcium in "leaky" adrenal medullary cells. FEBS Lett. 160, 98-100.

Knight, D.E. and Baker, P.F. (1987) - Exocytosis from vesicle view point. In cellular and molecular biology of hormone and neurotransmitter containing secretory vesicles. Ann. Newyork Acad.Sci., 493, 504-521.

Kobayashi, S. (1977) - Adrenal medulla: chromaffin cells as paraneurons. Arch. Histol. Jpn. 40,(suppl):61-79.

Kobayashi, S. and Coupland, R.E. (1977) - Two populations of microvesicles in the SGC(small granule chromaffin) cells of the mouse adrenal medulla. Arch. Histol. Jpn. 40, 251-259.

Kobayashi, R., Bradley, W.A., Bryan, J. and Field, J.B. (1983) - Identification and purification of calcium ion dependent modulators of actin polymerization from bovine thyroid. Biochemistry, 22, 2463-2469.

Kondo, H., Wolosewick, J.J.and Pappas, G.D. (1982) - The microtrabecular lattice of adrenal medulla revealed by polyetylene glycol embedding and stereo electron microscopy. J. Neurosci. ?, 57-65.

Kondo, H., Pappas, G.D. and Wolosewick, J.J. (1983) - The cytoskeletal

lattice of neurohypophysial cells. Biol. Cell. 49, 99 -108.

Korn, E. (1982) - Actin polymerisation and its regulation by proteins from nonmuscle cells. Physiol.Rev. Vol.62, No.2, 672-737.

Kuo, I.C.Y. and Coffee, C.J. (1976) - Purification and characterisation of a troponin-C like protein from bovine adrenal medulla. J. Biol. Chem. 251, 6315-6319.

Kwiatkowski, D.J., Janmey, P.A., Mole, J.E., and Yin, H.L.(1985) - Isolation and properties of two actin binding domains in gelsolin. J. Biol. Chem. 260, 15232-15238.

Lasek, R.J. (1982) - Translocation of neuronal cytoskeleton and axonal locomotion. Philos. Trans. R. Soc. Lond. b299, 313-327.
Le Douarin, N.M. (1982) - The neural crest. University Press, Cambridge, London Newyork.

Le Douarin ,N.M. and Teillet,M.A.(1973) The migraton of neural creast cells to the wall of the digestive tract in avian embryo. J. Embryol. Exp. Morphol. 30, 31-48.

Lee, R.W.H., Mushynski, W.E. and Trifaró, J.M. (1979) - Two forms of cytoplasmic actin in chromaffin cells. Neuroscience 4, 843-852.

Lee, R.W.H. and Trifaró, J.M. (1981) - Characterization of actin antibodies and their use in immunocytochemical studies on the localisation of actin in adrenal chromaffin cells in culture. Neuroscience 6, 2087-2108.

Lelkes, P.I., Friedman, J.E., Rosenbec, K. and Oplatka, A. (1986) - Destabilization of actin-filaments as a requirement for the secretion of catecholamines from permeabilised chromaffin cells. FEBS Lett., 208(2), 357-363.

Lever, J.D. (1955) - Electron microscopic observations on the normal and denervated adrenal medulla of the rat. Endocrinology 621.

Lin, S., Cribbs, D.H., Wilkins, J.A., Casella, J.F., Margagal, W.M. and Lin, D.C. (1982). The capactins, a class of proteind that cap the ends of actin filaments. Philos. Trans. R. Soc.Lond.Biol. Sci. 299, 263-273.

Lingg, G., Fischer-Colbrie, R., Schmidt, W. and Winkler, H. (1983) - Exposure of an antigen of chromaffin granules on cell surface during exocytosis. Nature 301, 610-611.

Livett, B.G. (1984) - The secretory process in adrenal medullary cells. In Cellbiology of the secretory process. M. Cantin (ed.)Karger, Basel. 310-358.

Livett, B.G., Boksa, P., Dean, D.M., Mizobe, F. and Lindenbaum, M.M. (1983). Use of isolated chromaffin cells to study basic release mechanisms. J.Auton.Nerv.Syst. 7, 59-86.

MacLean-Fletcher, S.D. and Pollard, T.D. (1980) - Viscometric analysis of the gelation of acanthamoeba extracts and purification of the two gelation factors. J. Cell Biol. 85, 414-428.

Matter, K., Dreyer, F. and Aktories, K. (1989) - Actin involvement in exocytosis from PC 12 Cells:Studies on the influence of Botulinum C2 toxin on Stimulated noradrenaline release. J. Neurochem., 52, 370-376.

McIntosh, J.R. (1982) - Mitosis and the cytoskeleton. In Developmental Order: Its Origin and Regulation, (Alan R. Liss, ed.), New York, NY. pp 77-115.

McPherson, G.A. (1983) - A practical computer based approach to the analysis of radioligand binding experiments.Comput. Programs Biomed. 17, 107-114.

Nagata, K. and Ichikawa, Y. (1984) - Changes in actin during cell differentiation. Cell Muscle Motil. 5, 171-193.

O'Farrell, P.Z., Goodman, H.M. and O'Farrell, P.H. (1977) - High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell, 12, 1133-1142.

Oliver, J.M. and Berlin, R.D. (1983) - Surface and cytoskeletal events regulating leukocyte membrane topography. Semin. Haematol. 20, 282-304.

Oliver, J.M., Senecal, J.L. and Rothfield, N. (1985) - Autoantibodies to the cytoskeleton in the human sera. Cell Muscle Motil. 6, 55-74.

Ornberg, R.L. and Reese, T.S. (1981) - Beginning of exocytosis captured by rapid freezing of Limulum amoebocytes. J. Cell Biol., 90(1), 40-54.

Overall, C.M. (1987) - A microtechnique for dialysis of small volume solutions with Quantitative recoveries. Anal. Biochem. 165, 208-214.

Pardee, J.D. and Spudich, J.A. (1982) - Purification of muscle actin. Methods in Cell Biol., 24, 271-289.

Payne, M.E. and Rudnick, S.E. (1985) - Tropomyosin: structural and functional diversity. Cell Muscle Motil. 6, 141-184.

Pearse, A.G.E.(1969) The cytochemistry and ultrastructure of polypeptide hormone binding cells of the APUD series and the embryologic, physiologic and patologic implications of the

concept. J.Histochem cytochem. 17, 303 - 313.

Perrin, D. and Aunis, D. (1985) - Reorganisation of α-Fodrin induced by stimulation in secretory cells. Nature 315, 589-592.

Petrucci, T.C., Thomas, C. and Bray, D. (1983) - Isolation of a Ca^{2+} dependent actin fragmenting protein from brain, spinal cord and cultured neurones. J. Neurochem. 40, 1507-1516.

Phillips, J.H. (1987) - The structure and dynamics of chromaffin granules. In Stimulus-Secretion Coupling in Chromaffin Cells, (K.Rosenheck and P.I.Lelkes, eds.,), CRC Press, Boca Raton, Florida, pp 55-86.

Phillips, J.H., Burridge, K., Wilson, S.P. and Kirschner, N. (1983) - Visualization of the exocytosis/endocytosis secretory cycle in cultured adrenal chromaffin cells. J. Cell Biol., 97, 1906-1917.

Poisner , A.M., Trifaró, J.M. and Douglas, W.W. (1967) - The fate of chromaffin granule during catecholamine release from the adrenal medulla. II Loss of protein and retention of lipid in subcellular fractions. Biochem. Pharmacol. 16, 2101-2108.

Pollard, T.D. and Cooper, J.A. (1986) - Actin and actin binding proteins : A critical evaluation of mechanisms and functions. Ann. Rev. Biochem. 55, 987-1035.

Pollard, T.D., Levy, J., Isenberg, G. and Aebi, U. (1980) - Two new acanthamoeba proteins involved with regulation of actin filament cross-linking. J. Cell Biol. 87,(2, Pt 2): 223a.(Abstr.)

Pollard, T.D. and Moosekar, M.S. (1981) - Direct measurement of actin polymerisation rate constants by electron microscopyof actin filaments nucleated by isolated microvillus cores. J. Cell Biol. 88, 654-659.

Potter, J.D., Strang-Brown, P., Walker, P.L. and Iida, S. (1983) Methods in Enzymology, 102, 135-143.

Prentice, F.D. and Wood J.G. (1985) - Adrenergic innervation of cat adrenal medulla. Anat.Rec. 181, 689-703.

Scatchard, G. (1949) Ann. N.Y. Acad. Sci., 51, 660-672.

Smith, A.D., and Winkler., H. (1972) - Fundamental mechanisms in the release of catecholamines. In Handbuch der experimentelle Pharmakologie, Vol 33, Catecholamines (H.Blaschko and E.M. Uscholl, eds.). Springer-Verlag, Newyork, 1972.

Sobue, K., Kanda,K., Adachi, J. and Kakiuchi, S. (1983) - Calmodulin

binding proteins that interact with actin filaments in a flip-flop manner: Survey in brain and secretory tissues. Proc. Natl. Acad. Sci. U.S.A. 80, 6868-6871.

Sobue, K., Muromoto, Y., Yamazaki, R. and Kakiuchi, S. (1985) - Ca^{2+} and calmodulin regulate microtubule associated protein-actin filament interaction in a flip-flop switch. BIochem. Biophys. Acta 845, 366-372.

Sontag, J.M., Aunis, D. and Bader, M.F. (1988) - Peripheral actin filaments control calcium mediated catecholamine release from stereptolysin O - permeabilised chromaffin cells. Eur. J. Cell Biol.46, 316-326.

Stossel, T.P., Chaponnier, C., Ezzell, R.M., Hartwig, J.H., Janmey, P.A., Kwiatkowski, D.J., Lind, S.E., Smith, D.B., Southwick, F.S., Yin, H.L. and Zaner, K.S. (1985) - Nonmuscle actin binding proteins. Ann. Rev. Cell Biol.1, 353-402.

Szent-Gyorgyi, A. (1951) - Chemistry of muscular contraction. Academic Press Inc. New York, NY. 148-151.

Taylor, D.L. and Condeelis, J.S.-(1979) - Cytoplasmic structure and contractility in amoeboid cells Int. Rev. Cytol. 56, 57-144.

Towbin, H., Staehelin, T. and Gordon, J. (1979) - Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. Proc. Natl. Acad. Sci. U.S.A.76, 4350-4354.

Trifaró, J.M. (1977) - Common mechanisms in hormone secretion. Annu. Rev. Pharmacol. Toxicol.17, 27-47.

Trifaró, J.M. (1982) - The cultured chromaffin cell: a model for the study of biology and pharmacology of paraneurones. Trends.Pharmacol.Sci. 3, 389-392.

Trifaró, J.M., Bader, M.F. and Doucet, J.P. (1985a) - Chromaffin cell cytoskeleton; its possible role in secretion. Can. J. Biochem. Cell Biol. 63, 661-679.

Trifaró, J.M., Bader, M.F., Côté, A., Kenigsberg, R.L., Hikita, T. and Lee, R.W.H. (1985b) In Alia, E.E., Arena, N. and Russo, M.A. (eds.), Contractile Proteins in Muscle and Non-Muscle Cell Systems: Biochemistry, Physiology and Pathology. Praeger Scientific, Philadelphia, pp. 459-472.

Trifaró, J.M. and Cubbedu, X.L. (1979) - Exocytosis as a mechanism of noradrenergic transmitter release.In Trends. Aut. Pharm. (Kalsner,

ed.),Urban and Schwarzenber, Baltimore, 195.

Trifaró, J.M. and Fournier, S. (1987) - Calmodulin and the secretory vesicle. In Cellular and Molecular Biology of Hormone and Neurotransmitter Containing Secretory Vesicles. Ann. N.Y. Acad. Sci.493, 417-434.

Trifaró, J.M., Fournier, S. and Doucet, J.P. (1988) - Calmodulin and the cytoskeleton in secretion. In Thorn, N.A., Treiman, M. and Petersen, O.H. (eds.), Molecular Mechanisms in Secretion. Alfred Benzon Symposium 25, Munksgaard, Copenhagen, pp. 632-651.

Trifaró, J.M. and Kenigsberg, R.L. (1983) - Microinjection of calmodulin antibodies (CM-AB) into chromaffin cells(CC) provides direct evidence for a role of calmodulin(CM) in the secretory process(SP). Fed.Proc., 42, 456.

Trifaró, J.M., Kenigsberg, R.L., Côté, A., Lee, R.W.H. and Hikita, T. (1984) - Adrenal paraneuron contractile proteins and stimulus-secretion coupling. Can. J. Physiol. Pharmacol. 62, 493-501.

Trifaró, J.M. and Lee, R.W.H. (1980) - Morphological changes and stimulus-secretion coupling in bovine adrenal chromaffin cell cultures. Neuroscience, 5, 1533-1546.

Trifaró, J.M., Lee, R.W.H., Kenigsberg, R.L. and Côté, A. (1982) - Synthesis, storage, and secretion of adrenal catecholamines. In Advances in the Biosciences,(Izumi, F., Oka, M. and Kumakura, K. eds.) Vol. 36. Pergamon Press, Oxford, pp. 151-158.

Trifaró, J.M., Novas, M.L., Fournier, S. and Rodriguez Del Castillo, A. (1989) - Cellular and molecular mechanisms in hormone and neurotransmitter secretion. In Proc. XII Latin-American Congress of Clinical Pharmacology and Therapeutics, (Israel, A. and Valazco, M. eds.) . Caracas, Oct. 2-7, 1988. Elsevier Science Publishers BV, The Netherlands

Trifaró, J.M. and Poisner, A.M. (1982) - Common properties in the mechanisms
of synthesis,processing and storage of secretory products. In "The secretory granule".The secretory process ,(Trifaró, J.M. and Poisner, A.M. eds.), Vol.1. Elsevier Biomedical press,Amsterdam. 387-403.

Trifaró, J.M. and Ulpian, C. (1975) - Actomyosin like protein isolated from the adrenal medulla. FEBS Lett. 57, 198-202.

Trifaró, J.M. and Ulpian, C. (1976) - Isolation and characterisation of myosin from adrenal medulla. Neuroscience 5, 1533-1546.

Trifaró, J.M., Ulpian, C. and Preiksaitis H. (1978) - Anti-myosin stains chromaffin cell. Experientia 34, 1568-1571.

Tucker, R.P., Edwards, B.F. and Erickson, C.A. (1985) - Tension in the culture dish: microfilament organization and migratory behaviour of quail neural crest cells. Cell Motil. 5, 225-237.

Ulpian, C. (1977) - Contractile proteins of the adernal medulla. Ph.D. Thesis (McGill University), Montréal, Canada.

Unsicker, K. (1976) - Comparative ultrastructural aspects of adrenal chromaffin cells in reptiles. In Chromaffin, Enterochromaffin and related cells, (Coupland, R.E., and Fugita, T. eds.). Elsevier Scientific Press, Amsterdam.

Vale, R.D., Reese, T.S. and Sheetz, M.P. (1985a) - Identification of a novel force generating protein, Kinesin, involved in microtubule based motility. Cell 42, 39-50.

Vale, R.D., Schnapp, B.J., Reese, T.S. and Sheetz, M.P. (1985b) - Organelle, bead, and microtubule translocations promoted by soluble factors from the squid giant axon. Cell 40, 559-569.

Vanderkerckhove, J. and Weber, K. (1979) - Atleast six different actins are expressed in a higher mammal: an analysis based on the mainoacid sequence ofn the amino terminal tryptic peptide. J. Mol. Biol. 126, 783-802.

Vanderkerckhove, J. and Weber, K. (1980) - Vegetative Dictyostelium cells containing 17 actin genes express a single major actin. Nature 284, 475-477.

Viveros, O.H., Arqueros, L. and Kirschner, N.(1968) - Release of catecholamines and dopamine β oxidase from adrenal medulla. Life Sci. 7, 609-618.

Wang, K., Ash, J.F. and Singer, S.J. (1975) - Filamin, a new high molecular weight protien found in smooth muscle and non muscle cells. Proc. Natl. Acad. Sci. U.S.A., 72, 4483-4486.

Wang, L.L. and Bryan, J. (1981) - Isolation of calcium-dependent platelet proteins that interact with actin. Cell 25, 637-649.

Wang, L.L. and Spudich, J.A. (1984) - A 45,000 molecular weight protein from unfertilised sea urchin eggs, severs aactin filaments in a calcium dependent manner and increases the steady state concentration of non-filamentous actin. J. Cell Biol., 99, 845-851.

Weber, K. and Osborn, M. (1982) - The cytoskeleton. Natl. Cancer Inst.

Monogr. 60, 31-46.

Weeds, A.G. (1982) - Actin-binding proteins - regulators of cell architecture and motility. Nature (London), 296, 811-816.

Weeds, A.G., Harriet, H., Gratzer, W. and Gooch, J.(1986) - Interactions of pig plasma gelsolin with G-actin. Eur.J.Biochem. 161, 77-84.

Wilkins, J.A. and Lin, S. (1981) - Association of actin with chromaffin granule membranes and the effect of cytochalasin B on the polarity of actin filament elongation. Biochim. Biophys. Acta 642, 55-66.

Yamamoto, K., Pardee, J.D., Reidler,J., Stryer, L., and Spudich, J.A. (1982) - Mechanism of interaction of Dictyostelium severin with actin filaments. J. Cell Biol., 95, 711-719.

Yin, H.L. (1988) - Gelsolin: Calcium and Polyphosphoinositide- Regulated actin- modulating protein. BioEssays 7, 176-179.

Yin, H.L., Albrecht, J.H. and Fattoum, A. (1981) - Identification of gelsolin, a calcium dependent regulatorry protein of actin gel-sol transformation and its intracellular distribution in a variety of cells and tissues. J. Cell Biol. 91, 901-906.

Yin, H.L., Ilida, K. and Janmey, P.A. (1988) - Identification of a phosphoinositide - regulated domain which mediates binding of gelsolin to the side of actin filaments prior to severing. J. Cell Biol. 106, 805-819.

Yin, H.L. and Stossel, T.P. (1979) - Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein . Nature 281, 583-586.

Yin, H.L. and Stossel, T.P. (1980) - Purification and structural properties of gelsolin, a calcium activated regulatory protein of macrophages. J. Biol. Chem. 255, 9490-9493.

Zackroff, P.V., Steinert, P.M., Anyardi-Whitman, M. and Goldman, R.D. (1981) -Intermediate filaments. In Cytoskeletal Elements and Plasma Membrane Organisation.(Poste, G. and Nicolson, G.L., eds). Elsevier/North-Holland, New York, NY. 55-97.