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MOLECULAR CLONING, FUNCTIONAL EXPRESSION AND CHARACTERIZATION OF  
SCINDERIN, A  $Ca^{2+}$ -DEPENDENT ACTIN-FILAMENT SEVERING PROTEIN  
PRESENT IN SECRETORY TISSUES

(C) MONICA GIANINA MARCU

Thesis submitted to the School of Graduate Studies and Research  
in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy

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September 1997



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## ABSTRACT

Chromaffin cells and platelets store amines in dense core vesicles whose contents are released by exocytosis. In chromaffin cells and probably in platelets, the cortical filamentous (F)-actin cytoskeleton represents a negative control for secretion, therefore it has to be locally disassembled (depolymerized) to allow vesicles movement toward the plasma membrane and exocytosis. Search for factors which might be involved in the regulation of F-actin networks resulted in the discovery in our laboratory of **scinderin (Sc)**, a  $\text{Ca}^{2+}$ -dependent F-actin severing protein present in tissues with high secretory activity. Sc is mainly cortically localized, it has two  $\text{Ca}^{2+}$ -binding sites ( $K_d$   $5.9 \times 10^{-7}\text{M}$ ,  $B_{\text{max}}$  0.8 mol  $\text{Ca}^{2+}$ /mol protein;  $K_d$   $2.8 \times 10^{-6}\text{M}$ ,  $B_{\text{max}}$  1.9 mol  $\text{Ca}^{2+}$ /mol protein), and interacts with actin and  $\text{PIP}_2$ . Its activity is regulated by  $\text{Ca}^{2+}$ , pH and  $\text{PIP}_2$ . Here we report the molecular cloning and sequence elucidation of Sc, identification and localization of two  $\text{PIP}_2$  and three actin binding sites.

Nucleotide and amino acid sequence analysis indicates that Sc has 6 domains, each containing 3 internal sequence motifs, 63% and 53% homology with gelsolin and villin, respectively, two other F-actin severing proteins. Sc has three actin binding sites, two of each in domains 1 and 2, two  $\text{PIP}_2$  binding sites with the same distribution. These two domains of Sc are of great importance for  $\text{Ca}^{2+}$ -dependent F-

actin severing activity and thus for regulation of exocytosis. A truncated form of Sc ( $Sc_{3-6}$ ) devoid of the putative actin binding sites had no effect on  $Ca^{2+}$ -induced exocytosis but nevertheless, it could bind to actin in a  $Ca^{2+}$ -independent manner. This fact led to the discovery of another important actin binding site in the  $NH_2$  terminal of domain 5 of Sc. This actin binding site is involved in nucleating actin polymerization and does not contribute directly to the severing activity of scinderin, though, its presence in the molecule is necessary for proper positioning of the active severing sites of the domains 1 and 2 of scinderin when binding actin.

Functional studies involving recombinant Sc indicated that this protein potentiates  $Ca^{2+}$ -induced exocytosis in both, digitonin-permeabilized chromaffin cells and platelets. Recombinant  $Sc_{1-715}$  potentiated  $Ca^{2+}$ -induced F-actin disassembly and exocytosis in permeabilized chromaffin cells and platelets, an effect blocked in the presence of peptides Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub> (with sequences corresponding to the first two actin binding sites of Sc), exogenous  $\gamma$ -actin or the addition of PIP<sub>2</sub>. The inhibitory effect of PIP<sub>2</sub> was blocked by peptide Sc-PIP<sub>2</sub>BP (with the sequence corresponding to a PIP<sub>2</sub>-binding site present in domain 1 of Sc).

The results suggest that Sc-evoked cortical F-actin disassembly is required for secretion and that Sc is an important component of the exocytotic machinery.

## DEDICATION

To my beloved husband, Mihai, for his continuous understanding, encouragement and patience, to my wonderful mother Armida and in memory of my dear father Vasile, to my sister Doina Manuela and my nephew Bogdan and all my friends and relatives who supported and encouraged me along this difficult but fulfilling path of scientific and self discovery. I love you all !

*If I can stop one heart from breaking,  
I shall not live in vain;  
If I can ease one life the aching,  
Or cool one pain  
Or help one fainting robin  
Unto his nest again,  
I shall not live in vain.*

*Emily Dickinson*

*(1830-1886)*

## AKNOWLEDGEMENTS

First and foremost, I would like to express my appreciation to Dr. J-M Trifaró for his thoughtful supervision, advice and for allowing me to do science in his laboratory. I am grateful for all the excellent workshops he allowed me to attend, for the freedom of decision and expression that he always granted me, without which I could have never fully developed my scientific skills. I "acknowledge" his humor and unbelievable but predictable "talent" to stress me beyond the word in most situations.

I extend my appreciation to members of my graduate committee, namely Drs. Cristine Pratt, Martin Tenniswood and John Bell for their advice and direction whenever needed.

I am eternally grateful to Dr. I.Skerjanc for her advice and guidance, for never saying "I am busy now" when I needed her. I also thank all members of the Ottawa Cancer Research Group, among them R.Marius, M.Dube, J.Coulombe for their kindness and help.

I would like to thank from all my heart Drs. Pavel Hrdina, Mary Morris and Balwant Tuana for their trust, advice and support.

Special thanks to Dr. Paul Cizdziel (BRL, LTI Training Center, Germantown, MD) for the job offer and his friendship; his excellent teaching skills have helped me understand molecular biology. I also acknowledge and thank Dr. N.Nelson (Roche Institute of Molecular Biology, Nutley, NJ) for useful comments and for providing us with the primers for sequencing.

I am grateful to Dr. A.Rodriguez Del Castillo for his humor, guidance and real help during the tedious work of sequencing

scinderin cDNA, to Dr. M.L. Vitale for her peaceful and wise presence, for her advice and friendship. I appreciate the help and great collaboration with Dr. Li Zhang during the last three years, his financial and business advice that turned me into an eager (though not rich, yet) investor.

Countless thanks to Ms. Sara Dunn for her perfect typing, warm and sincere smiles, great help and understanding, patience and just for existing in our department; it was a privilege to meet and work with her. Many thanks to all our secretaries, C.Lalonde, D.Drapeau and A. Peetsma for their professional presence and help, we were indeed lucky to have you around.

I am grateful to Ms. Rainy Tang for her advice, for technical assistance in the preparation of chromaffin cell cultures.

I would also like to thank Mr. A.Bergeron in Biochemistry Dept. for his technical assistance and for the high quality of the primers provided. My gratitude goes as well to Mrs. Simon Nsonwah and Pierre Drouin for their friendship and technical support. I am grateful to all my friends, especially to my dear Isabela Roy, and colleagues from the Pharmacology department as well as the Ottawa University.

I acknowledge the substantial financial support from the Medical Research Council of Canada and the School of Graduate Studies, University of Ottawa. I am grateful to the wonderful Canadian people for all I have experienced, for all I was granted since I live in this country.

## PREFACE

All the molecular biology (from construction of the cDNA library and different truncations of scinderin to the PCRs, sequencing of scinderin cDNA and expression of the different proteins) and most of the other biochemical data (all the purification methods, study of the scinderin-actin and -PIP<sub>2</sub> interactions, scinderin properties, and part of immunohistochemistry work, etc) presented in this thesis are entirely my own work. Dr. Antonio R. Del Castillo has sequenced about 25% of the scinderin cDNA. The release experiments in platelets and chromaffin cells were performed in collaboration with Dr. Li Zhang. Part of the immunohistochemistry and video-enhanced image processing work, were performed together with Dr. Maria L. Vitale, Ms. Rainy Tang and Dr. Kirstin N. Staudt.

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## LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
bp	base pairs
Ca <sup>2+</sup>	cytosolic free calcium
DMEM	Dulbecco's modified Eagle's medium
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FITC	fluoresceine iso-thiocyanate
GST	glutathione S-transferase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Ig	immunoglobulin
IPTG	isopropyl-B-D-thiogalactopyranosid
Kb	kilobase pairs
KDa	kilodaltons
mRNA	messenger ribonucleic acid
NA	noradrenaline
RNase	ribonuclease
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PI	phosphatidyl inositol

PIP <sub>2</sub>	phosphatidyl inositol 4,5 bis-phosphate
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethyl-sulphonylfluoride
TRIS	tris (hydroxymethyl) aminomethane
TRX	thioredoxin

**CHAPTER 1**  
**INTRODUCTION**

## 1.1 ADRENAL MEDULLA AND ADRENAL CHROMAFFIN CELLS

Adrenal medulla is the core of the adrenal glands (Nagel, 1886), which can be defined as endocrine, ductless glands, as well as a part of the sympathetic nervous system (one of the two branches of the autonomic nervous system).

The autonomic nervous system is the portion of the nervous system that controls the visceral functions of the body; it is activated mainly by centers located in the spinal cord, brain stem and hypothalamus. The autonomic signals are transmitted to the body through two major subdivisions: the sympathetic and parasympathetic systems.

The sympathetic nerves originate in the spinal cord between the segments T1 and L2 and pass from here to the sympathetic paravertebral chains (containing ganglia), and then to the organs that are innervated by the sympathetic nerves (DeQuatro, 1979; Guyton, 1987; Keese et al., 1988). Each sympathetic pathway is comprised by two fibers: a preganglionic and a postganglionic neuron. The sympathetic fibers originating in different parts of the spinal cord are distributed to various organs in the body. Some preganglionic sympathetic nerve fibers pass from the intermediolateral horn cells of the spinal cord, through the sympathetic chains, through the splanchnic nerves into the **adrenal medullae** (in adrenal glands), directly on special cells that secrete epinephrine / adrenaline and norepinephrine / noradrenaline

(catecholamines) straight into the blood (Fujita, 1977; Guyton, 1987; Landsberg, 1980). These secretory cells are derived embryologically from nervous tissue (from neural crest), they are analogous to postganglionic neurons (Fujita, 1977; Kobayashi and Coupland, 1977; Pearse, 1969;). They also have rudimentary nerve fibers, it is these fibers that secrete the hormones (Prentice and Wood, 1975) .

- The adrenal glands are paired structures localized anterior or medial to the kidney, in quadrupeds. In Latin, adrenal means adjacent to the kidney. In humans, suprarenal glands would be the proper name due to the fact that they lie on the top of the kidneys at the level of the 11-th or 12-th vertebra (embedded in adipose tissue and encapsulated by the renal fascia) (DeQuatro, 1979). The medulla (in Latin, marrow) is the central part of the gland and it is surrounded by an adrenal cortex. The correct anatomical name is glandula suprarenalis medulla (DeQuatro, 1979; Guyton, 1987). The adrenal medulla is an endocrine gland, it affects other organs by discharging hormones into the blood, but it can also be seen as part of the sympathetic nervous system; the main adrenal hormone, adrenaline, is closely related to noradrenaline, the main sympathetic neurotransmitter (Benchimol and Cantin, 1977; DeQuatro, 1979; Landsberg, 1980). Furthermore, the adrenal medulla itself secretes noradrenaline, some dopamine as well as some neuropeptides

(Hillarp, 1953; Landsberg, 1980; Unsicker, 1976).

The combined weight of the two human glands is about 1 g. The central part of the gland, medulla, comprises mainly of chromaffin cells (historically, they have been characterized to react and produce a colour with dichromat salts - Kohn, 1902) which look like irregularly shaped polyhedrons. These cells are surrounded by nerves, connective tissue and blood vessels. Inside the chromaffin cells, the catecholamines are stored in numerous chromaffin granules (100-300 nm diameter), which are electron-dense vesicles resembling the dense core vesicles of sympathetic nerve endings (Blaschko et al., 1953; Hillarp et al., 1953). A variety of putative noncatecholamine mediators have been identified in adrenomedullary chromaffin cells (Benchimol et al., 1977). The cells contain one or the other of the catecholamines in the vesicle stores. There are three main types of chromaffin cells : noradrenaline (NA - representing 15% of the cells), adrenaline (A - representing 85% of the cells) and dopamine containing cells (about 1%) (Benchimol and Cantin, 1977; Hillarp et al., 1953; Landsberg, 1980) .

The arteries to the adrenal glands are numerous: the superior adrenal arteries are derived from the inferior phrenic artery, the middle artery comes from the aorta above the origin of the renal artery, and the inferior adrenal artery arises from the renal artery. The main venous drainage is via the adrenal vein, arising from the the central vein

within the gland (DeQuatro, 1979; Yeasting, 1986).

- **Chromaffin cells** of adrenal medulla are arranged in groups separated by connective tissue, around blood vessels (Carmichael, 1979). Isolated cells are spherical, with a diameter of 10-20  $\mu\text{m}$  and a nucleus of 5  $\mu\text{m}$ . In 1955, Lever was the first to study the adrenal medulla at the electron microscope level. He demonstrated the presence of electron-dense membrane bound vesicles as a characteristic feature of these cells. The chromaffin vesicles (often called "chromaffin granules", a term hinting to the image in the light microscope) were the first secretory organelle to be isolated (Blaschko et al., 1953; Hillarp et al., 1953). The discovery of these vesicles characterized for the first time in endocrine systems a mode of storage which is now known to be common to most of the secretory cells. There are between 17,000 and 30,000 vesicles per cell (Nordmann, 1984; Vitale et al, 1995). Eranko (1955) distinguished adrenaline containing vesicles from the noradrenaline (more dense) containing vesicles. In most mammals, A-containing cells comprise 80-90% of the chromaffin cell population, with large species variations (Benchimol and Cantin, 1977). As mentioned above, biochemical data, together with morphological observations have demonstrated the presence of three types of secretory granules, subsequently three types of chromaffin cells: A-containing cells, NA-containing cells and small granule

chromaffin cells (SGC) (Hillarp et al., 1953; Unsicker, 1976). The first two types are represented by the large dense-core vesicles (750-1,000 Å), related to the LDCV of the sympathetic neurons, while SGC is comprised of the small dense-core vesicles (400-500 Å), also found in sympathetic nerves (Trifaró et al., 1992). Electron-translucent vesicles have been described in chromaffin cells (Navone et al., 1989) but their function is not clear yet. At the electron microscopy level, SGC (which contain dopamine) seem to look as they would contain both A and NA (Coupland et al., 1989).

## 1.2 CULTURED CHROMAFFIN CELLS AS A MODEL FOR NEUROSECRETORY STUDIES

A major breakthrough in understanding the neuronal function, endocrine mechanisms and stimulus-secretion coupling was the development of the techniques to isolate and culture the adrenal chromaffin cells. Once isolated, these cells provide a relatively homogenous population for the study of secretion by morphological, biochemical and electrophysiological methods (Douglas et al., 1967; Trifaró et al., 1978). Adrenal chromaffin cells store their secretory products in membrane-bound organelles. Upon stimulation by acetylcholine, a cascade of events is triggered in the cell, leading to the secretion of catecholamines and other vesicle components by

exocytosis. Many of the important aspects of these mechanisms are still poorly understood. The possibility of isolating and maintaining chromaffin cells in culture for long periods of time, has provided a better opportunity to study the mechanisms of membrane fusion during exocytosis, site of action of cellular  $Ca^{2+}$ , fate and origin of secretory vesicles, turnover of membrane and receptor proteins, etc (Douglas, 1975; Trifaró et al., 1980). For the neuroscientist, the chromaffin cells have also the advantage of sharing primordial origin with sympathetic neurons, even more, they have some common properties with neurons (voltage-dependent  $Na^+$  and  $Ca^{2+}$  channels, neuronal specific proteins, both chromaffin cells and sympathetic neurons synthesize, store and release catecholamines in response to cholinergic stimulation, etc) as well as with the endocrine cells (Fujita, 1977; Kobayashi and Coupland, 1977). Another advantage of chromaffin cells is their availability in relatively pure form after isolation from the adrenal gland, unlike the sympathetic neurons which are difficult to isolate (being scattered through the whole organism) and study. In other words, adrenal chromaffin cells are a great practical laboratory model of neurons.

Different research groups have described methods for isolation and culturing chromaffin cells of various origins (Douglas et al., 1967; Hochman et al., 1976; Brooks, 1977; Trifaró et al., 1978, 1980). There are significant species differences between chromaffin cells, however, most of the

work in this type of cells was carried on bovine-derived cultures. This is the best studied system and most of the literature deals with data originated here from. In our laboratory, we also use a bovine-derived, primary chromaffin cell type of culture developed by Trifaró and Lee, 1980. Chromaffin cells change functionally during time in culture, they begin to develop processes (neurite-like in nature) after several days, therefore, they should be used after short culture periods. The lengths of these processes are linearly related to the age of the culture; the axon-like processes display varicosities containing catecholamines (Trifaró and Lee, 1980). Another morphological feature of bovine chromaffin cell culture is the presence of body-body or process-body cell contacts (Trifaró and Lee, 1980). The relevance of this phenomenon to the formation of new synapses, for instance, remains to be elucidated.

A large number of technical approaches are enabling us to examine the mechanisms of exocytosis in chromaffin cells in culture. Here there are some of them:

- single cell **membrane capacitance** measurements with the **patch-clamp** method for determining the extent of membrane fusion in exocytosis (Neher et al., 1982) and / or  $Ca^{2+}$  transients measurements (Penner et al., 1988), as well as single cell **video imaging** of topographical dynamics of  $Ca^{2+}$  signal.
- single cell staining with antisera against certain secretory

vesicle components appearing at the surface of the cell after exocytosis and membrane fusion (Cheek et al., 1989; Vitale et al., 1991).

- single cell **voltametric measurement** of catecholamine release from a single chromaffin granule (Leszczyszyn et al., 1990)

- simultaneous independent measurement of endocytosis (capacitance minus fluorescence) and exocytosis (fluorescence) (Smith et al., 1996).

-**biochemical and immunocytochemical methods after cell permeabilization** and direct manipulation of intracellular factors involved in exocytosis. Permeabilized chromaffin cells have proven to be an excellent model for studying the role of these factors (Knight et al., 1982). Various substances (streptolysin O, saponin, staphylococcal alpha toxin, digitonin) have been used to permeabilize the membrane; they produce different membrane lesions. Membrane holes should be small enough to impede the exit of large molecules but, at the same time they should allow the slow entering or leaving of proteins . Electron microscopy has shown that **digitonin** permeabilized chromaffin cells release catecholamine by exocytosis but intact granules are not released, and the morphology of the cells is not changed by permeabilization (Schafer et al., 1987; Ito et al., 1991). In other words, the digitonin permeabilization of the membrane preserves the secretory process (Dunn, 1983; Wilson, 1983). Following digitonin treatment, soluble cytosolic proteins and other

components are lost at a relative slow rate, the responsiveness to  $\text{Ca}^{2+}$  challenge decreases with time and the secretory process is slowed down (Ito et al., 1991). Upon addition of the necessary leaked proteins the exocytosis can be reinstated (Dunn, 1983). Thus, the constituents which are proven vital for exocytosis can be identified and studied.

The release experiments described in this thesis were carried out on 2-3 day old cultures of bovine chromaffin cells. Catecholamine stores were well maintained during this period of time (cytochemical and fluorometric determinations) (Trifaró and Lee, 1980). Chromaffin cells are characterized by the presence of a high affinity uptake system for catecholamines (Kenigsberg et al., 1979; Kenigsberg, 1980) which allows the study of the release of [ $^3\text{H}$ ]noradrenaline from cells preloaded with labelled noradrenaline. The spontaneous release of catecholamines from cultured chromaffin cells (several days old) is much lower than that of freshly isolated chromaffin cells, this might be due to the experimental conditions during isolation of the cells i.e.: collagenase treatment,  $\text{Ca}^{2+}$ -free and  $\text{Mg}^{2+}$ -free media (Trifaró and Lee, 1980). The response to acetylcholine or depolarizing concentrations of  $\text{K}^+$  (56 mM) seem to be enhanced in cultured cells compared to freshly isolated cells (Trifaró & Lee, 1980). The cholinergic receptor of bovine cultured chromaffin cells appears to be nicotinic, catecholamine release is stimulated by nicotine and carbamylcholine but not by

pilocarpine (Trifaró & Lee, 1980).

In conclusion, adult bovine chromaffin cells in culture display many of the morphological and functional characteristics of sympathetic neurons, and constitute an excellent model for studying secretion.

### 1.3 EXOCYTOSIS AND THE ROLE OF CHROMAFFIN CELL CYTOSKELETON

#### 1.3.1 General Organization of the Cytoskeleton: Actin Microfilaments and Actin-Binding Proteins.

The eukaryotic cytoskeleton is a dynamic filamentous network of microfilaments (thin filaments consisting mainly of actin), microtubules (hollow, noncontractile structures assembled from tubulin dimers) and intermediate filaments (built of different peptide polymers) involved in the control of cell shape, motility, intracellular organization and cell division (Darnell, 1990). Traditionally, the cytoskeleton was mainly viewed as a supporting web but recent findings point to the fact that this mechanical support and its force-carrying connections which can reach all the way from the membrane to the genome, can affect signal transduction, the way some proteins bind to the DNA and even the fate of malignant cells (Glanz, 1997). The cytocontractile and cytoskeletal elements also play an important role in certain pathological processes

such as fibromatosis, liver cirrhosis (and other fibrotic lesions), formation of hypertrophic scars, retinitis pigmentosa, Alzheimer's disease, different muscular dystrophies as well as tumor invasion and metastasis (Rungger-Brandle et al., 1983). One of the most important components of the cytoskeleton is actin in the form of microfilaments (5-7 nm diameter) which are present in all eukaryotic cells (Burgoyne, 1987). Bundles of micro-filaments (stress fibers) exert an important structural role by anchoring the cytoplasmic matrix to the substrate, are responsible for the gel-like consistency of the marginal cytoplasm and are characteristic for compartments that display active motility and movement (Rungger-Brandle et al., 1983).

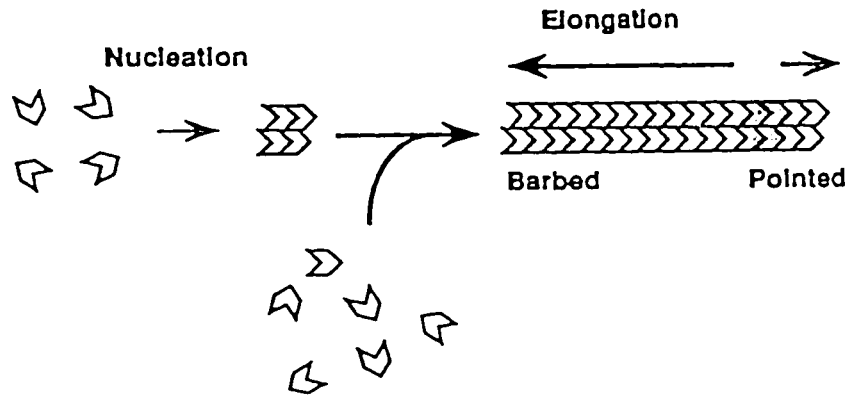
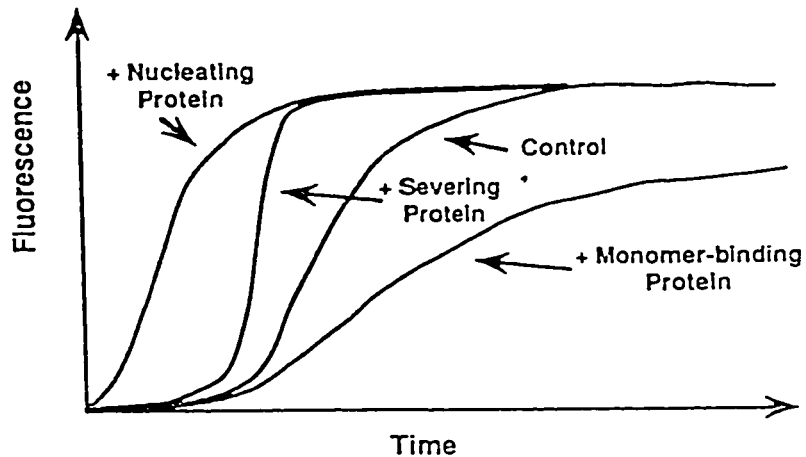
**ACTIN** - MW= 43,000 - is the most abundant protein in typical eukaryotic cells (Darnell et al., 1990). In the cytoplasm most of the actin is in the form of randomly cross-linked network of filaments - remarkable stable polymers - (the concentration of actin in the cytoplasm favors full polymerization) which can behave as either gels or liquids, depending on filament length and degree of cross-linking. Rapid interconversions of the polymeric states of actin change the viscoelastic properties of the network - these changes are the structural fundamentals of cell shape, motility and intracellular organelles movement (Burgoyne, 1987). The actins in nonmuscle cells are the products of different genes, therefore differ slightly in some of their properties (Darnell

et al., 1990). At least six different actins have been identified in eukaryotes, however many multicellular organisms contain multiple actin genes (maybe not all of them expressed). Actins are also posttranslationally modified by acetylation of the N-terminus and methylation of a histidine, leading thus to multiple functional species in excess of the actual number of genes (Darnell et al., 1990). Microfilaments, also called F-actin, are polymers of the globular (G-actin) subunit. Viewed in the electron microscope, they appear as double stranded helices. In the filament, the actin subunit has a certain polarity. After interaction with the microfilaments, the head fragments of myosin form arrowheads (Ishikawa et al., 1969), thus the filaments are polarized as well, and all subunits "grow" or point in the same direction (Woodrum, 1975). The two ends of the microfilament have been referred to as "pointed" (- end) and "barbed" (+ end) (Huxley, 1963), as determined by the presence of heavy meromyosin-labelled filaments. The overall rates of monomer assembly at the two ends are different, the barbed end (+ end) is preferred for monomer addition over the pointed end (- end) where the disassembly is more favoured (Woodrum, 1975). Polymerization of globular actin monomers is induced by  $Mg^{2+}$  and  $K^+$  at concentrations similar to those found in the cytosol. The double-helical structure of the actin filament requires that each actin monomer within the filament is in contact with four adjacent monomers (Tellam, 1989).

Hydrophobic and electrostatic forces contribute to the noncovalent bonds between the actin subunits (Stossel et al., 1985). The extent of polymerization is determined by the thermodynamics of the microenvironment and the average filament length (which is inversely proportional to the number of filaments) is influenced by the kinetics of polymerization (Stossel et al., 1985). Under ionic conditions favourable for polymerization actin dimers are thermodynamically unstable, they dissociate easily. But when aggregates consisting of a critical number monomers assemble, addition of new monomers is more probable than dissociation, therefore polymerization is enhanced by these "nuclei" (Stossel et al., 1985). Nucleation is viewed as the rate limiting step in the polymerization reaction (Stossel et al., 1985). A "lag" phase (which precedes detectable polymerization of an actin solution) in the beginning of the polymerization reaction is ascribable to the time necessary for nucleation. In vitro, nucleation and polymerization from monomers and their time courses can be monitored by the fluorescence of a labelled (pyrene) actin (Carraway et al., 1992). Fluorescence is plotted against time. The initial lag phase (most likely accounted for by nucleation) is followed by an exponential phase (addition of subunits to the filament end) and a plateau phase (Carraway et al., 1992). Figure 1.3.1 depicts the actin polymerization process and a schematic representation of the action of different actin-binding proteins.

Given the ionic environment within cells, one would expect to find most of the actin in filamentous form. This is not the case, about half the actin is present in the G-form due to the action of various small actin-binding proteins such as profilin and actin depolymerizing factor (ADF) which bind and sequester individual actin monomers (Tellam, 1989). In vivo, cell activation does not appear to change the amount of total actin but leads to an increase in the relative amount of F-actin (Carlsson et al., 1979). Associated with the actin filaments are a great number of other actin-binding proteins. These bind to actin monomers, cross-link actin filaments into bundles, gel or sever the actin filament and cap its growing ends (See Figure.1.3.2). Actin binding proteins can be placed into families which share similar properties but most of the time these proteins share domains with actin-binding proteins of other families; these domains are responsible for actin binding itself and/or for various controlling and structural functions (Stossel et al., 1985).

Chromaffin cells are among the non-muscle cells from which a great number of cytoskeletal proteins have been isolated and characterized, their cellular localizations were revealed by immunocytochemical, biochemical and ultra-structural techniques.

**A****B**

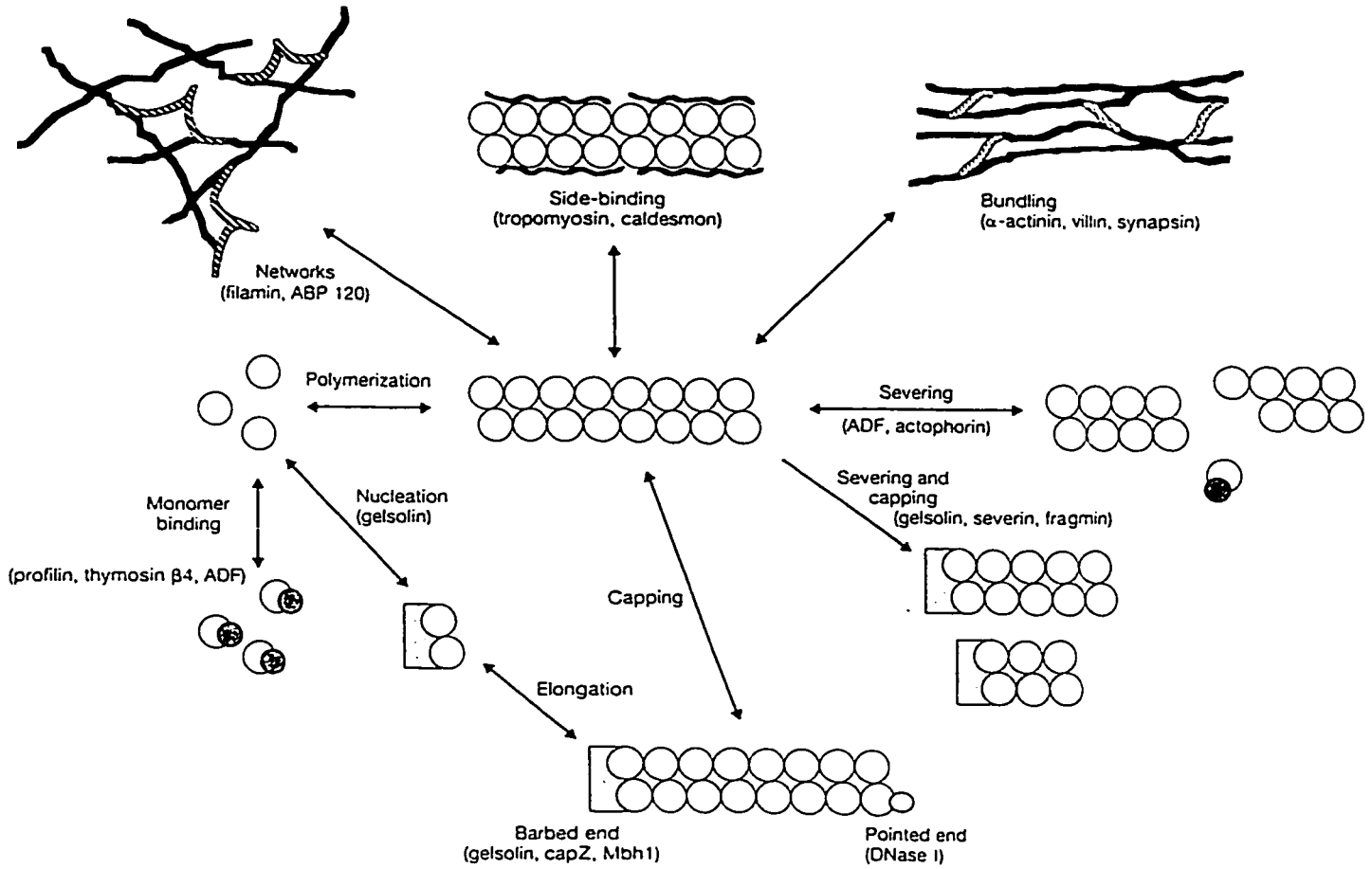
**FIGURE 1.3.1.1 A) Actin Polymerization.** Subunits (drawn as chevron-shaped monomers) associate with each other to form nuclei. The filaments have barbed and pointed ends. Elongation is favoured at the barbed end. All of the reactions drawn in the forward direction also occur in the reverse direction.

**B) Effect of Actin-Binding Proteins on the Time Course of Actin Polymerization from Monomers.** The control is actin alone. The data are hypothetical. (Taken from Carraway et al., 1992).

**ACTIN-BINDING PROTEINS** - ( See Figure 1.3.1.2.). The list of actin-binding proteins continues to increase but it is clear now that most of them can be placed into three main families:

1. Actin cross-linking proteins - join the actin filaments together and possess at least two F-actin-binding sites (Darnell, 1990). They have different potencies and are sensitive to pH, Ca<sup>2+</sup> concentrations, temperature, ionic strength, and vary widely in molecular weight, subunit composition, etc but they all seem to have the shape of long, flexible rods (which probably facilitates cross-linking and rigidity before formation of actin bundles and aggregates takes place) (Craig et al., 1982). Spectrin (fodrin) (Bennet et al., 1990), villin (Bretscher et al., 1980), vinculin (Burridge et al., 1988), alpha-actinin (Maruyama et al., 1965) and caldesmon (Sobue et al., 1988) are just a few of the actin-crosslinking proteins but other non-specific gelation factors are known as well : histones, RNase, aldolase, lysozyme (Craig et al., 1982).

2. Actin filament depolymerizing proteins (predominantly sequester actin monomers)- such as DNaseI (Lazarides et al., 1974) and profilin (Carlsson et al., 1976) bind to actin monomers but do not form stable complexes with the filaments. As a consequence, the polymerization in vitro is retarded due to the delay in the incorporation of actin monomers into filaments (Stossel et al., 1985). These sequestering proteins might bind to the same end of actin where the addition to the



**FIGURE.1.3.1.2. The Different Roles of Actin-Binding Proteins** (Taken from Way M. & Weeds A., 1990, Nature 344, p.292).

filament should occur, therefore impeding the growing of the filament length or can even extract an actin monomer from the middle of the filament (Stossel et al., 1985). Actin depolymerizing proteins decrease the amount of polymer rather than the filament length, they bind with greater affinity to monomeric actin than monomeric actin binds to the end of a filament. Therefore monomers dissociate from the filament ends in order to restore the equilibrium concentration of free monomers (Craig et al., 1982).

3. Actin filament length regulators and capping proteins- can shorten the length of actin filaments and influence both the nucleation and the elongation steps of actin polymerization through their binding preferentially to one of the two polar ends of the filament, thus preventing the exchange of monomers with that end. If actin polymerization (and the effects of these capping proteins) is assayed by shear viscosity, the lag phase of viscosity increase (which is reduced by nucleation) is eliminated in the presence of these proteins, therefore they should be able to favour the nucleation process (Craig et al., 1982). The capping proteins bind to the (+) end of actin filaments, they can bind to one or two actin monomers accelerating the nucleation process. But if these proteins are added in vitro to preformed F-actin, the filaments are shortened by severing along their length through a pure mechanical displacement of a unit from neighbouring units (Stossel et al., 1985). The end of the filament remains

"capped" after the binding of the capping protein, and monomer addition is impeded. Severing is a nonenzymatic, nonproteolytic process dependent on  $\text{Ca}^{2+}$  (Matsudaira et al., 1988). In vitro, severing can be assayed by a number of methods, one of the most frequently used - falling ball viscometry - is based on simple rheologic measurements of the viscosity of F-actin solutions. The apparent viscosity of these solutions is proportional to the number, concentration and length of the actin filaments (MacLean Fletcher et al., 1980).

**Gelsolin** (MW=87,000) was the first protein described in this category (Yin et al., 1979) which also includes, among others, villin (Bretscher et al., 1980), fragmin (Hasegawa et al., 1980),  $\beta$ -actinin (Maruyama et al., 1977) and scinderin (Rodriguez Del Castillo et al., 1990). Gelsolin is a ubiquitous multidomain protein with at least three actin- and two phosphatidylinositol 4,5-bisphosphate - binding sites which might be activated alone or in combination, cooperating to the various effects on actin assembly and disassembly (Janmey et al., 1992). Gelsolin binds avidly ( $KD=10^{-5}$  mol/liter) to the barbed end of F-actin, capping it (Yin et al., 1980), and can also bind to the sides of the filaments, breaking the noncovalent actin-to-actin bonds (Yin et al., 1988). The overall effect is the depolymerization of F-actin and an exponential increase in the number of filaments (Yin et al., 1988). Interestingly, a plasma form of gelsolin - brevin - plays vital roles in protection (by severing) against plasma

actin filaments, which can reach 10  $\mu\text{m}$  in length: these F-actins can alter the microcirculation flow and possibly lead to disseminated intravascular coagulation in patients with several disorders characterized by severe tissue damage : fulminant hepatic necrosis, septic shock, acute respiratory distress, myocardial infarction, etc (Lee et al., 1992).

Our attention was focused on gelsolin family of  $\text{Ca}^{2+}$ -dependent actin severing proteins, to which scinderin is a member (Rodriguez Del Castillo et al., 1990; Marcu et al., 1994). A regulatable actin severing protein such as gelsolin (which shortens actin filaments) introduces breaks into the cross-linked structures (Janmey, 1993). Gelsolin requires elevated  $\text{Ca}^{2+}$  (Yin et al., 1979) or  $\text{H}^+$  (Lamb et al., 1993) concentrations. Recent studies suggest that gelsolin would be likely to become active only in regions of very high free  $\text{Ca}^{2+}$ , such as occur in the proximity of  $\text{Ca}^{2+}$  channels (Llinas et al., 1992). In fact, most of the effects on actin assembly (severing, nucleation, capping, bundling and linkage to organelles or cell membrane) are regulated by  $\text{Ca}^{2+}$ .

The severing process (as well as nucleation, monomer binding) can be reversed by binding of certain polyphosphoinositides (PPI) such as phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) to the severing protein (Janmey et al., 1988; Matsudaira et al., 1988). One of the strongest evidence that PPI regulate actin assembly was brought by Lassing et al., (1985), who demonstrated that complexes of actin with the

monomer-sequestering protein profilin were dissociated by  $\text{PIP}_2$ , thus leading to actin polymerization. Gelsolin activity is also regulated by  $\text{PIP}_2$  (Janmey et al., 1987). During cell stimulation,  $\text{PIP}_2$  (even in low concentrations and in the presence of 200-1000  $\mu\text{M}$   $\text{Ca}^{2+}$ ) can inhibit the F-actin severing and nucleating ability of gelsolin (Janmey et al., 1987). The site affected by the  $\text{PIP}_2$  is located in N-terminal half of gelsolin, as well as the actin-binding site involved in severing. The interaction between gelsolin and the micelles of  $\text{PIP}_2$  is of high affinity and can be reversed by hydrolysis with phospholipase A2 or C, thus suggesting that the acyl chains of  $\text{PIP}_2$  form contacts with a hydrophobic site of gelsolin (Janmey et al., 1987). It is not known exactly how the interaction occurs in vivo but the levels of  $\text{PIP}_2$  during cell activation are sufficient to alter the activity of gelsolin (and other proteins) especially if these components are spatially close. Dissociation of the gelsolin- $\text{PIP}_2$  complexes would favour actin nucleated assembly of actin (Janmey et al., 1987). Briefly, an initial calcium-induced nucleation and severing of F-actin by gelsolin and related proteins (following the stimulation of certain cell receptors) would increase the number of nuclei on which filaments of actin could grow (Janmey et al., 1987). Thus, gelsolin family of actin-severing proteins is placed between receptor activation and cytoskeletal remodelling (Stossel, 1989).

What are the conditions that dictate whether a dual-

function severing protein will function as a stimulant or inhibitor of actin polymerization ? The answer is related to the presence and concentrations of calcium ( $Ca^{2+}$ ) and polyphosphoinositides (mainly  $PIP_2$ ). The mechanisms underlying actin binding and its regulation by gelsolin, scinderin and related proteins can be partially deduced from the sequences of these proteins and the binding properties of their actin,  $PIP_2$ ,  $Ca^{2+}$  - binding domains.

### 1.3.2 Chromaffin Cell Exocytosis : Cytoskeleton Dynamics During the Neurotransmitter Release.

Over the last few years, remarkable progress has been made in understanding the organization and function of the exocytosis apparatus (exocytosis = the fusion of the membrane of an intracellular vesicle with the plasma membrane), new protein-effectors involved in vesicle transport and fusion have been identified (Kelly, 1993); Zucker, 1996). Nevertheless, many questions remain to be answered and little is known about the fundamental mechanisms at the basis of exocytosis and how it is regulated by the several interacting second messenger systems.

Ultrastructural and molecular organization of the cytoskeletons of neurons and secretory cells (such as chromaffin cells) differ significantly but there are also

similarities between the two systems (for instance,  $\text{Ca}^{2+}$  plays a vital role in the control of cytoskeleton dynamics, [Douglas et al., 1961, 1982]). Neurons are specialized for the fast release of neurotransmitters, clusters of synaptic vesicles are closely positioned to the presynaptic membrane (readily available for release) but another "reserve" pool could be recruited for release upon cell stimulation (Hirokawa, 1991). In secretory cells the vesicles are positioned at a distance of about 250 nm from the plasma membrane, thing that might suggest the presence of an impediment (a physical barrier) in the cortical area of these cells (Burgoyne, 1982; Cheek, 1991; Vitale et al., 1995). In resting chromaffin cells, 1-3 % of the total secretory vesicles are either docked to the plasma membrane or within about 50 nm from it (Vitale et al., 1995); this represents the release-ready pool (Neher et al., 1993), to differentiate it from the reserve pool (the rest of 97-99% of the vesicles) which remains behind the cytoskeletal F-actin barrier (Vitale et al., 1995).

**Role of calcium :** As it was discussed in chapter 1.2 , adrenal chromaffin cells have been extensively studied for many reasons. The main physiological stimulus for release of the secretory material in these cells is a significant rise in  $[\text{Ca}^{2+}]_i$  entry (Douglas et al., 1961, 1982: Llinas et al., 1982) through the nicotinic receptor-associated channel itself and mainly through voltage-dependent  $\text{Ca}^{2+}$  channels, from a resting

level of 0.1  $\mu\text{M}$  to the range of 0.5-10  $\mu\text{M}$  (Baker and Knight, 1981). It was demonstrated by many researchers that, under physiological conditions, it is the  $\text{Ca}^{2+}$  entry and not release from internal stores that activates exocytosis in chromaffin cells (Burgoyne, 1991; O'Sullivan et al., 1988;). A large variety of  $\text{Ca}^{2+}$  channels (L, N, P, Q, T) have been identified in chromaffin cells but their precise contribution to the regulation of exocytosis remains to be established (Burgoyne, 1991; Lopez et al., 1994). Activation of a number of receptors (such as angiotensin II, ATP, bradykinin, endothelin, muscarinic, nicotinic, etc.) of chromaffin cells will stimulate catecholamine secretion by different mechanisms. For instance, nicotinic stimulation and depolarization with high  $\text{K}^+$  leads to the production of  $\text{Ins}(1,4,5)\text{P}_3$  through  $\text{Ca}^{2+}$ -dependent activation of PLC (Eberhard et al., 1987).

In recent years it has been suggested that in certain secretory systems, such as insulin-secreting cells (Blondel, 1995) but also in chromaffin cells (Yoo et al., 1990),  $\text{Ins}(1,4,5)\text{P}_3$  - elicited  $\text{Ca}^{2+}$  release from secretory granules could play a role in controlling secretion and neurotransmitter release. In any case, exocytosis requires very high local  $\text{Ca}^{2+}$  concentrations (Neher et al., 1993). They can be achieved by clustering of plasma membrane  $\text{Ca}^{2+}$  channels near the zones where exocytosis occurs (Llinas et al., 1992) or by  $\text{Ca}^{2+}$  release from the granules themselves (Blondel, 1995;

Ravazzola et al., 1996) or both. Calcium has, without a doubt, a pivotal role in secretion due to its second messenger activity (i.e. PKC, calmodulin), control of the cytoskeleton dynamics (actin filament disassembly), vesicle-plasma membrane fusion process, etc.

**Cortical cytoskeleton** (and its associated proteins) plays an important role in regulating the exocytosis by forming a cortical barrier (Cheek et al., 1986, 1987; Lee and Trifaró, 1981; Trifaró et al., 1982, 1984) which is disassembled upon  $Ca^{2+}$  entry and activation of actin-filament severing proteins such as scinderin (Trifaró et al., 1993; Vitale et al., 1991). Reorganization of actin filaments in certain subplasmalemmal areas leads to low viscosity cytoplasmic spaces where chromaffin granules have a high mobility and they can move toward the release sites on the plasma membrane. At present, little can be said about which of the many components are really essential to the exocytosis process.

Secretion by cells can take two forms: constitutive (unregulated, follows the rate of synthesis of secretory products) and regulated (the secretory products are stored in membrane bound secretory vesicles or granules, the contents are released in fixed amounts upon cell stimulation) (Tartakoff et al., 1978; Gumbiner et al., 1985). The regulated form of secretion is characteristic of neurons and endocrine (with the exception of steroid-secreting cells) and exocrine cells. The

membrane bound vesicles allow the cell to store large amounts of material which is protected from enzymatic degradation, transport and release of quanta of secretory material (Trifaró, 1977; Trifaró and Poisner, 1982). Secretory cells containing secretory vesicles, such as chromaffin cells, have been named "paraneurons", they are considered to be closely related to neurons in structure and function (Fujita, 1977). The release of secretory products by exocytosis is a highly complex mechanism whereby the membrane of the vesicles fuses with the plasma membrane, the contents of the secretory vesicles are thus released to the cell exterior. As described above, the regulated secretion is triggered by an increase in cellular  $Ca^{2+}$ . In chromaffin cells, catecholamines are released along with other secretory granule components such as ATP, chromogranin A, dopamine  $\beta$ -hydroxylase and other neuropeptides (Banks and Helle, 1965; Viveros et al., 1968; Lastowecka and Trifaró, 1974). It was shown that in adrenal medulla the entire soluble content of the granule is released to the cell exterior meanwhile the granule membrane components are retained within the cell after exocytosis. Exocytosis was therefore considered to be an all-or-none release phenomenon (Viveros et al., 1969; Trifaró, 1977). Endocytosis of empty vesicles and regeneration of fresh vesicles follow exocytosis. The synaptic vesicle cycle is very fast and tightly regulated.

The cell cytoskeleton plays a crucial role in this cascade of events, it determines the positioning of secretory

vesicles within the cell (Burgoyne, 1990; Trifaró, 1990), the actin filaments interact with the inner surface of the plasma membrane and also with the cytoplasmic surface of secretory vesicles through certain anchorage proteins such as  $\alpha$ -actinin, (Jokush, 1977; Trifaró et al., 1985) synapsin I (expressed mainly in neurons; Greengard, 1993) and II (which is expressed in chromaffin cells; Haycock, 1988) but its interaction with chromaffin vesicles has not been demonstrated, and fodrin (Aunis et al., 1984; Perrin, 1985). Caldesmon (calmodulin-dependent actin-binding protein) is another protein associated with chromaffin vesicles (Burgoyne et al., 1986). Caldesmon binds and cross-links actin filaments in a  $Ca^{2+}$ -dependent manner. Synapsin I is a phosphoprotein associated with neuronal synaptic terminals (DeCamilli et al., 1983) and it is functioning as a substrate for cAMP kinase (DeCamilli and Greengard, 1986) that can bind to spectrin (an actin binding protein) and actin (Baines and Bennet, 1985). Synapsin I serves as an anchor between synaptic vesicles and cytoskeleton (DeCamilli and Greengard, 1986).

These cytoskeleton associated proteins and many others could be involved in the control of cell viscosity and vesicle mobility ; for instance fodrin cross-links the actin filaments resulting in a mesh (Perrin et al., 1985) but, in certain conditions, (cell stimulation and  $Ca^{2+}$  increase) actin-severing proteins such as scinderin decrease the local cell viscosity allowing free movement of the vesicles toward the release

sites on the plasma membrane (Rodriguez Del Castillo et al., 1990; Trifaró et al., 1993). As it will be discussed in the following chapters, it seems that scinderin plays a more significant role in neurotransmitter release than its close counterpart - gelsolin. During chromaffin cell stimulation, subplasmalemmal scinderin, but not gelsolin, is redistributed together with F-actin disassembly, this  $Ca^{2+}$ -dependent redistribution precedes exocytosis and exocytosis sites are preferentially localized to areas of F-actin disassembly (Vitale et al., 1991).

It is obvious now that a large number of proteins is involved in exocytosis and membrane recycling; molecular cloning of these proteins is leading to functional studies in order to define their specific role.

#### 1.4. SCINDERIN : CHARACTERISTICS AND ROLE IN CHROMAFFIN CELL SECRETION

**Scinderin Is a Key Player in the Control of Cortical Cytoskeleton Dynamics During Exocytosis.**

Adrenal medulla chromaffin cells possess a mesh of actin filaments (F-actin) underneath the plasma membrane: a dynamic reorganization of this cortical actin network must take place for exocytosis to occur (Trifaró et al., 1982, 1984; Cheek et al., 1986; Burgoyne et al., 1987, 1991). It is demonstrated that disassembly of actin filaments facilitates exocytosis (Lelkes et al., 1986; Vitale et al., 1992). Following nicotinic receptor stimulation of chromaffin cells, a decrease in F-actin concentration (Cheek et al., 1986; Burgoyne et al., 1989) together with disassembly of the cortical F-actin network (Cheek et al., 1986; Trifaró et al., 1989) can be clearly noticed.  $Ca^{2+}$ -dependent actin-binding proteins, such as gelsolin and scinderin regulate the dynamics of actin filament length (Trifaró et al., 1985; Bader et al., 1986), playing an important role in the reorganization of cortical actin filaments brought about by cell stimulation. The two proteins are different in many ways, the tissue distribution is one of them: gelsolin (MW=87,000) is a widely distributed  $Ca^{2+}$ -dependent actin filament capping and severing protein (Yin et al., 1979, 1981; Stossel et al., 1985), meanwhile, scinderin, also a  $Ca^{2+}$ -dependent actin-filament-severing

protein, which was first described by us in chromaffin cells (Bader et al., 1986; Rodriguez Del Castillo et al., 1990), is expressed only in tissues with high secretory activity (Tchakarov et al., 1990). Immunocytochemical studies have shown that nicotinic receptor stimulation of chromaffin cells induces cortical F-actin disassembly and simultaneously, redistribution of subplasmalemmal scinderin (Vitale et al., 1991). These nicotine-induced effects are  $Ca^{2+}$ -dependent, precede exocytosis and, even more, during nicotinic receptor stimulation there is a spatial relationship between scinderin and F-actin cortical redistribution: exocytotic sites are preferentially localized to cortical areas devoid of scinderin and F-actin (Vitale et al., 1991). The distribution of gelsolin, on the other hand, is not affected by either nicotinic receptor stimulation or  $K^+$ -evoked depolarization (Vitale et al., 1991). Therefore, the effect of cell stimulation seems to be specific for scinderin (cell stimulation and  $Ca^{2+}$  entry bring about activation of scinderin with a consequent disassembly of cortical actin-filament networks). Previous studies from our laboratory have also demonstrated that PKC seems to be involved in the regulation of scinderin cellular distribution (Rodriguez Del Castillo et al., 1992). Other different characteristics of the two proteins (scinderin and gelsolin) include: different molecular weights, isoelectric points, amino acid composition and different peptide maps after limited proteolytic digestion

(Rodriguez Del Castillo et al., 1990). Both proteins have an actin filament severing activity which is  $\text{Ca}^{2+}$ -dependent and inhibited by phosphatidylinositol 4,5 bisphosphate (Yin et al., 1988; Maekawa et al., 1990). Immunocytochemical studies showed that, in chromaffin cells, scinderin has a diffuse cytoplasmic and a more dense subplasmalemmal distribution, meanwhile, gelsolin only showed a diffuse cytoplasmic distribution (Vitale et al., 1991; Rodriguez Del Castillo et al., 1990). Gelsolin does not leak out from permeabilized chromaffin cells whereas scinderin does (Vitale et al., 1992). All these experimental data were suggesting that gelsolin and scinderin were two distinct  $\text{Ca}^{2+}$ -dependent F-actin severing proteins, differing in their chemical properties and fine regulation by intracellular messengers.

As described before, the cortical F-actin cytoskeleton represents a negative control for secretion and it must be locally disassembled to allow chromaffin vesicle exocytosis. Immunofluorescence cytochemical studies have described the presence of this mesh of filamentous actin (F-actin) underneath the chromaffin cell plasma membrane (Lee et al., 1981; Trifaró et al., 1984; Cheek et al., 1986; Sontag et al., 1988). In resting chromaffin cells, 1-3% of the total chromaffin vesicles are either docked to the plasma membrane or within 50 nm from it (Vitale et al., 1995), constituting the release-ready vesicle pool (Neher et al., 1993; Vitale et al., 1995). The majority of the chromaffin vesicles (97-99%)

form a reserve pool and remain behind a barrier of cortical F-actin (Vitale et al., 1995). Chromaffin cell stimulation is accompanied by a focal and transient disassembly of the cortical F-actin network (Cheek et al., 1986; Vitale et al., 1991, 1995), allowing the movement of additional secretory vesicles from the reserve pool to release sites on the plasma membrane (Vitale et al., 1995). It was concluded that the cortical actin network dynamics can control the size of the release-ready vesicle pool and, consequently, the initial rate of exocytosis (Vitale et al., 1995). It has also been suggested that cortical actin network dynamics is controlled by scinderin, a  $Ca^{2+}$ -dependent F-actin severing protein (Rodríguez Del Castillo et al., 1990, 1992; Vitale et al., 1991).

## 1.5. THE SECRETORY PROCESS IN PLATELETS

In addition to chromaffin cells, we have focused our attention on the role of scinderin in other secretory systems, as well.

### 1.5.1 Platelets: General Description

Platelets (thrombocytes) are small (2-5  $\mu\text{m}$  diameter), disc-shaped cells that are involved in the early response to blood vessel damage and bleeding. They are fragments of the cytoplasm of megakaryocytes, large granular cells of the bone marrow which derive from pluripotent hematopoietic stem cells that also give rise to granulocytes and erythrocyte precursors (Wright, 1910). Platelets are anucleate but their cytoplasm contains a large variety of organelles (mitochondria, glycogen deposits, secretory granules) and chemical mediators that are potent initiators of hemostasis and coagulation. The plasma membrane, about 8 nm thick, communicates with a canalicular system of tubules which goes into the interior of the cell. A well developed cytoskeleton of microtubules lies beneath the plasma membrane. Upon tissue damage, a fast release of materials will promote platelet clumping; the resulted plug acts as a barrier, impeding further blood loss. Even more, the platelet surface facilitates the coagulation cascade which leads to the formation of fibrin clot that stabilizes the initial platelet mass. Platelets have also been implicated in inflammatory disease, atherosclerosis and thrombosis

(Ginsberg, 1981).

After formation and maturation, platelets leave the marrow, get into the venous stream where they are circulated to the spleen. Here they remain for 2-4 days before being distributed throughout the body. Platelet concentration in the blood varies between 200,000 and 450,000 / $\mu$ l. About 20-30% of the peripheral platelet mass is found at any time in the spleen. In response to injury or exposure to different substances, the blood vessel wall secrete collagen, which in turn, stimulate platelets to undergo activation (a reversible process) which consists of shape changes, formation of cellular pseudopodia, aggregation and secretion. During activation, the platelets undergo a dramatic change in shape, from the flattened disc to a sphere with long "arms". These changes involve movements of the microtubules and other cytoskeleton elements, and are accompanied by cycles of cytoskeletal actin depolymerization and polymerization. At this time, many of the granules and other organelles become positioned close to the canalicular system (an invagination of the plasma membrane which provides access for cellular contents to the cell exterior). Upon release of the granular contents, the platelets aggregate forming a haemostatic plug. Other platelets are attracted to form an aggregate and strengthen the plug. Aggregation is a response to thromboxane, ADP and thrombin produced by the platelets. These agents, as well as collagen, activate platelets, alter their metabolism and structure.

**Platelet Secretory Granules .** The cytoplasmic granules are classified into two groups (Powers, 1989) according to their appearance on transmission electron microscopy of thin sections:

- Alpha granules which consist of two types : lysosomes (with high content of acid hydrolases) and proper alpha granules (with a variety of products such as albumin, Factor V, VIII, fibrinogen, fibronectin, thrombospondin, platelet-derived growth factor-PDGF-, platelet factor 4 etc).

- Dense bodies (dense core vesicles) are thicker walls, opaque bodies containing ADP, GTP, adrenaline, calcium, serotonin (5-HT).

### **1.5.2 The Cytoskeleton of Human Platelets : Activation and Secretion**

The term "cytoskeleton" coins the detergent-resistant elements of the cytoplasm; this lattice-like structure, the cytoskeleton, can also be named cytomatrix (Darnell et al., 1991). Electron microscopy has revealed a complex matrix inside platelets (Wolpers and Ruska, 1939), consisting of microtubule bundles, surface-associated submembrane filaments, cytoplasmic actin and the intrinsic cytoskeleton of the plasma membrane. The term "actin" refers to the submembrane filaments and this actin is different physically and chemically from the actin associated to the cell membrane

(Boyles et al., 1985). The estimated actin concentration of platelets is about 20% among the soluble proteins, in other terms, 0.25 - 1 mM (Fox, 1986). This is 1,000-2,000 times higher than the critical concentration above which actin monomers polymerize spontaneously in vitro (Pollard et al., 1986). When activated, platelets change shape by polymerizing actin into various structures, such as stress fibers and filopodia (these are not really actin structures although they contain actin). There must be many player proteins participating in the promotion and initiation of actin polymerization. Indeed, Bearer (1995) identified more than 30 different actin-binding proteins in ADP-stimulated platelets, by using a filamentous (F)-actin affinity chromatography. Some of these proteins were known, other appeared to be novel. Bearer also demonstrated that four distinct actin filament structures were formed during the first 15 min of activation: filopodia, lamellopodia, a contractile ring surrounding degranulating secretory granules and thick actin bundles (resembling stress fibers). The precise interaction and regulation of actin by the actin-binding proteins, their spatial distribution during these processes are not known. Among the most studied actin-binding proteins are gelsolin (severing and capping activity) and profilin (forms binary complexes with actin monomers). The actin stress fibers in platelets contain alpha-actinin (Bertagnolli et al., 1994), whereas microvilli contain villin (Mooseker, 1985) and fimbrin

(Bretscher, 1991).

The platelet membrane skeleton (coating the lipid bilayer) is associated with extracellular glycoproteins and intracellular cytoskeletal elements, and seems to play a major role in regulating signal transduction processes. This membrane cytoskeleton could localize signalling enzymes that might be activated upon binding of ligands to their corresponding membrane receptors; one such signalling enzyme has been detected in submembranous actin scaffold - tyrosine kinase pp60<sup>c-src</sup> (Ferrell et al., 1990).

- **Platelet Secretory Activity.** The content of platelet storage granules is secreted after membrane stimulation, these secreted molecules interact with plasma proteins, with other cells (including platelets) extracellular matrix components in the walls of the blood vessels. Secretion is initiated in response to agonists acting on specific membrane receptors. There are at least three activation pathways in human platelets that can be initiated individually or simultaneously (Hawiger, 1989) :

- phospholipase A<sub>2</sub> and C pathway, which generate messenger molecules derived from phospholipids, arachidonic acid, diacylglycerol and inositol-1,4,5-triphosphate,
- diacylglycerol pathway, which activates protein kinase C,
- Ca<sup>2+</sup>/calmodulin complex that activates myosin light chain kinase, this is phosphorylated in the early steps of platelet activation and precedes the secretory response.

Extracellular  $\text{Ca}^{2+}$  is required for many aspects of platelet activity. The activation by agonists such as ADP, adrenaline and others requires  $\text{Ca}^{2+}$ , which in turn could activate extracellular proteins (fibrinogen) or might bind to and alter the function of the platelet membrane components (Hawiger, 1989). Extracellular  $\text{Ca}^{2+}$  enters the platelet and influences intracellular  $\text{Ca}^{2+}$ -dependent reactions, all three above mentioned pathways involve  $\text{Ca}^{2+}$ -dependent steps leading to platelet activation and secretion (Hawiger, 1989). Most of the calcium is found in the dense storage granules, the rest is either sequestered within intracellular organelles or bound to membranes and cytoplasmic proteins (Hawiger, 1989). In our experiments, we stimulated the permeabilized platelets with  $10 \mu\text{M}$   $\text{Ca}^{2+}$ . Human platelets were isolated (plasma contains factors that depolymerize actin filaments, careful separation methods should be used in order to avoid activation!) and maintained in the conditions that preserve their functions. The study of platelet secretion has at its core the serotonin release: among all other substances found in the secretory granules, serotonin is the only one that readily exchange with its cytoplasmic or extracellular counterpart. Serotonin crosses the plasma membrane with its transport system and it is subsequently taken up into granules (Holmsen, 1989), this ability of platelets to take up, store and release serotonin enables us to study platelet secretion activity.

### **- Scinderin in platelets**

Our attention was focused on the role of scinderin in platelet activation, actin-cytoskeleton dynamics and serotonin release. Previous work from our laboratory has demonstrated the presence in platelets of gelsolin and scinderin, two  $\text{Ca}^{2+}$ -dependent F-actin severing proteins that control actin network dynamics (Rodriguez Del Castillo et al., 1992). The concentration of scinderin in platelets was estimated to be 75 fmol/mg total protein (Rodriguez Del Castillo et al., 1992, a). This might represent 11% of the total actin-filament severing activity if both proteins are equally potent, on molar basis, in severing actin filaments. Taking into consideration our results in chromaffin cells, we have considered, and opposite to common accepted ideas, that F-actin disassembly and not its polymerization (at least at certain specific sites) might be required for platelet secretion and scinderin could be an important component of the exocytotic machinery in platelets as well.

## Statement of the Problem

The purpose of this project was to further study scinderin, its molecular biology and the specific functions of its domains. As discussed above, adrenal chromaffin cells possess a network of actin filaments underneath the plasma membrane, this physical barrier prevents the free movement of the secretory vesicles and exocytosis. Release of the granule contents to the cell exterior through exocytosis is possible only when the cortical actin barrier is removed, that is to say when the actin filaments are severed at least locally. The existence of endogenous actin-binding and severing proteins (gelsolin, scinderin) which regulate actin network dynamics suggests an important role for these proteins in the stimulus-evoked disassembly of F-actin and exocytosis. In our view, scinderin seemed to be a better candidate than gelsolin for the regulation of secretion. Scinderin is mainly present in secretory tissues whereas gelsolin is a ubiquitous protein, scinderin (more concentrated in the subplasmalemmal region), but not gelsolin (which has a diffuse cytoplasmic presence) is redistributed (and the redistribution precedes) during F-actin disassembly and exocytosis, and the exocytotic sites are preferentially localized to cortical areas devoid of F-actin and scinderin. Therefore, the effects of cell stimulation appear to be specific for scinderin.

The present project was undertaken for the following purposes:

1. To clone and sequence the cDNA of scinderin, to analyse its amino acid sequence in order to better understand its structure, function and relation with the other known  $\text{Ca}^{2+}$ -dependent severing proteins.
2. To express and purify recombinant scinderin in high amounts so it can be applied to two different biological systems (permeabilized chromaffin cells and platelets) to study its mechanism of action and relevance for the neurosecretory process. In particular we were interested **a:** to determine the role, number, localization and function of actin binding sites (if present) and **b:** to study the role of  $\text{PIP}_2$  binding sites (if present) in the context of scinderin activation.

CHAPTER 2  
METHODS AND MATERIALS

## 2.1 Chromaffin Cell Culture

**Isolation of cells:** Bovine adrenal glands were obtained from a local slaughterhouse and chromaffin cells were isolated by collagenase digestion and further purified using a percoll gradient. A homogeneous population of bovine adrenal chromaffin cells was obtained as described by Trifaró and Lee (1980). The glands were rinsed at the slaughterhouse with buffer I ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -free Locke's buffer, in millimolar: NaCl, 154; KCl, 2.6;  $\text{K}_2\text{HPO}_4$ , 1.25;  $\text{KH}_2\text{PO}_4$ , 0.5; glucose, 10; pH 7.0; antibiotics: penicillin, 200 mg/l; streptomycin, 50 mg/l; gentamycin, 50 mg/l; 5 mM HEPES and Phenol red, 15 mg/l. On arrival at the laboratory, glands were rinsed again and perfused with the same buffer via a polyethylene tube inserted in the main adrenal vein for 10 min. The glands were then dissected and cortexes were removed. Each gland was perfused in a closed circuit perfusion chamber for 60 min with 30 ml enzyme mixture (DNAseI, 5.3 mg/l; collagenase, 548.13 U/gland and mycostatin, 25,000 U/l in 400 ml buffer I) previously sterilized through a millipore filter at 37°C at the rate of 10 ml/min. Once the gland flaccid, the remaining cortex was removed and the medullae were minced. The minced medullae were then transferred into a "trypsinizing flask" containing the same enzyme mixture pre-warmed and stirred for 30 min in a water bath at 37°C. The tissue was filtered and rinsed through a 44  $\mu\text{m}$  sterile cloth mesh into a graduated cylinder containing buffer II (1.2 mM  $\text{MgCl}_2$ , 2.2 mM  $\text{CaCl}_2$  added to

buffer I, pH 7.2). Cell number was determined by using a haemocytometer (Neubauer, Levy chamber Cat.500), then the number of the Percoll gradients to be used was estimated. The above solution was centrifuged for 10 min (50 x g) at room temperature, the sediment was washed again with 100 ml buffer II and centrifuged at 50 x g for 15 min at room temperature. The sediment was resuspended in Eagle's Balance Salt Solution (EBSS 1x) containing antibiotics : streptomycin, 100 mg/l; penicillin, 100 mg/l; mycostatin, 25,000 U/l; gentamycin, 50 mg/l. Taking into consideration the number of Percoll gradients to be used, the suspension was mixed with Percoll - colloidal silica coated with polyvinylpyrrolidone at pH 7.2 - and EBSS 10x in the ratio 8:9:1. The mixture was centrifuged at 20°C, 45,000 x g, for 20 min. After discarding the top of the gradient, corresponding to cortical cells and debris, the bands corresponding to the desired chromaffin cell population were collected. The gradient fractions were then diluted with five volumes of EBSS 1x containing antibiotics and centrifuged again at 50 x g, room temperature, for 15 min. The supernatant was discarded and the pelleted cells were washed with EBSS 1X /antibiotics. An aliquot was removed, the cell number was estimated and the whole yield was calculated prior the plating.

**Cell culture:** The cell sediment was suspended in Dulbecco's modified Eagle's medium (DMEM: 10 % fetal calf serum, 0.1 mM ascorbic acid, 0.1 % glucose and 15 mM HEPES, pH 7.2)

including penicillin, 100  $\mu\text{g/ml}$ ; gentamycin, 50  $\mu\text{g/ml}$ ; streptomycin, 100  $\mu\text{g/ml}$ ; nystatin, 25,000 U/l; 10 mM 5-fluorodeoxyuridine and 10 mM cytosar. Cells were plated on collagen-coated plastic Petri dishes at the density of  $0.3 \times 10^6$  cells/35mm dish for studies of immunohistochemistry,  $1 \times 10^6$  cells/35 mm diameter dish for studies of catecholamine output and RNA extraction. Cells were grown at  $37^\circ\text{C}$  in a humidified incubator (NAPCO 6300) under  $\text{CO}_2$  + air atmosphere. After 24 h (period of initial attachment), cells were fed every 2-3 days with the medium previously described. Cultures were periodically examined by phase contrast optics. The age of the cultured chromaffin cells utilized in all studies described in my thesis was between 48 and 72 h.

## **2.2 Source of Antibodies**

Polyclonal antibodies were raised in rabbits against purified bovine chromaffin cell scinderin as previously described (Rodriguez del Castillo et al., 1990). Scinderin antiserum #6 (which was used for screening the cDNA library, immunocytochemistry and most of the Western blots performed for the experiments presented here) thus obtained does not recognize gelsolin, another  $\text{Ca}^{2+}$ -dependent actin severing protein, and does not cross-react with any cytoplasmic or cytoskeletal proteins from chromaffin cells (Rodriguez del Castillo et al., 1990; Vitale et al., 1991). Moreover, scinderin was the only protein immunoprecipitated from an

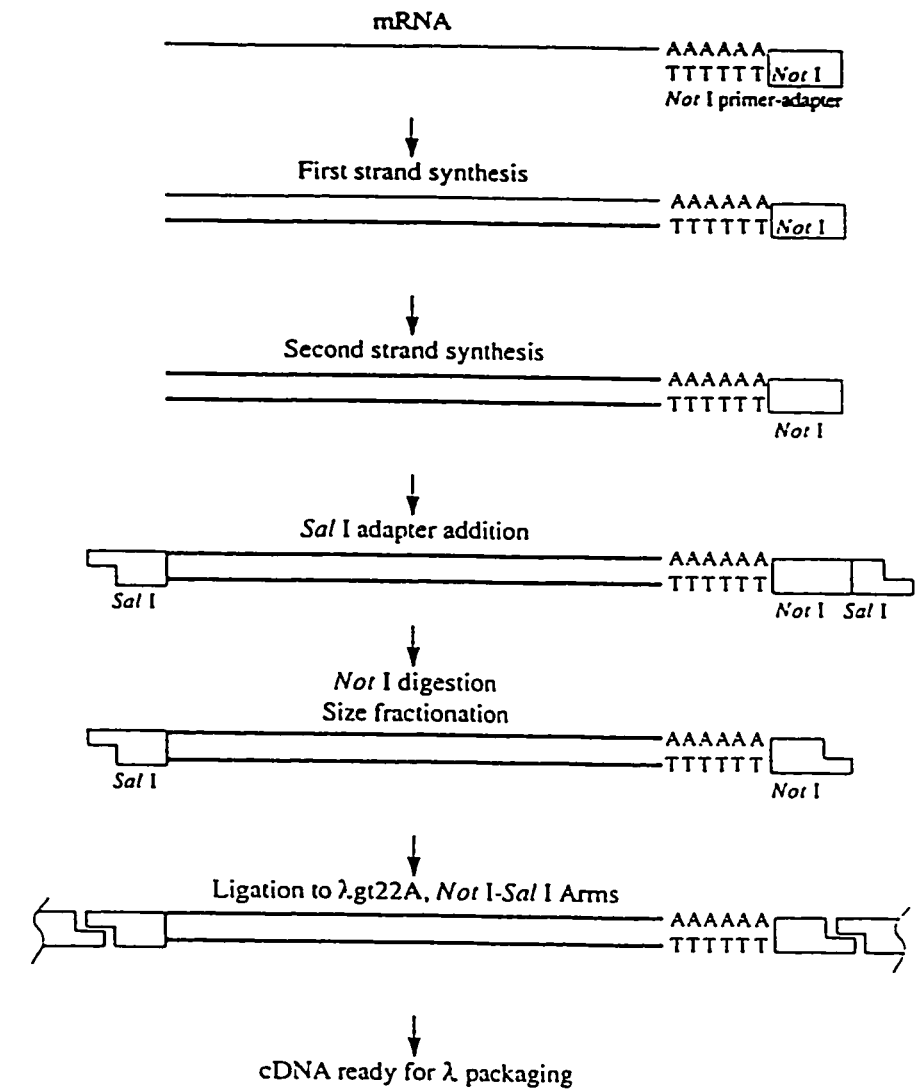
adrenal medullary cytosol preparation by antiserum #6 (Tchakarov et al., 1990). Antibodies against fusion protein F-Sc6 ( GST-Sc6), the first scinderin fusion protein obtained in our laboratory, were raised in white New Zealand rabbits by intramuscular injection of 200-250  $\mu$ g protein /rabbit in complete Freund adjuvant. Rabbits were boosted 15 days later with 100  $\mu$ g protein/rabbit. Four weeks later the titer of the antibody was high enough to give positive Western blots at 1:5000 dilution.

### 2.3 Construction of a Bovine Chromaffin Cell cDNA Expression Library

Total RNA was isolated from 2-day-old cultured chromaffin cells using the lithium/urea/phenol method described by Birnboim et al. (1988). Poly (A<sup>+</sup>) RNA was then isolated by oligo (dT) column chromatography (Aviv et al., 1972). Using 5  $\mu$ g of poly (A<sup>+</sup>) RNA a directional cDNA library was constructed in a  $\lambda$ gt22A using the "SuperScript Lambda System for cDNA Synthesis and Cloning" (Bethesda Research Laboratories, BRL). A summary of the whole procedure together with the sequences of the two adapters used is presented in Fig. 2.3.a. A NotI primer-adapter was used to prime the first strand synthesis. SuperScript reverse transcriptase lacking RNaseH activity was employed to synthesize the first strand cDNA. The cDNA was labeled with [ $\alpha$ -<sup>32</sup>P]dCTP, 1  $\mu$ Ci/ $\mu$ l (Amersham). Following the second strand synthesis which is

catalyzed by *E. coli* DNA polymerase I in combination with *E. coli* RNase H and DNA ligase, the *Sal*I adapter is added to the DNA. Size fractionation of cDNA, following adapter addition and digestion with *Not*I is required in order to remove residual adapters, contaminating fragments, small (<500 bp) inserts. Cerenkov counting and agarose gel analysis of the collected cDNA samples identified the fractions to be used in subsequent steps. Fig.2.3.b presents an autoradiogram of the dried agarose gel containing aliquots of the various column chromatography fractions collected and further used for ligation to the  $\lambda$ gt22A vector. This phage can be used to express cloned genes by inducing the *lac* promoter when is introduced into cells containing the *lac* repressor gene such as *E. coli* Y1090 *r*<sup>-</sup>. Transcription of cDNA inserts from the promoter is effectively repressed unless the promoter is induced by the addition of 1 mM isopropylthio- $\beta$ -galactoside, IPTG, a lactose analog, to the media. When the agar contains the noninducing chromogenic substrate X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) the blue-colored product provides a marker for monitoring the transfer of genes. The phage is supplied predigested with *Sal*I and *Not*I and has been prepared to provide a low background of nonrecombinant colonies. Fifty ng of cDNA was used for ligation with the vector which was packaged *in vitro* and then introduced into *E. coli* Y1090 (*r*<sup>-</sup>) by infection. The culture medium and plates for *E. coli* Y1090 were made with rich medium -LB- and contained ampicillin, 100

$\mu\text{g/ml}$ . For initial analysis of the cDNA library, the ratio of recombinants to nonrecombinants may be estimated by blue/white screening (add IPTG and X-gal to the agar plates). The estimated sizes of the 3 best libraries thus obtained were 500,000 , 350,000 and 750,000 plaque forming units (p.f.u.). The 3 libraries were combined and plated on IPTG/X-gal agar to generate a density suitable for screening, 15-20,000 p.f.u. per 150 mm dish. The entire library was screened the following days. A previous attempt to screen an amplified 500,000 p.f.u. cDNA library lead to negative results despite the fact that amplification was successful.



5' -pGACTAGTTCTAGATCGCGAGCGGCCGCC (T)<sub>15</sub>-3'  
*Spe I Xba I Nru I Not I*

Sequence of the *Not I* Primer-Adapter.

5' -TCGACCCACGCGTCCG -3'  
 3' - \_\_\_\_\_GGGTGCGCAGGCp-5'  
*Sal I Mlu I*

Sequence of the *Sal I* Adapter.

FIGURE 2.3.a Summary of SuperScript Lambda System Procedure and Sequences of the Adapters (taken from the Instruction Manual, BRL).

Kbp

10 11 12 13 14 15 16 17

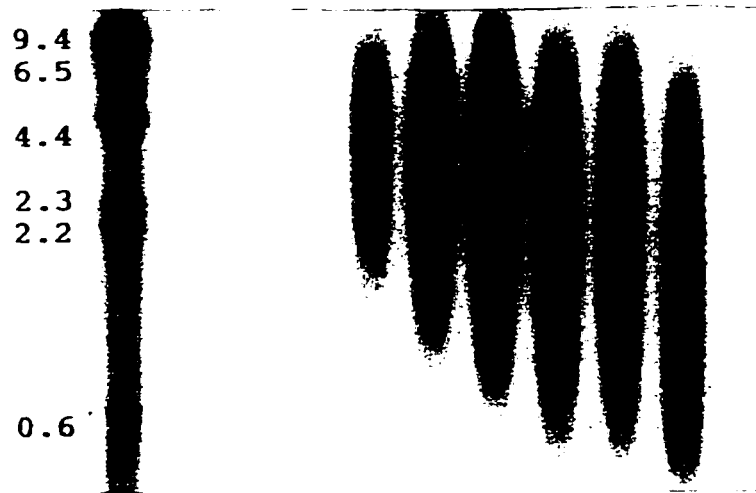


FIGURE 2.3.b An Autoradiogram of Fractionated Second Strand Chromaffin Cell cDNA. Each lane represents aliquots of individual fractions that were collected from the Sephacryl S 500 HR sizing column. Fractions 12-17 were used for further ligation to the phage.

#### 2.4 Screening of the cDNA Library

The  $\lambda$ gt22A cDNA library was screened with the Sc polyclonal antibody #6. However, prior to screening, the antibody preparation was immuno-absorbed by incubation with E-coli Y1090 lysate (Sambrook et al., 1989). The library was plated on 0.7% top agarose/LB in 15 mm dishes and incubated at 37°C for 8 h, then IPTG-soaked nitrocellulose filters (Amersham) were placed on the top of the dishes and further incubated at 42°C for other 3 h. Two replicas of nitrocellulose filters were made from each plate in order to eliminate artifacts. The filters were then screened according to Sambrook et al., 1989. Sc polyclonal antibody was diluted 1:1,000. For details see Chapter 2.8 - Electrophoresis and Immunoblotting. Antigen-antibody complexes were detected by alkaline phosphatase goat anti-rabbit IgG (BioRad) 1:3,000, followed by treatment with nitro-blue tetrazolium chloride and bromochloride indolylphosphate toluidine chromogenic reaction (BioRad) as described by Sambrook et al., 1989. The positive clones were further identified, isolated, replated and rescreened five times until phage stocks were pure (Sambrook et al., 1989). The final stocks had a titer of aprox. 5,000 p.f.u./ $\mu$ l. Eight positive clones were isolated from a total of  $1.6 \times 10^6$  p.f.u. These were labelled as Sc6, Sc2 and Sc8 = 2.7 Kb; Sc9 and Sc21 = 2.3 Kb; Sc19 = 2.1 Kb; Sc16 = 1.6 Kb and Sc20 = 1.3 Kb.

## 2.5 DNA Sequencing

All positive clones were then sub-cloned in pBluescript II KS M13+ plasmid (Stratagene) using the *SalI* and *NotI* restriction sites present both in clones and vector. The lambda phage DNA was obtained by precipitation with a cationic detergent, CTAB, using a Stratagene kit. Once restricted, the phage DNA was purified using GeneClean II (Bio101 Inc., CA) and ligated to the phagemid DNA ("DNA ligation kit", Stratagene). The ligation mix was further transformed into the host, *E. coli* XL1-blue, by  $\text{CaCl}_2$  method (Sambrook et al., 1989). The partial sequencing of the 3' end of all 8 clones was carried out by the dideoxy method (Sanger et al., 1977) using ds DNA Cycle Sequencing System (according to manufacturer, BRL). The results obtained revealed the common identity of the clones. Two different approaches were then used to sequence the full length of the longest clone (clone Sc6 = 2.7 Kb): a) a restriction map showed the presence of one *HindIII* and three *EcoRI* sites; the fragments obtained by digestion with these enzymes were subcloned again in the same plasmid and separately sequenced using ds DNA (both strands) in a cycle sequencing method using  $^{32}\text{P}$ , as previously described by the manufacturer (BRL). The ds DNA was also prepared according to BRL. The 5' end of the 8 clones was sequenced employing the same method; b) the entire length of the clone Sc6 was, again, sequenced at least twice, but this time by the  $^{35}\text{S}$  dideoxy sequencing method using Sequenase version II DNA polymerase

(US Biochemicals). The ss DNA was recovered from pBluescript II in the presence of VCS-M13 helper phage (Stratagene). Gene specific, synthetic oligonucleotide primers (18-20 bp) were constructed (Beckman Instruments Oligo 1000 DNA Synthesizer) and they were used in the above reactions. The 5' cDNA end obtained by "anchored" PCR was sequenced using ds and ss DNA. Fig. 2.5 presents a map of the phagemid vector.

## 2.6 "Anchored" Polymerase Chain Reaction Preparation of the 5' end of Scinderin cDNA

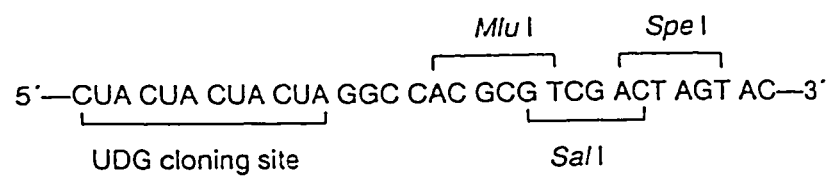
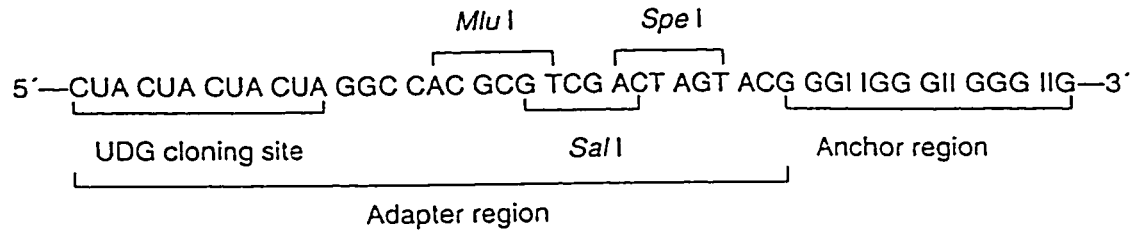
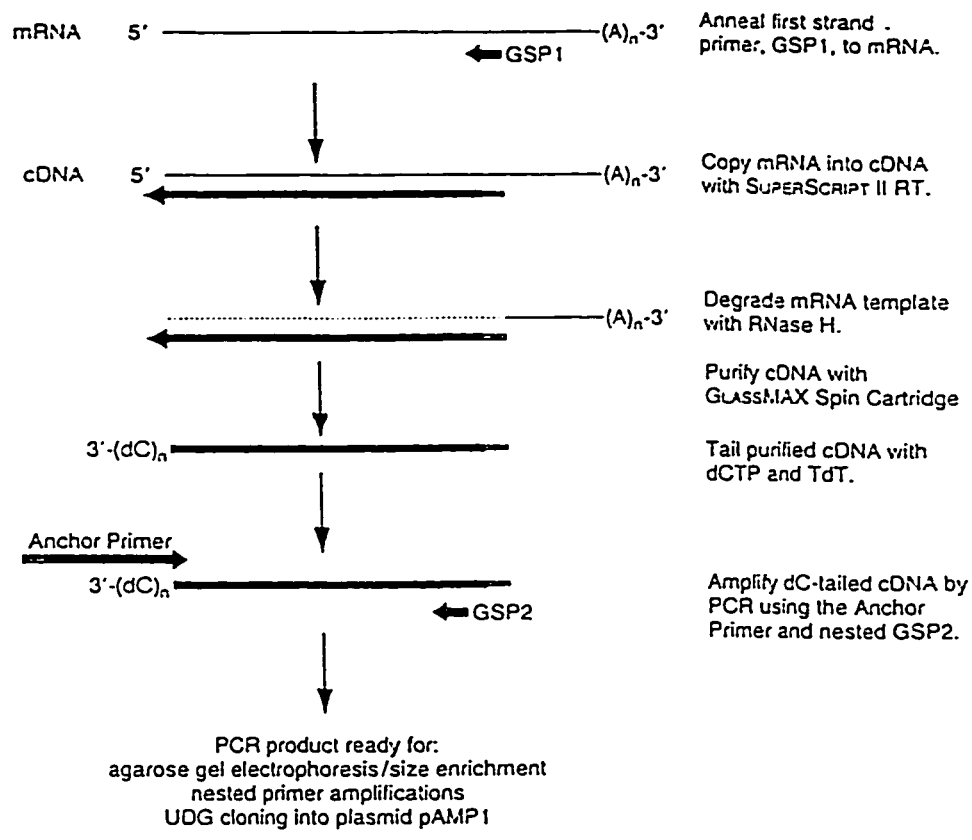
The "Rapid Amplification of cDNA Ends" method (RACE) developed by BRL was employed (Frohman et al., 1988). Using 1  $\mu$ g of the same chromaffin cell mRNA and 2 gene specific primers designed on the basis of the N-terminal sequence of clone Sc6 (GSP1a and a nested GSP2a, the last one carrying a designed sequence for the restriction enzyme *BamH I*), a RACE anchor primer (provided by BRL), cDNA was synthesized and amplified by PCR. A single clear DNA band of the expected size was visualized by agarose gel electrophoresis. This DNA was purified using Gene Clean (Bio101 Inc., CA), reamplified using a Sc-derived primer (GSP2) and the "universal amplification primer", UAP) and then sub-cloned in pBluescript II KS M13+ (Stratagene) using the restriction sites *BamHI* and *SalI*. Six pools of DNA coming from 6 different colonies were sequenced and the results indicated that all had the same bp sequence. Fig.2.6. presents an overview of the 5' RACE procedure.



The sequences of the "anchor" primer (provided by BRL) and the two Sc-derived primers are also presented in Fig.2.6. Unfortunately, the 5' DNA thus obtained did not contain any restriction site to be used for ligation with the longest clone, Sc6. Another RACE was performed using two nested Sc-derived primers closer to the 3' end - GSP1b (5' - CGT GAC CCA CTT TTT CTG 3') and GSP2b (5' - ACG CGA ATC CGC CTG CCA CCA GAC TCA CTT TCA TGC 3') - in order to amplify a longer Sc DNA, this time including a known *Hind*III site. The resulting Sc 5' end, ScR, was further ligated to the *Hind*III restricted Sc6. The whole scinderin cDNA was then subcloned in pBluescript II KS M13 and sequenced. Fig.3.1 (Chapter 3) presents a diagram of the Sc6, ScR and the whole scinderin cDNA -Sc1. For convenience, all the following data will use Sc cDNA to name Sc1.

## 2.7 Northern Blot Analysis

Bovine chromaffin cell total RNA and mRNA, rat and rabbit brain total RNA purified as described by Birnboim et al. 1988, were electrophoresed in 1% agarose-formaldehyde (Sambrook et al., 1989) and transferred onto nylon membranes (Magna M.S.I.). The position of 28S and 18S rRNA was visualized by ultraviolet illumination of the gel due to the addition of ethidium bromide to the RNA loading buffer. Hybridization was performed for 24 h in 50% formamide, 5 x SSC (750 mM sodium chloride, 75 mM sodium citrate), 5 x Denhardt solution (0.1% Ficoll 400, 0.1% PVP, 0.1% BSA) and 0.5% SDS at 42°C



**FIGURE 2.6 Overview of the 5' RACE Procedure, Sequences of the BRL Primers** (taken from BRL Instruction Manual).

containing  $1 \times 10^5$  CPM of  $^{32}\text{P}$  labelled random prime DNA probe per ml. This was followed by high stringency washes in 5 x SSC, 0.5% SDS at 65°C, 0.1 x SSC, 1% SDS at 50°C and 2 x SSC at room temperature. The filter was exposed to X-ray film (Kodak X-OMAT-AR) for 24 h at -70°C using a cassette with intensifying screen.

#### **Preparation of a probe**

The ds DNA restriction fragment (500 bp) produced by treatment of Sc6 with *SalI* and *HindIII* was chosen as probe. Twenty-five ng of DNA were labelled with ( $\gamma^{32}\text{P}$ ) dCTP (Amersham) using the "Random Primers DNA Labelling System" (BRL, specific activity =  $1 \times 10^9$  CPM/ $\mu\text{g}$ ).

#### **2.8. Electrophoresis and Immunoblotting**

Monodimensional SDS-PAGE was performed according to Doucet and Trifaró, 1988. Gels will usually run at 60 V overnight in a Bio-Rad Protean I apparatus (Bio-Rad Laboratories Inc., Richmond, CA). The protocol for the immunoblotting was as described by Towbin et al. 1979. After electrophoresis proteins were electrotransferred onto nitrocellulose membranes (Hoefer Scientific Instruments, San Francisco, CA). Membranes were first blocked with 3% BSA in TNT buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH=8) and then incubated with scinderin antiserum #6 (1 : 1,000 dilution) or with the antisera raised against fusion Sc, F-Sc (1 : 1,000 dilution). Membranes were next incubated with goat anti-rabbit

immunoglobulin G - alkaline phosphatase conjugate (1 : 3,000 dilution) for another 60 min. Colour was developed by treatment with a mixture of p-nitro blue tetrazolium chloride and 5-bromo-4-chloride-3-indolylphosphate toluidine salt.

For Western Blots analysis the TRX-Sc deletions (fusion proteins) a monoclonal anti-thioredoxin antibody, dilution 1:5,000 (Invitrogen), was used. The second antibody, goat anti-mouse, was diluted 1:3,000 in the same buffer as above, PBS. Washings, colour development were done as above.

## **2.9 Amino Acid Sequence Analysis**

Cyanide bromide peptides prepared from purified scinderin were isolated by HPLC. The amino acid sequence analysis of the peptides was carried out by the Edman degradation method on a gas-phase sequenator (model 470 A, Applied Biosystems), coupled in line to a HPLC analyzer (Henvick et al., 1981). The sequence analyses were performed by Dr. Max Bluom (according to Henvick et al., 1981), Department of Biochemistry, University of Toronto, according to the procedure described by Henvick et al., 1981.

## **2.10 Bacterial Expression; Purification of Expressed Proteins**

### **2.10.1 Preparation and Purification of Fusion GST-Scinderin :**

The glutathione S-transferase (GST) gene fusion system was used for the expression, purification and detection of fusion

proteins (Smith et al., 1988). Clones Sc1 and Sc6 were separately subcloned in expression vectors pGEX 4T-2 and pGEX 4T-3 respectively (Pharmacia) using restriction sites Sall and NotI. Recombinant plasmids were then transfected into the host E-coli JM 105 (Pharmacia). Transformed E-coli were grown for 24 h at 37°C in 5 ml of Luria-Bertani's (LB) medium containing 100 µg ampicillin/ml. The culture was diluted 10 times with LB medium and grown (approximately 4 h) to a density given a reading of 0.8 at the absorbance of 600 nm. Cells were then induced for 4 h by addition of isopropyl β-D thiogalactopyranoside (IPTG) at the final concentration of 0.1 mM. An aliquot of the bacterial culture was centrifuged at 3,000 g for 10 min and the sediment was suspended in 1 ml 50 mM Tris, 2% SDS and 1 mM EDTA (pH 8.5). Aliquots (150 µl) were tested for the presence of fusion protein by immunoelectrophoresis using scinderin antiserum #6. Two hundred ml of LB medium containing 100 µg ampicillin/ml were inoculated with transformed E-coli, grown for 12 h at 30°C under strong agitation. The culture was then diluted 10 times (2 L) with LB medium and incubated with agitation at 30°C for 4 h. IPTG (0.1 mM final concentration) was added and incubation continued for an additional 4 h period. Cells were then centrifuged at 3,000g for 10 min and the sediments were resuspended in 100 ml of 125 mM Tris-HCl and 1 mM EDTA (pH 8.0). The suspension was then sonicated in a Fisher 300 apparatus (setting 5). Triton-X100 was added to obtain a

final concentration of 1% and incubation continued for an additional 30 min at room temperature. The preparation was then applied to a Glutathione-Sepharose 4B column. The scinderin fusion protein was cleaved from GST on the column matrix by treatment with 20 units of thrombin (10 units/mg protein) for 16 h. GST-Sc proteins were eluted with PBS buffer (in mM: 130, NaCl; 100, Na-phosphate, pH 7.0) following the instructions of the manufacturer (Pharmacia). The presence of the proteins in the eluate was determined by immunoblotting using scinderin antiserum #6. Fig. 2.10.1 presents a map of the GST fusion vectors. The purified GST-Sc6 was used to produce antibodies as described in Chapter 2.2.

#### **2.10.2. Preparation and Purification of Fusion TRX-Scinderin :**

The Thioredoxin ThioFusion System (Invitrogen, San Diego, CA) was used for the expression and purification of the fusion proteins, full-length scinderin and various truncations of it. This system allows expression and purification of large amounts of soluble heterologous proteins. The fusion of thioredoxin to foreign proteins may confer a unique cellular location allowing purification by osmotic shock. Native thioredoxin localizes at particular sites on the cytoplasmic side of the inner membrane known as "adhesion zones", which create an "osmotically sensitive" cellular compartment. The fusion scinderin thus obtained appeared to have full

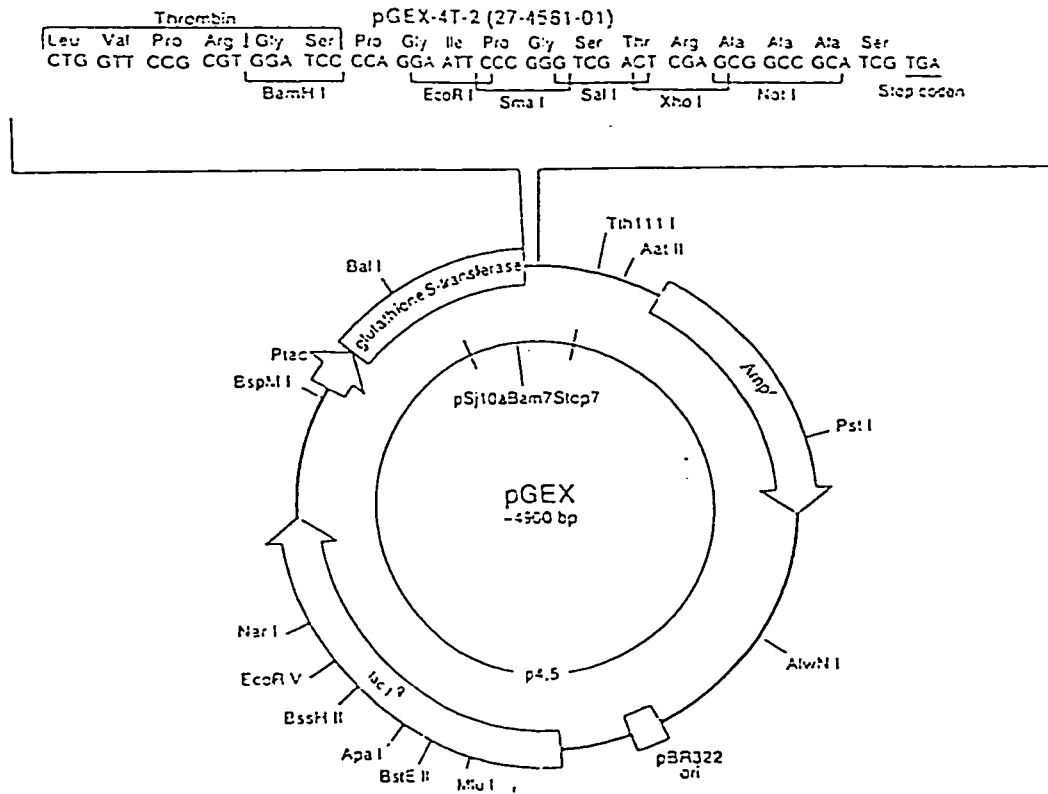


FIGURE 2.10.1 Map of the Glutathione S-Transferase Fusion Vectors (taken from Pharmacia Instruction Manual). Scinderin was subcloned between *SalI* and *NotI* restriction sites.

biological activity, therefore it was not necessary to release thioredoxin from the fusion. The digestion of the fusion protein with enterokinase could not be achieved, while the required conditions lead to the rapid proteolysis of scinderin. The fusion proteins were partially purified by osmotic shock (for actin- and phospholipid-binding studies) and highly purified by actin-affinity chromatography for the catecholamine release studies (Sc full length and Tr-Sc = Sc3-6).

**2.10.2.1 Full-length Scinderin** cDNA was subcloned in expression vector pTrxFus using Sma I restriction site (blunt ends) of the vector and SmaI and NotI (Klenow filled in) of scinderin cDNA previously cloned in pGEX 4T2 (Pharmacia). Recombinant plasmids were then transfected into the host E-coli GI698. Transformed E-coli were grown overnight at 29°C in 100 ml of RM medium containing 100 µg ampicillin/ml. The culture was diluted 10 times with induction medium and grown for 4 h before induction with tryptophan (100 µg/ml; final concentration). After 2 h incubation at 29°C, the OD<sub>550</sub> was measured and cells were sedimented. Using osmotic shock solution 1 (20 mM Tris-HCl, pH 8; 2.5 mM EDTA; 20% sucrose) the cells were resuspended to an OD<sub>550</sub> of 5, incubated for 30 min on ice and centrifuged at 10,000 g for 10 min. The supernatant was decanted and the cell pellet was resuspended

in osmotic shock solution 2 (20 mM Tris-HCl, pH 8; 2.5 mM EDTA), incubated on ice for 30 min and centrifuged at 10,000 g for 10 min. The supernatant ("shock fluid") was dialyzed against buffer A (20 mM Tris-HCl, pH 7.5; 1 mM DTT; 1 mM Ca<sup>2+</sup>; 1 mM PMSF; 1 mM NaATP) and then applied to an actin-DNAse I-Sepharose 4B affinity column (Marcu et al., 1994; Bader et al., 1986). The binding of scinderin to actin allowed the retention of the fusion protein complex thioredoxin-scinderin in the affinity column. The actin-DNAse I-Sepharose 4B affinity column was prepared as described previously (Bader et al., 1986). The column was washed with buffer A prior the application of the dialyzed shock fluid. The recombinant protein was eluted from the column with buffer B (Ca<sup>2+</sup>-free buffer A containing 10 mM EGTA (Marcu et al., 1994). The preparation was dialyzed against EGTA-free K<sup>+</sup>-glutamate buffer (for release experiments) or water (for the rest of experiments) and then lyophilized. Purified thioredoxin (TRX) as control, was prepared using expression vector pTrx (Invitrogen, San Diego, CA). This plasmid encoding wild type TRX was transfected into E-coli GI698 (CaCl<sub>2</sub> method, Sambrook et al., 1989). Transformed E-coli GI698 was processed to obtain "shock fluid" as described above. All protein samples were analyzed by SDS-PAGE and immunoblotting using an antibody against native scinderin. Monodimensional (SDS-PAGE) sodium dodecylsulfate-polyacrylamide gel electrophoresis were performed according to Doucet and Trifaró, 1988.

Fig.2.10.2.1 presents a map of the pTrx vectors and an overview of the purification procedure.

**2.10.2.2 Truncated Scinderin, Tr-Sc (Sc3-6)- fusion protein,** consisting of the last four domains ( $S_3$ ,  $S_4$ ,  $S_5$ ,  $S_6$ ) starting at aminoacid 254, valine, was obtained by PCR. Vent<sub>5</sub> DNA polymerase (New England Biolabs, Beverly, Mass.), Ultra-pure dNTP set (Pharmacia) and two specifically designed primers carrying exogenous sequences of restriction enzymes *Bam*HI (in 5'ACT GCA GGA TCC ATT GGT TTC AGA TGC CAG TGG 3') for the 5'-end of Tr-Sc and *Sal*I (in 5'GTC GTG TCG ACA GTC AGC ATC CAG CAG AGC TA 3') for 3'end of Tr-Sc were used to amplify the desired sequence of scinderin. The primers used in PCR were synthesized with Beckman Instruments Oligo 1,000 DNA Synthesizer, Microbiology dept. Sc DNA was further purified (GeneClean) and cloned in the same vector, pTrxFus. Transformed E.coliGI698 were grown and harvested as described for full-length scinderin. The protein was purified by osmotic shock, dialyzed and lyophilized as indicated above.

**2.10.2.3 Various Deletions of Scinderin expressed as TRX-fusion proteins.** The TRX Thio-Fusion System (Invitrogen, San Diego, CA) was used for the expression and purification of other fusion proteins derived from Sc. Different truncations (deletions) of Sc were obtained by PCR (for details see chapter 2.10.2.2). To facilitate subcloning, the primers were

designed to include a *Bam*HI restriction site for 5' end and a *Sal*I site for 3' end. The obtained PCR products were digested with *Bam*HI and *Sal*I and subcloned in expression vector pTRX Fus using the same restriction sites, taking into consideration the reading frame. For Sc1-4,6 a different strategy was used: a DNA fragment of the first 4 domains was obtained by PCR using two primers, one containing *Kpn*I restriction site (for 5' end) and the other - *Bam*HI (for 3' end). This Sc1-4 DNA was ligated to Sc6 previously obtained in the same pTRX Fus vector, which was first cut with *Kpn*I and *Bam*HI. One new aminoacid was introduced just in front of the amino end of segment 6 of Sc. Recombinant plasmids were then transfected into the host E.coli GI698. The sequences of all clones were confirmed by dideoxy sequencing. The PCR primers used to construct the fusion proteins are as follows:

5' ACT GCA GGA TCC ATT GGT TTC AGA TGC CAG TGG 3' forward  
for: TrSc, Sc3-4, Sc3-<sub>1/2</sub>4,

5' GTC GTG TCG ACA GTC AGC ATC CAG CAG AGC TA 3' reverse  
for : TrSc, Sc5-6, Sc<sub>1/2</sub>5-6, Sc<sub>1/2</sub>5-6, Sc6.

5' GTC GTG TCG ACT TGC TCC TTG CCA GGT GTA G 3' reverse  
for: Sc 3-<sub>1/2</sub>4

5' ACT GCA GGA TCC AGA AGG TCA GGC ACC AGC C 3' forward  
for: Sc5-6

5' CTC GTG TCG ACC TGG TGC CTG ACC TTC TTT C 3' reverse  
for: Sc3-4

5' ACT GCA GGA TCC AGA GAA AGG AGC AGA GTA CG 3' forward

for: Sc<sub>1/2</sub>5-6

5' ACT GCA GGA TCC AGG CCT GGC TTC GAT CAC CAG 3' forward

for: Sc<sub>2</sub>5-6

5' ACT GCA GGA TCC AGC ATC CCA GGC TGA AGA CCA TC 3'

forward for: Sc6.

5' ACT TGG TAC CAA TGG CCC AGG GGC TGT AC 3'

forward for: Sc1-4,6.

5' TAG CAG GAT CCA CTG GTG CCT GAC CTT CTT TC 3' reverse

for: Sc1-4,6.

Transformed E.coli were grown, induced, harvested, the proteins were purified by osmotic shock, dialyzed against water and lyophilized as described above.

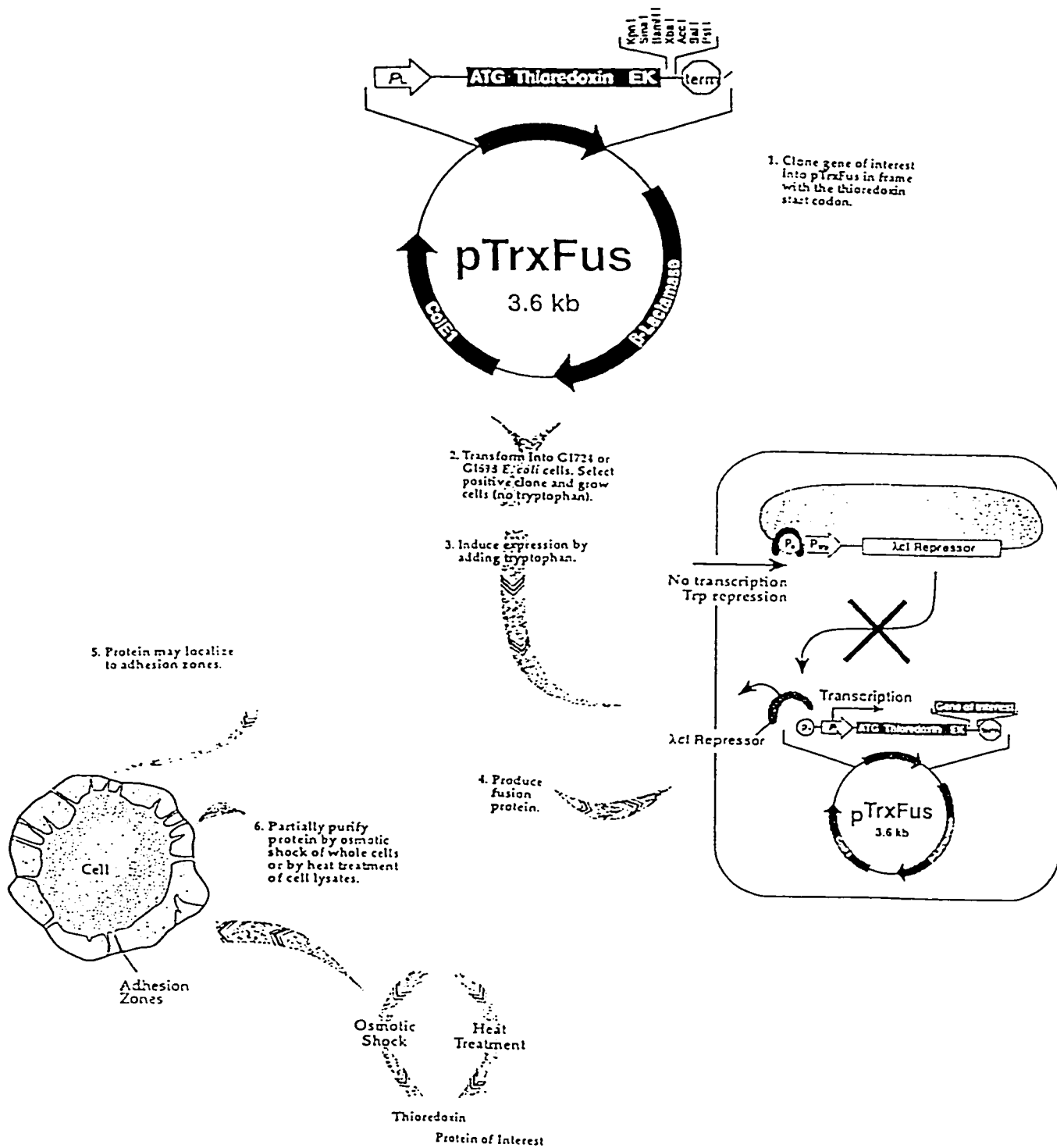


FIGURE 2.10.2.1 Map of pTRXFus. Purification of Fusion Proteins, Flow Chart (taken from Invitrogen Instruction Manual). Full length scinderin (blunt ends) was subcloned in *Sma*I, Tr-Sc as well as the smaller deletions were subcloned between *Bam*HI and *Sal*I. Sc1-4,6 was subcloned between *Kpn*I and *Bam*HI.

## **2.11 Protein Determination**

Protein assays in the range of 1-20  $\mu\text{g}$  were performed according to Bradford, 1976, using a commercially available product (Bio-Rad protein assay). Bovine serum albumin (Sigma) was used as a standard for the establishment of calibration curves. Whenever necessary, Coomassie stained SDS-PAGE gels were densitometrically scanned (Laser Densitometer, Ultro ScanXL, Bromma).

## **2.12 Interaction with Actin; Actin Binding Studies**

### **2.12.1 Actin-DNaseI-Sepharose 4B Binding Studies**

**2.12.1.1 Actin-binding of GST-Sc fusion protein:** Actin-DNaseI-Sepharose4B (Sigma) affinity column was prepared according to Bader et al., 1986. Chromaffin cell actin was used in the preparation of the column (Bader et al., 1986). The column was extensively washed with buffer A: 20 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM EGTA, 5 mM DDT and 1 mM phenylmethyl-sulfonyl fluoride (PMSF). Overnight cultures of E-coli JM 105 transformed with either parental (non-recombinant) or recombinant pGEX 4T2 plasmid were diluted 10 times with fresh LB medium (1 l final volume) and subsequently grown for 3 h at 30°C before adding 0.1 mM IPTG. This was followed by 3 h of growth and cells were then centrifuged at 3,000g for 10 min and resuspended in a 100 ml 100 mM Tris-HCl,

and 1 mM EDTA. Cells were lysed under mild sonication, 1% (final concentration) Triton-X100 (BDH) was added and the suspension was incubated at room temperature with agitation for 30 min. After centrifugation at 5,000g for 10 min, the supernatant was dialyzed overnight against buffer A. Before the supernatant was applied to the affinity column,  $\text{CaCl}_2$  was added to a final concentration of 1 mM. After washing the column with buffer A containing  $\text{Ca}^{2+}$ , the column was eluted with  $\text{Ca}^{2+}$ -free (5 mM EGTA) buffer A. The diluted proteins were analyzed by SDS-PAGE and immunoblotting using scinderin antiserum #6. See also 2.10.1 .

#### 2.12.1.2 Actin-binding of TRX-Sc fusion proteins:

See 2.10.2.1 for studies concerning purification of full-length Sc and truncated Sc (TrSc) TRX-fusion protein.

Different deletions of scinderin were tested for their ability to bind actin. The actin-DNaseI-Sepharose-4B affinity beads were prepared as described before (Marcu et al., 1996). The binding experiments were performed "in bulk", in tubes, rather than using columns. The rabbit skeletal muscle  $\gamma$ actin (G-150) was purchased from Cytoskeleton (Denver, CO). The buffers used for binding assay were either containing 1 mM  $\text{Ca}^{2+}$  in 20mM Tris-HCl (pH=7.5), 150 mM KCl, 1mM DDT, 1 mM NaATP, 1 mM PMSF, leupeptin 1  $\mu\text{M}$ , or 10 mM EGTA (Ca-free) in the same buffer as above. The washings were made with either buffer including this time 600 mM instead of 150 mM KCl. For the

EGTA -elution based experiments, the actin binding was performed in CaCl<sub>2</sub> buffer and elution was with EGTA buffer. For every different deletion of scinderin, 300  $\mu$ l actin-DNaseI-resin beads were incubated at 4° C for 1 h on a rotary shaker with 50  $\mu$ g of fusion proteins, then washed and centrifuged for recovery. The actin-DNaseI-Sepharose 4B was eluted with EGTA buffer for the EGTA-based experiments (when binding was done in Ca<sup>2+</sup> -based buffer). For all conditions, the actin-DNaseI-Sepharose 4B (pellet) and supernatants (EGTA eluates) were analysed by SDS PAGE and Western blot.

#### 2.12.2 Actin Cosedimentation Experiments:

The ability of all deletions of TRX-Sc fusion proteins to bind actin was also investigated by an actin-cosedimentation assay (Fechheimer et al., 1984). Monomeric actin (0.5 mg/ml, Cytoskeleton (Denver, CO) was induced to polymerize in 10 mM TrisHCl, 100 mM KCl, 2 mM MgCl<sub>2</sub>, 1mM CaCl<sub>2</sub>, 1 mM ATP-Na salt, 1mM DTT, 1  $\mu$ M leupeptin, pH=7.4. Proteins (final concentration 60  $\mu$ g/ml) were mixed at room temperature with actin and incubated for 90 min. Filamentous actin was sedimented by centrifugation for 60 min at 100,000 g. The supernatants were collected and tested for the presence of actin-binding proteins. The pellets were washed and the presence of fusion proteins and actin was tested by SDS-PAGE and Western blots. For determination of the K<sub>d</sub> (dissociation constant), various molar ratios between the truncated proteins and actin

(constant concentration of 4  $\mu\text{M}$ ) were used: 0.15:1 , 0.35:1 , 0.5:1 , 0.75:1 , 0.95:1 , 1.2:1 . The relative amounts of truncated scinderins (Sc3-4, Sc5-6) in the supernatants (free) and pellets (bound to actin) were determined by densitometric scanning of Coomassie Blue-stained SDS-gels. A Scatchard analysis (plotting bound/free on Y axis and bound to actin on X axis) was done in order to calculate the  $K_d$  (Scatchard, 1949).

### **2.12.3 Viscometry Analysis :**

Apparent viscosity was measured at low shear rates by the falling ball technique as described by MacLean-Fletcher and Pollard, 1980. This technique gives a semiquantitative characterization of the actin polymer solutions, which are non-Newtonian fluids. G-actin was induced to polymerize (4mg/ml) for 1 h at 22°C, then it was diluted to 1 mg/ml (0.22  $\mu\text{M}$ ) in the following buffer: 100 mM KCl, 2 mM  $\text{MgCl}_2$ , 5 mM EGTA, 0.005%  $\text{NaN}_3$ , 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$  (4.6 mM  $\text{CaCl}_2$ ) 1 mM NaATP, 1 mM PMSF, 1 mg Leupeptin/ml, 40 mM PIPES, pH= 6.8. Actin alone or mixed (at 0°C) with fusion proteins in different ratios was drawn into 100  $\mu\text{l}$  glass capillaries, sealed with plasticine at one end, and held horizontally at room temperature for 2 h before determination of the apparent viscosity. One stainless steel ball was used per capillary, one measurement per tube, and 3 measurements per condition were done. Capillary tubes were placed at an angle of 45°,

and the time for the ball to fall between 2 points was recorded. The viscosity is inversely proportional to the velocity. Apparent viscosity was calculated from a calibration curve obtained by measuring ball falling times through glycerol solutions (from 0-100%) of known viscosity and the results expressed in centipoises.

#### **2.12.4 Actin Nucleation Analysis :**

Six micromolar G-Actin (G150 Actin, Cytoskeleton, Denver, CO), containing 5% pyrene-iodoacetamide-labelled actin (Cytoskeleton, Denver, CO) were dissolved in low salt buffer: 0.2 mM NaATP, 0.5 mM DTT, 0.2 mM CaCl<sub>2</sub> and 5 mM Tris-HCl, pH=8, and left on ice for 30 min to allow complete depolymerization of small oligomers that might form during defrosting. The solution was then centrifugated at 100,000g for 1 h, at 4°C to remove any actin aggregates and small actin oligomers. The actin present in the clear supernatant was polymerized alone, in the presence of recombinant full length scinderin (r-Sc), recombinant Sc5-6, Sc1-4,6 or recombinant Sc<sub>2</sub>5-6 (the molar ratio of actin to recombinant protein was 400:1) by addition of 1/50th volume of polymerization inducer buffer: 2.5 M KCl, 100 mM MgCl<sub>2</sub> and 50 mM NaATP. Actin polymerization was performed at 22°C and was monitored by measuring the change in fluorescence, using a 10 nm band width, excitation and emission wavelengths of 365 and 407 nm respectively (Perkin Elmer LS5 Fluorescence Spectrophotometer). Fluorescence was

measured for 120 min.

## 2.13 Phospholipid-Binding Studies

2.13.1 **GST-Scinderin Fusion Protein, F-Sc1** (full-length) purified from transformed E-coli JM 105 as indicated above was dialyzed overnight at 4°C against the buffer: in mM: Tris-HCl, 20; NaCl, 100; MgCl<sub>2</sub>, 2; dithiothreitol (DDT), 1; PMSF, 1; EGTA, 5; pH 7.1. Phosphatidylinositol 4,5-bisphosphate (sodium salt, PIP<sub>2</sub>, Sigma) was dissolved in buffer B at the concentration of 1 mg/ml. Phosphatidylserine (sodium salt, PS, Sigma) was dissolved in the same buffer at the concentration of 10 mg/ml. Phospholipid suspensions were mixed by shaking in a vortex for 15 min and further sonicated for 20 min (Mettler Electronic Corporation, Anaheim, CA). Liposomes thus obtained (85 µl) were incubated with 200 µl protein samples (1 µg F-Sc1 per sample) in a final volume of 300 µl. Free Ca<sup>2+</sup> concentrations (from 10<sup>-7</sup> to 10<sup>-5</sup> M) were adjusted according to Caldwell et al., 1970. The mixtures were incubated at 37°C for 30 min, centrifuged at 78,000 for 30 min and supernatants and sediments were separated. Sediments were dissolved in 200 µl 50 mM Tris-HCl (pH 8.5), 2% SDS and 1 mM EDTA. The presence of F-Sc1 (GST-Sc) in supernatants and sediments was analyzed by SDS-PAGE.

**2.13.2 TRX-Scinderin Fusion Protein .** Ten  $\mu\text{l}$  (10  $\mu\text{M}$ ) of protein samples were incubated with 200  $\mu\text{l}$  PIP<sub>2</sub> (sodium salt, Sigma) liposomes prepared as described above, with or without 10  $\mu\text{M}$  Sc-PIP<sub>2</sub> BP in a final volume of 220  $\mu\text{l}$ . The incubation, centrifugation and analysis were performed as above. SDS-PAGE was followed by Western blots using a scinderin antibody (#6).

## **2.14 Fluorescence Microscopy**

### **2.14.1 Localization of Scinderin in Control and Stimulated Chromaffin Cells. Comparison Between the Antisera Raised Against Native Scinderin and Fusion Protein F-Sc6.**

(Antisera was raised against fusion protein GST-Sc6; at that time Sc6 was the longest Sc clone obtained). Chromaffin cells were plated on collagen-coated glass coverslips contained within plastic Petri dishes at a density of  $0.3 \times 10^6$  cells/35 mm dish. After 48 h in culture, cells were rinsed with Locke's solution (in mM: NaCl, 154; KCl, 2.6; K<sub>2</sub>HPO<sub>4</sub>, 1.25; KH<sub>2</sub>PO<sub>4</sub>, 0.50; MgCl<sub>2</sub>, 1.2; CaCl<sub>2</sub>, 2.2; and D-glucose 10.0; pH 7.2) and incubated under different conditions and then fixed in 3.7% formaldehyde in Locke's solution for 20 min and processed for fluorescence microscopy as described previously by Lee et al., 1981. Cells were permeabilized by three successive exposures of 5 min each to 50, 100 and 50% acetone. Coverslips were washed with phosphate-buffered saline (PBS, in mM: NaCl, 130; Na-

phosphate 100; pH=7.0), and incubated for 60 min at room temperature with a blocking solution consisting of 1% BSA in PBS. Chromaffin cells were stained for scinderin with either scinderin antiserum #6 (1/200 dilution in 0.1% BSA in PBS) or antiserum raised against fusion protein F-Sc6 (1/350 dilution in 0.1% BSA in PBS). Incubation with the primary antibodies was carried out for 60 min at 37°C. Next, coverslips were thoroughly washed with PBS and further incubated with goat antirabbit IgG-fluorescein isothiocyanate conjugate (FITC-IgG, SIGMA, MO) 1:250 dilution in 0.1% BSA in PBS) for 60 min at 37°C. Preparations were washed with PBS and mounted in glycerol-PBS (1:1). Slides were observed with a Leitz Ortholux fluorescence microscope equipped with a 200 W high-pressure lamp and a Ploemopack II incident light illuminator equipped with an L-filter block (KP 490 plus 1 mm GG 455 exciting filter, TK dichroic beam splitting mirror, K 515 suppression filter). One hundred single-rounded chromaffin cells per coverslip were examined. Each cell was classified as having either a "continuous" or "discontinuous" cortical fluorescent ring (Vitale et al., 1991). The percentage of chromaffin cells showing cortical scinderin redistribution (discontinuous fluorescent ring) was calculated for each experimental condition. In order to avoid personal bias, code numbers were given to each coverslip. The cells were examined and classified without knowing whether they were from control or treated preparations or whether they had been stained with

scinderin or fusion protein antisera. Only after all coverslips were examined and the results recorded were the codes revealed to identify the experimental conditions used (single-blind design). Photographs were taken with Kodak Tri-X pan films (400 ASA).

#### **2.14.2 Effect of PIP<sub>2</sub>, Recombinant TRX-Scinderin, Sc-ABP<sub>1</sub> and Sc-PIP<sub>2</sub>BP on Cortical F-actin**

Chromaffin cells were plated on collagen-coated coverslips contained within plastic petri dishes as a density of  $3 \times 10^5$  cells per 35mm dish. Cultured cells were rinsed with Locke's solution and then they were permeabilized by treatment with 20  $\mu$ M digitonin in K<sup>+</sup>-glutamate buffer for 5 min at room temperature. Cells were then incubated for 2 min with K<sup>+</sup>-glutamate buffer (139 mM K glutamate, 20 mM PIPES K salt, 5 mM EGTA, 2mM MgCl<sub>2</sub>, 2 mM ATP Na salt; pH = 6.6) in the absence (control) or presence (stimulated) of Ca<sup>2+</sup> and different compounds. Following these incubations, chromaffin cells were fixed in 3.7% formaldehyde and processed for fluorescence microscopy as described previously (Lee et al., 1981). Cells were permeabilized with acetone, washed with phosphate-buffered saline (PBS: 130 mM NaCl, 100 mM Na-phosphate, pH=7), and incubated for 60 min at room temperature with a blocking solution consisting of 1% bovine serum albumin (BSA) in PBS. Chromaffin cells were stained for F-actin with rhodamine-phalloidin (0.25 U/ml; Molecular Probes, Eugene, OR) for 40

min in the dark at room temperature. Finally, coverslips were rinsed with PBS and mounted in glycerol-PBS (1:1). Slides were observed with a Leitz Ortholux fluorescence microscope equipped with a 100 watt high pressure lamp and a Ploemopak II incident light illuminator (Vitale et al., 1991). Photographs were taken with Kodak-Tri-X pan films (400 ASA). To study the effect of several treatments on the percentage of cells showing cortical F-actin disassembly, 100 single-rounded chromaffin cells per coverslip (usually 8 coverslips per experimental condition) were examined. Each cell was classified as having either a "continuous" or "discontinuous" cortical rhodamine (F-actin) fluorescent ring. The percentage of chromaffin cells showing cortical F-actin disassembly (discontinuous rhodamine fluorescent ring) was calculated for each experimental condition. To avoid personal bias, code numbers were given to each coverslip. The cells were examined and classified without knowing whether they were from control or treated preparations. Only after all coverslips were examined and the results recorded were the codes revealed to identify the experimental conditions used (single-blind design).

### **2.15 Video-Enhanced Image Processing**

Quantitative analysis of cortical rhodamine fluorescence (F-actin) was performed by using a Hamamatsu Photonic KK Argus-50/CL Image Processor (Hamamatsu Photonic Systems, Bridgewater,

NJ) as described by Vitale et al., 1995. The fluorescence microscope was coupled to the video camera (Carl Zeiss, TV3M model), which was connected to the Argus 50-Image processor. Video camera control parameters (offset, sensitivity) were adjusted by using the image of a resting chromaffin cell on the monitor. Control parameters were set up to obtain a clear image of the cell on the monitor and a fluorescence intensity of 250 (arbitrary units) in the cortical region of the cell. Image analysis of other cells was carried out without modifying video camera control parameters. The three dimensional graphic analysis represents the coordinates of the equatorial plane of the cell as the X and Y axes and the fluorescence intensity of this plane as the Z axis. The image stored in the memory is constructed of 16 bits containing 512 horizontal pixels by 483 vertical pixels.

#### **2.16 Chromaffin Cell Permeabilization and Catecholamine Release**

Chromaffin cells plated on collagen-coated plastic 24 multiwell dishes at a density of  $5 \times 10^5$  cells per well were cultured for 48-72 hr. Experiments were started by rinsing the wells three times with 0.5 ml of amino-acid-free DMEM containing 10% fetal calf serum (Kenigsberg et al., 1980). Chromaffin cells were then incubated in the same medium containing this time  $10^{-7}$  M [ $^3$ H]NA (specific activity 43.7 Ci/mmol) at room temperature for 5 min. After this labelling

step, cells were incubated with six changes of 0.5 ml of regular Locke's solution (in millimolar: NaCl, 154; KCl, 2.6; K<sub>2</sub>HPO<sub>4</sub>, 1.25; KH<sub>2</sub>PO<sub>4</sub>, 0.5; MgCl<sub>2</sub>, 1.2; CaCl<sub>2</sub>, 2.2; glucose, 10; pH=7.2) over a 60 min period. Finally, cells were washed once with Ca<sup>2+</sup>-free Locke's solution and permeabilized by treatment for 5 min with 20 μM digitonin in K<sup>+</sup>-glutamate buffer (in millimolar: K<sup>+</sup>-glutamate, 129; EGTA, 5; MgCl<sub>2</sub>, 3; ATP, 2 and PIPES, 20; pH 6.6). Following permeabilization, media were removed and catecholamine secretion was determined by incubating the cells with 250 μl of K<sup>+</sup>-glutamate buffer for 2 min in the absence (basal) or in the presence of Ca<sup>2+</sup> (stimulated). Incubation media were collected and assayed for [<sup>3</sup>H]NA. The cell content of [<sup>3</sup>H]NA was determined by treating the cells with 250 μl of 10% trichloroacetic acid (TCA) for 10 min followed by two washes with 250 μl of 6% TCA; the three aliquots were combined. Total [<sup>3</sup>H]NA cell content was calculated by adding the [<sup>3</sup>H]NA secreted during the releasing step plus the [<sup>3</sup>H]NA extracted with TCA. Basal values (0.5-0.8%) were subtracted from the data obtained after stimulation. Radioactivity in the samples was measured using a liquid scintillation spectrophotometer (Model LS-7800, Beckman Instruments, Fullerton, CA,). Previous published experiments have demonstrated a simultaneous and parallel release of both endogenous catecholamines and [<sup>3</sup>H]NA from cells previously loaded with [<sup>3</sup>H]NA and subsequently permeabilized with digitonin (Vitale et al, 1992). Thus, the

use of [<sup>3</sup>H]NA-labelled cells increases the sensitivity of the catecholamine assay and allows the use of fewer cells per experiment. Therefore, only [<sup>3</sup>H]NA output was monitored in the experiments described here.

### **2.17 Preparation of Scinderin-Derived Peptides**

Peptides with sequence corresponding to actin-binding sites of scinderin (Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub>, Sc-ABP<sub>3</sub>) and PIP<sub>2</sub>-binding (Sc-PIP<sub>2</sub>BP) sites, as well as scramble peptides of these active sites were prepared by solid faced peptide synthesis (Merryfield, 1963), the sequence checked by Edman chemistry in a gas-liquid solid phase peptide sequenator (Henvick et al., 1981) and their purity determined by mass spectroscopy (Edmonds et al., 1990).

### **2.18 Source of Platelets**

Platelet-rich plasma was obtained from the blood bank of Ottawa Red Cross and centrifuged at 200 g for 15 min to eliminate red blood cells. The supernatant thus obtained was centrifuged at 800 g for 15 min to obtain a platelet sediment.

### **2.19 Platelet Permeabilization and Labelling of Serotonin Stores**

The platelet pellet was resuspended in Ca<sup>2+</sup>-free Locke's solution (mM: NaCl, 154; KCl, 2.6; K<sub>2</sub>HPO<sub>4</sub>, 2.14; KH<sub>2</sub>PO<sub>4</sub>, 0.85; MgCl<sub>2</sub>, 1.2; glucose, 10 and EGTA, 2.0; pH=7.2). After a wash

with Locke's solution, the platelet concentration was adjusted to  $7.5 \times 10^8$ /ml. Platelets were then incubated at 37° C for 90 min with 0.6 nmol [ $^3$ H]5-HT/ml (specific activity= 25.4 Ci/mmol, DuPont, Boston, MA) according to Lapetina et al., 1985. After incubation, the [ $^3$ H]5-HT labelled platelets were washed by incubation with 6 changes of 1 ml  $\text{Ca}^{2+}$ -free Locke's solution over a 60 min period before the experiments were commenced. [ $^3$ H]5-HT labelled platelets were permeabilized by treatment during 5 min with 15  $\mu\text{M}$  digitonin in  $\text{K}^+$ -glutamate buffer (mM:  $\text{MgCl}_2$ , 12.5;  $\text{K}^+$ -glutamate, 160; EGTA, 2.5; EDTA, 2.5; ATP, 5; HEPES, 20; pH=7.4). After permeabilization, platelets were centrifuged at 900 g for 2 min (4°C) and then resuspended in  $\text{K}^+$ -glutamate buffer.  $\text{Ca}^{2+}$  concentrations required to give appropriate pCa values were calculated as previously described (Fabiato et al., 1979).

## 2.20 Serotonin Release Studies

The samples (100  $\mu\text{l}$ ) containing  $7.5 \times 10^7$  permeabilized platelets in  $\text{K}^+$ -glutamate buffer were stimulated with 10  $\mu\text{M}$   $\text{Ca}^{2+}$  for 45 s in the absence or presence of recombinant scinderin, thioredoxin or scinderin-derived actin binding peptides. Release experiments were terminated by addition of an equal volume of 6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Preparations were centrifuged at 900 g for 2 min. Sediments were extracted with 200  $\mu\text{l}$  of 10% trichloroacetic acid (TCA) and radioactivity in supernatants and TCA extracts

was measured in a liquid spectrometer (Beckman Instruments, CA). Total [<sup>3</sup>H]5-HT platelet content was determined by adding the radioactivity present in the incubation medium and the TCA extract. [<sup>3</sup>H]5-HT output was expressed as a percentage of total content after subtraction of values for spontaneous (absence of Ca<sup>2+</sup>) release. A minimum of 5 samples per condition were measured and mean ± SEM were plotted.

**CHAPTER 3**

**RESULTS**

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF SCINDERIN: A  
PROTEIN BELONGING TO THE FAMILY OF  $Ca^{2+}$ -DEPENDENT F-ACTIN  
SEVERING PROTEINS

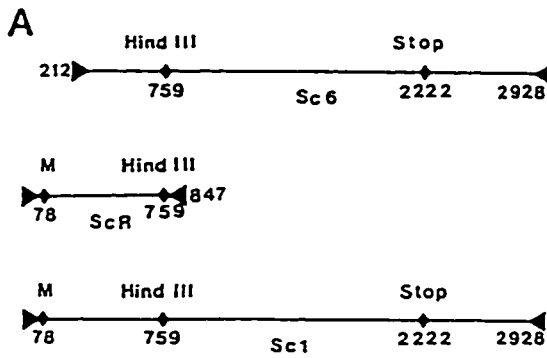
3.1 cDNA Cloning and Sequence of Scinderin

Starting from bovine chromaffin cell culture, mRNA was extracted and a directional cDNA expression library was constructed as indicated in Chapter 2 (Methods and Materials). The library thus obtained was screened with a scinderin polyclonal antibody (#6). This antibody has been previously characterized in our laboratory and it is known that it does not cross-react with gelsolin or any other chromaffin cell cytoskeleton proteins (Vitale et al., 1991). The best three libraries were pooled together giving a total of about  $1.5 \times 10^5$  p.f.u. Eight Sc cDNA clones of different lengths were isolated (Sc2, Sc8 and Sc6 = 2.7 Kb; Sc9 and Sc21 = 2.3 Kb; Sc19 = 2.1 Kb; Sc16 = 1.6 Kb and Sc20 = 1.3 Kb). The sequence analysis of the clones elucidated almost the entire Sc nucleotide sequence with the exception of the first 218 bases at the amino-terminal. These were obtained by an "anchored" PCR technique, the "Rapid Amplification of cDNA Ends" (RACE). ScR, obtained with the "RACE" method and clone Sc6, had each a common *HindIII* restriction site. This restriction enzyme together with *NotI* were used to cut the vector pBluescript II KS M13+ containing Sc6 which was ligated to ScR, also previously cut with *HindIII* and *NotI*. The result of this ligation was a vector containing the full length clone

Sc1 (ScR + Sc6). Figure 3.1 shows the entire nucleotide sequence of scinderin (Sc1) and the amino acid sequence deduced therefrom. For convenience, full length scinderin (Sc1) will be called Sc. The cDNA consists of 2928 nucleotides and contains a putative polyadenylation signal as well as the beginning of a poly-A tail. When the first possible start codon of the open reading frame is taken as a translational start site, the polypeptide consists of 715 amino acids with a calculated molecular weight of 80,800 daltons. The molecular weight derived from the sequence agrees with the value of 79,600 derived from SDS-PAGE analysis of purified scinderin (Rodriguez del Castillo et al., 1990). The polypeptide contains 28.5% of charged amino acids of which 14.1% are basic amino acids. Earlier studies from our laboratory have demonstrated that a 40 kDa fragment obtained from scinderin by limited proteolytic digestion with chymotrypsin binds one molecule of actin in a  $Ca^{2+}$ -dependent manner (Trifaró et al., 1992). Amino acid sequences for three cyanobromide peptides obtained from this fragment are present in the N-terminal half (positions: 248-254, 261-271 and 310-317) of the sequence shown in Fig.3.1. On the basis of the functional properties ( $Ca^{2+}$ -binding, F-actin severing activity) previous work from our laboratory suggested that scinderin belongs to the family of filamentous actin severing proteins (Rodriguez del Castillo et al., 1990). Scinderin sequence analysis also shows 63% and 53% homology with gelsolin and

**FIGURE 3.1 Cloning and Complete Sequence of a Full-Length Bovine Chromaffin Cell Scinderin Clone.**

- A) Construction of the bovine chromaffin cell scinderin clone. The initial clone Sc6 was isolated from a bovine chromaffin cell cDNA library constructed in  $\lambda$ gt22A using a polyclonal antibody raised against native scinderin as a probe (see Materials and Methods). The 5' region of the sequence, ScR, was produced by the "Rapid Amplification of cDNA Ends" method (RACE, BRL) and amplified by PCR using two primers designed on the basis of the N-terminal sequence of clone Sc6 and a primer supplied by the manufacturer (BRL). Ligation of Sc6 and ScR at the indicated Hind III site produced clone Sc1. This latter clone was subcloned in pBluescript I IKS M13+ and subsequently sequenced.
- B) Nucleotide and predicted amino acid sequence of bovine chromaffin cell scinderin. The numbers on the right indicate the positions of amino acids and nucleotides. Bold letters correspond to the amino acid sequence of 3 peptides obtained from cyanobromide digestion of purified scinderin followed by sequencing by the Edman degradation method. These sequence data are available from EMBL Data Library under accession number X78479.



**B**

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AATCGGTCUUGTTCAGTCC 20
GACTCCCAGGTCAGCGAGACCCCGCTCCACTCCC CGGAACATCCGTCGCCGAGTC 77
ATGCCCAGGGCTGTACCAACGAGGAGTTCGCCCGCGCGGCAAGCGGGGGGGCTG 134
M A G L I V H E E F A R A G K R A G L 139
CAGGTCGAGAAATTGAGAAGCTGGAGCTGGTGC CGGTGCCCGAGCGCGGTATGCG 191
Q V R E K L E L V P V P E S A Y G 38
AACTTCTACGTCCGGGATGCCCTACCTGGTGTCCACACGACCGCAGCGCCAGCCGGGC 248
N F Y V G D A Y L V L H I T T O A S R G 57
TTCACCTACC CGCTGC ACTTCTGGCTGGGAAAGSAGTGTACTCAGGATGAAAGCACA 305
F T Y R L H F W L G K E C T T O D E S T 76
GCAGCTGCCATCTTTACTGTTT CAGATGGATGACTATTTGGGTGGCAAACTGTGCAG 362
A A A I F T V O M D D Y L G G K P V O 95
AACAGAGAACTTCAAGCTATGAGTCTACGGATTTGTTGGCTACTTAAAGAGCT 419
H R E L O G Y E S T D F V G Y F K G G 114
CTGAAATACAAGCTCCGGGTGGCGTCTGACTCAA TCAATGTGCTTACAAATGAC 476
L K Y K A G G V A S G L N H V L T N D 133
TTGACTGCTCAGAGCCTCTGCAATGTGAAAGGTCGGAGACTCGTCAGGGCCAGGAA 533
L T A O R L L H V K G R R R V V R A T E 152
GTTCCCTTAAGCTGGGACAGTTTCAACAAGGGTGACTGCTTCATCATGACCTTGGC 590
V P L S W D S F N K K G D C F I I D L G 171
ACTGAAATTTACAGTGGTGTGGATCTTCTTGAACAAGTACGAGCGCTGAAAGGCC 647
T E I Y O W C G S S C N A M K Y S E R L K A 190
AGCCAGGTTGCCATCCGCTTCCGGCAATGAAAGGAAAGCGCAGCTCAGCTGAT 704
S O V A I G I R D N E R K K G R A O L I 209
CTGGTAGAAGAGGGAGTGAACCATCAGAGCTTACAAGGATATAGGGGAAAGCCA 761
V V E E G S E P S E L T K V L G E K P 228
AAGCTTAGGATGAGAAAGATGATGATGACATCAAAGCAGATATAACTAATAGGAAA 818
K L R D G E D D D D I K A D I T N R K 247
ATGCTAAACTCTACATGGTTT CAGATGCCAGTGGCTCCATGAAAGTGAAGTCTGGTG 875
M A K L Y M V S D A S G S H K V S L V 266
GCAGAAAGAAACFCCTTCTCCATGGCGATGCTTCTGTCTGAAAGATGCTTCAATTTG 912
A E E N P F F S M A H L L S F E C F L L 285
GACTACGGTCTGCAMACAGATTTTGTATGAAAGTAAAGTAAAGTAAAGTAAAGTAAAG 989
D H H A A K O I F V W K K G K D A N P O 304
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E R K A A M K T A E E P L Q Q M N Y S 323
ACGAATCCCAATTCAGTCTTCCAGAAAGGAGTGAACACCAATCTTCAACAG 1103
T N T O I O V L P E G G E T P I F K O 342
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F F K D W R D R D O S D G F G K V Y V 361
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S P O M A O H V V D D G S G K V O 399
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: W R V E H N G R V E I D R H S Y G E 418
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F Y G G D C Y I I L Y T Y P R G O I I 437
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Y T W O G A N A T R D E L T T S A F L 456
ACTTTCAGTTGGATAGATCCCTCGGGGACAGGCTGTGCAGATTGAGTCTCCAA 1502
T V O L D R S L G G G O A V O I R V S O 475
GGCAAGAACTGCTCACCTGCTGAGTTTGTCAAACAGCAAGCCCTCATTATTTAC 1559
G K E P A H L L S L F K D K P L I Y 494
AAGACCGAACATCAAAGAAGAGGTCAGGCACCAACCCCTTATAGGCTCTTT 1616
K H G T S K K E G O A P A P P I R L F 513
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G V R R R N L A S I T R I M E V D V D A 532
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N S L N S N D V F V L K L R O H N G Y 551
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: W I G K G S T O E E E K G A E Y V A 570
AGCTTCTCAAATGCAAACTTCAGCATTCAGGAAGGCAAGGACCCAGAGGATTT 1844
S V L K C K T S T I O E G K E P E E F 589
GGAAATCCCTTGGAGGAAAGAACTACCAGACTCTCTCTCTGATAGAAATCCCG 1901
W N S G G K K D Y O T S P L L E S O 608
GCTGAAGCATCCACTCGGCTTACGGCTGCTCCAACAAACTGGAAGATTCATT 1958
A E D H P P R L Y G C S N K T G R F I 627
ATTGAAGAGTTCAGAGAGTTCACCCAGGATGATTAGCAGAAGATGATGTCATG 2015
I E E V P G E F T O D D L A E D D V M 646
CTGTTAGATCTTGGGACAGATTTTATTTGGATTGGAAAAGATGCCAATGAAAT 2072
L L D A W E O I F I W I G K D A N E V 665
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E K S I S L K S A K I Y L E T D P S G 684
AGAGACAAAGGACCCAAATGTCATCATAAAACAGGGTCATGACCACTACTTTC 2185
R D K H T I V I I K O G H E P P T F 703
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T G W F L G W D S S R W < 715
AAAACAAATATAATGGGCGAGCTGCCCGGGGGAAGGAGGAGCTTGTAACTTT 2300
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CTGCTAAGCATCTTCCTAAGAAAGCTTACCTTTTGGCTTTAAGAGCCTT 2756
TGAAGTACAGCTCTTCTGACAAATGATTTATATCTTACCCACTTTATATAC 2813
ATCCATATTTTACAGTACCTGTTTAAACATCACAAAAGATACATACAAAAGGCT 2870
TCACTAATACTGCTTTATTATTAATAATATATCTTTAAAAGGTGAAAAAAA 2927
A 2928

```

FIGURE 3.1

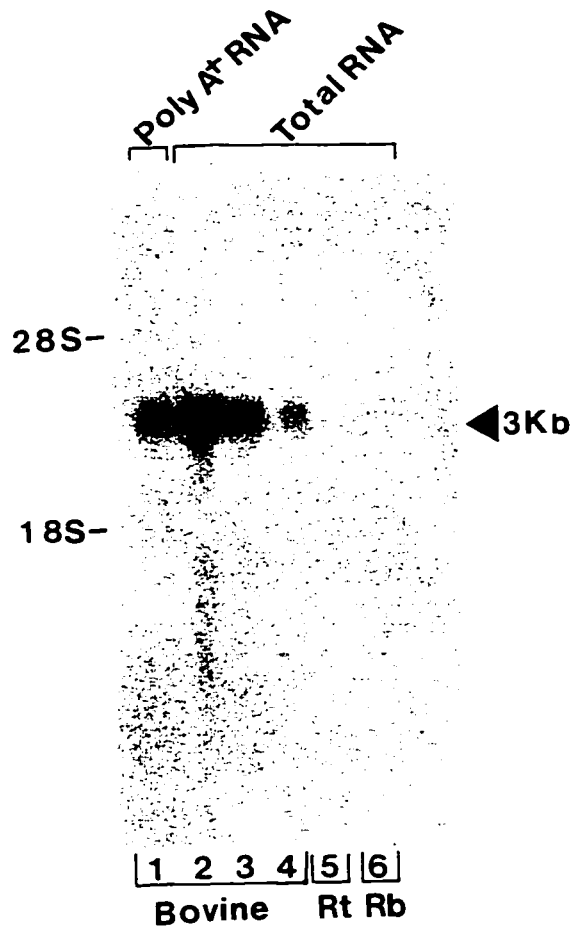
villin respectively, two other Ca<sup>2+</sup>-dependent F-actin severing proteins.

### **3.2 Northern Blot Analysis**

The main purpose of the Northern blot was to determine the size of the Sc transcript; the longest clone in the cDNA library was about 2.7 Kb and, clearly, it did not encode the N-terminal of scinderin. The RNA blot indicated a size of 3 Kb. Northern blot analysis, performed under high stringency conditions, also demonstrated that scinderin transcript is highly expressed in chromaffin cells but it was not detected in rat and rabbit brain. Previous work from our laboratory (Western blot analysis of Sc expression) has pointed to the presence of scinderin in bovine brain, pituitary glands, kidney and adrenal medulla (Tchakarov et al., 1990). Fig.3.2 presents the results of the Northern blot.

### **3.3 Comparison Between the Antisera Raised Against Native Scinderin and Fusion Protein F-Sc (GST-Sc). Scinderin Distribution in Resting and Stimulated Chromaffin Cells, Detected by Antibodies Against a Fusion Protein.**

The identity of the scinderin cDNA was further confirmed by expressing the gene as a fusion protein with glutathione S-transferase (GST) in E.coli. The protein thus obtained was purified and the corresponding antibody was compared with the antibody against native scinderin in immunoblotting and



**FIGURE 3.2 Northern Blot Analysis of Scinderin mRNA Expression.** Bovine chromaffin cell mRNA (10  $\mu$ g, lane 1) and total RNA (lanes 2-6), rat (lane 5) and rabbit (lane 6) brain total RNA were fractionated on 1% agarose formaldehyde gel, transferred onto nylon membrane and hybridized with a radio-labelled probe (500 bp) from scinderin clone Sc6. The figure shows the autoradiograph of the membrane. Lanes 2, 3, 4, 5 and 6 contained 50, 20, 10, 20 and 20  $\mu$ g of total RNA. RNA ladder (BRL) was also run on the gel. The positions of 28S (4.8 Kb) and 18S (1.9 Kb) are indicated on the left side.

immunocytochemical studies. Sc clone (Sc1) was subcloned into expression vector pGEX 4T2 using restriction sites *SalI* and *NotI*. The recombinant vector was transfected into E-coli JM 105 in order to express the corresponding fusion protein. This was purified by affinity binding to a glutathione-Sepharose 4B column as described in Chapter 2. and subsequently used in the immunocytochemistry and phospholipid binding experiments. The major problem encountered with this system was the insolubility of the fusion protein which was found as inclusion bodies after cell disruption. Despite all attempts (manipulating growth and induction temperatures and conditions) most of the fusion protein remained insoluble. Small amounts of GST-Sc were solubilized with Triton X-100 in an EDTA based buffer and bound to the column.

- The GST-Sc antibody was first tested for its **specificity**. Fig.3.3.A shows a Western blot performed with total chromaffin cell cytosolic proteins. Only one reactive band was obtained with anti-GST-Sc when reacted with the proteins (Fig.3.3.A lane 3). This band had the same mobility as that achieved with native scinderin antibody (Fig.3.3.A lane 2). The native scinderin antibody had been obtained and characterized earlier in our laboratory (Vitale et al., 1991; Rodriguez del Castillo et al., 1990). The gel was loaded with 200  $\mu$ g of protein and the results demonstrated no cross-reactivity of GST-Sc antibody with any other chromaffin cell protein. **Immunofluorescent studies** also showed no difference between

localisation of scinderin with one or the other antisera:

- Chromaffin cells cultured for 48 h were incubated in regular Locke's solution alone or in the presence of  $10^{-5}$  M nicotine for 40 s. At the end of the incubation, cells were processed for immunofluorescence using anti-scinderin #6 or anti-GST-Sc. Control resting cells stained with either antibody showed a bright and continuous fluorescent ring and a less intense and diffused cytoplasmic fluorescence (Fig.3.3.B, a and a'). As shown before (Vitale et al., 1991), nicotine stimulation caused a fragmentation of the bright fluorescent ring due to redistribution of cortical scinderin (Fig.3.3.B, b and b'). Patches of scinderin appear clear as a fragmented fluorescent ring. Staining with either antibody shows a similar pattern. To eliminate possible errors, scinderin redistribution induced by nicotinic receptor stimulation was measured in a large number of cells. Fig.3.3.C shows that 40 s stimulation with nicotine increased significantly the number of cells showing scinderin redistribution. Here, again, there was no difference in the percentage of cells with scinderin redistribution in preparations stained with either antibody. The results described here indicate that polyclonal antibodies raised against GST-Sc fusion protein cross-react only with native scinderin and behave as if they were raised against the native protein. In conclusion, the cDNA cloned and expressed in E.coli JM 105 was indeed scinderin.

**FIGURE 3.3 Comparison Between the Antisera Raised Against Scinderin and Fusion Protein F-Sc6 (GST-Sc6)**

**A. Specificity of the antisera.** Chromaffin cells cytosolic proteins (100,000xg supernatant, 200  $\mu$ g/lane) were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Lane 1 was stained with Amido Black. Lanes 2 and 3 are the immunoblots of the same preparations incubated with antiserum raised against either native scinderin (1/1,000 dilution, lane 2) or fusion protein F-Sc6 (1/1,000, lane 3). The presence of scinderin is indicated by an arrowhead.

**B. Localization of scinderin by fluorescence microscopy in control and nicotine-stimulated chromaffin cells.** Two-day old cultured chromaffin cells were incubated with Locke's solution alone or containing  $10^{-5}$  M nicotine for 40 s. Following this incubation period, cells were fixed and stained for scinderin using either scinderin antiserum #6 (1/200 dilution, a and b) or antiserum against the fusion protein F-Sc6 (1/350 dilution, a' and b'). Preparations were next incubated with FITC-IgG (1/250 dilution). In resting cells (a and a'), scinderin redistribution consisted of a bright cortical ring (open arrowheads) and a diffuse cytoplasmic staining. Upon nicotinic receptor stimulation (b and b'), the cortical fluorescent ring appeared fragmented (some fluorescent patches are indicated by arrowheads). Scinderin distribution under resting conditions or during nicotinic stimulation as revealed by staining with scinderin antiserum was similar to the distribution observed in cells stained with the antiserum against F-Sc6. Bar: 5 $\mu$ m.

**C. Quantitative analysis of nicotine-induced cortical scinderin redistribution.** Chromaffin cells were treated as described above. Following staining of the cells with either anti-scinderin or anti-fusion protein F-Sc6, cells were viewed under the fluorescence microscope. One hundred cells per coverslip were examined and classified as having either a continuous or a discontinuous cortical fluorescent ring. This procedure was conducted without knowing whether cells were from control or treated preparations or had been stained with anti-scinderin or anti-F-Sc6 (single-blind design, see Materials and Methods). Once all preparations had been examined the percentage of cells displaying a discontinuous fluorescent ring (scinderin redistribution) was calculated. The data plotted represent the mean  $\pm$  SEM of results from examination of 500 cells per experimental condition.

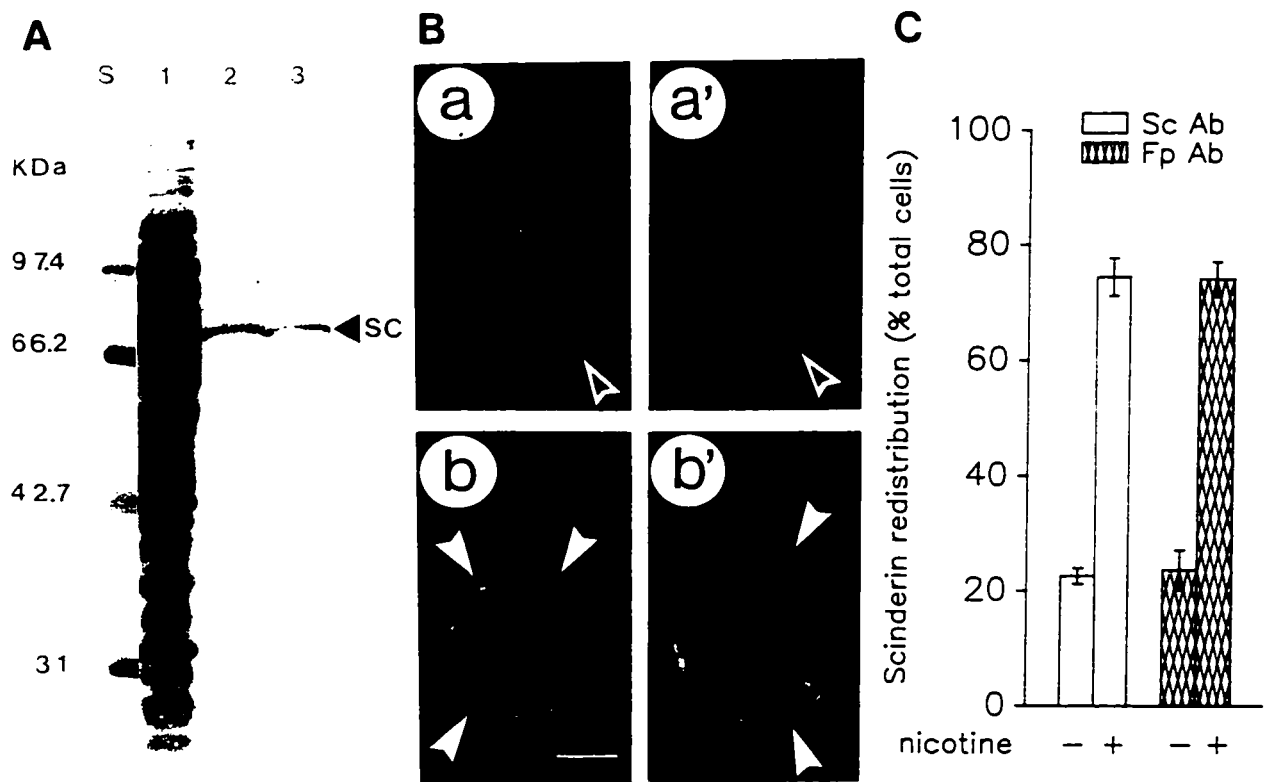


FIGURE 3.3.

### 3.4 GST-Sc Fusion Protein Binds Actin

Native scinderin has the ability to bind actin (in order to sever it), phosphatidylserine (PS) and phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) of the plasma membrane, in a Ca<sup>2+</sup>-dependent manner (Rodriguez del Castillo et al., 1992). The fusion protein GST-Sc (F-Sc1), which was obtained from a full-length scinderin cDNA clone, should therefore have actin and phospholipid binding sites.

Actin-DNaseI-Sepharose 4B columns prepared as described in Materials and Methods were used to test the ability of GST-Sc to bind actin. GST-gene fusion system was employed for the production of GST-Sc (F-Sc1) fusion protein. E-coli JM 105 were transformed with parental (non-recombinant) or recombinant pGEX 4T2 vectors. Transformed E-coli JM 105 were grown either in the absence or in the presence of the inducer isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG). E-coli JM 105 were collected by centrifugation and lysed. Proteins were further analyzed by SDS-PAGE (Fig.3.4). Bacteria transfected with parental (non-recombinant) vectors only, upon induction with IPTG showed the presence of glutathione S-transferase (GST), a protein of 26 kDa molecular mass (Fig.3.4.A, lane 2). When transformed E-coli containing recombinant GST-Sc vectors were tested, only lysates obtained from IPTG-induced preparations showed in SDS-PAGE the new protein band (GST-Sc) of approximately 110 kDa (Fig.3.4.A, lane 4). Furthermore, immunoblots of this SDS-PAGE showed that only the 110 kDa

protein cross-reacted with antiserum #6, which was obtained against native scinderin (Fig.3.4.B,4'). The fusion protein is mainly found in insoluble inclusion bodies. A small amount of GST-Sc could be solubilized with Triton X100 and applied in the presence of  $\text{Ca}^{2+}$  1 mM, to actin-DNaseI-Sepharose 4B columns. Columns were subsequently eluted with a  $\text{Ca}^{2+}$ -free (EGTA) buffer. SDS-PAGE and immunoblots of the eluates show that only the 110 kDa protein was retained by the affinity column and was subsequently eluted by perfusion with  $\text{Ca}^{2+}$ -free buffers (Fig.3.4.A, lane 6). The eluted protein (GST-Sc) showed cross-reactivity with scinderin antiserum #6 (Fig.3.4.B, lane 6').

In conclusion, full-length scinderin fusion protein (GST-Sc) was immunologically identical to native scinderin and bound to actin in a  $\text{Ca}^{2+}$ -dependent manner. These results are identical to those obtained earlier (Rodriguez del Castillo et al., 1992) and they clearly suggest that GST-Sc interacts with actin in a similar manner to native scinderin.

### 3.5 GST-Sc Fusion Protein Binds Phosphatidylserine (PS) and Phosphatidylinositol 4,5-Bisphosphate ( $\text{PIP}_2$ )

Native scinderin binds to the phospholipids of the plasma membrane in resting cells. To test the binding of protein GST-Sc to PS and  $\text{PIP}_2$  a protocol similar to that used for the binding studies of native scinderin was followed (Rodriguez del Castillo et al., 1992). GST-Sc fusion protein, purified

**FIGURE 3.4 Expression of Fusion Protein F-Sc1 (GST-Sc) and its Binding to Actin.**

E-coli JM 105 transfected with parental (non-recombinant) or recombinant (full-length Sc1 insert) pGEX 4T3 vectors were grown either in absence or presence of 0.1 mM IPTG. A) 100  $\mu$ g protein/well were used in the SDS-PAGE. Lanes 1 and 2 contain proteins from E-coli JM 105 transfected with parental vectors and incubated in absence (Lane 1) or presence (lane 2) of IPTG. The presence of the 26 kDa GST in lane 2 is indicated by an asterisk. Lanes 3 and 4 contain proteins from E-coli JM105 transformed with recombinant vectors incubated in absence (lane 3) or presence (lane 4) of IPTG. The presence of a 110 kDa GST-Sc (F-Sc1) fusion protein in lane 4 is indicated by arrowheads. This protein cross-reacted with the antiserum (#6) against native scinderin as shown in the Western blot (B, Lane 4'). Aliquots of lysates of preparations shown in A), lanes 3 and 4, were applied in presence of  $Ca^{2+}$  to actin-DNase Sepharose 4B columns (see Materials and Methods). Columns were washed and eluted with  $Ca^{2+}$ -free buffers and collected fractions were subjected to SDS-PAGE (A). Lanes 5 and 6 correspond to aliquots from protein preparations shown in lanes 3 and 4 respectively. Only the 110 kDa GST-Sc (F-Sc1) protein depicted in lane 6 was retained by the affinity column. This protein cross-reacted with scinderin antiserum #6 as shown in the Western blot (B, lane 6').

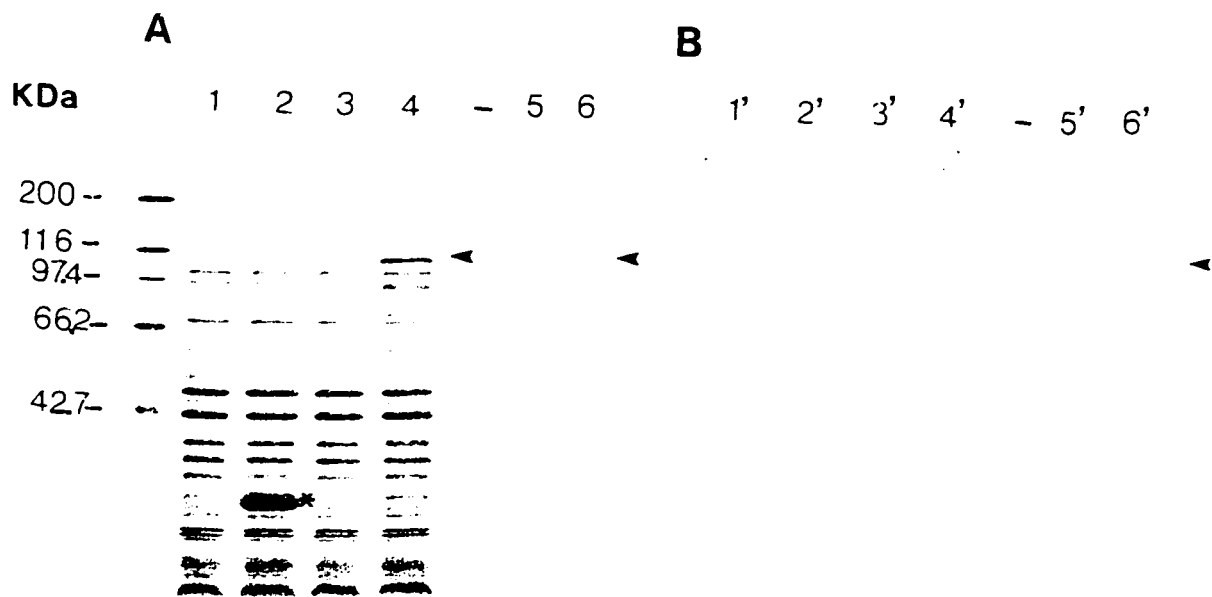


FIGURE 3.4

by affinity chromatography, was incubated with PS or PIP<sub>2</sub> liposomes at pH 7.1 in the presence of increasing free-Ca<sup>2+</sup> concentrations (10<sup>-7</sup> - 10<sup>-5</sup> M). After incubations, liposomes were separated by centrifugation. Pellets (liposomes) and supernatants were subjected to SDS-PAGE. At all Ca<sup>2+</sup> concentrations tested, GST-Sc was found to bind to PS and PIP<sub>2</sub> liposomes (Fig.3.5), results similar to those obtained earlier with native scinderin (Rodriguez del Castillo et al., 1992). In conclusion, both proteins (native scinderin and GST-Sc) interact with the phospholipids in a similar manner.

### 3.6. Sequence Analysis : Domain Organization of Scinderin

Scinderin sequence presents a 63% and 53% homology with gelsolin, a ubiquitous Ca<sup>2+</sup>-dependent F-actin severing protein, and villin, a protein of the same family, present in the intestinal villi of the brush border. As shown above, GST-Sc behaves very similar to the native scinderin in all studies of actin and phospholipids interaction. Comparison of scinderin sequence with the two above mentioned proteins (EMBL Data Library) revealed some very similar sequence motifs corresponding to either actin or PIP<sub>2</sub> binding domains. Figure 3.6.1.A presents a comparison between one of the actin binding regions of gelsolin /villin and that of scinderin. This sequence of scinderin shows an 87% and 80% homology with either gelsolin or villin; there are only 1 and 3

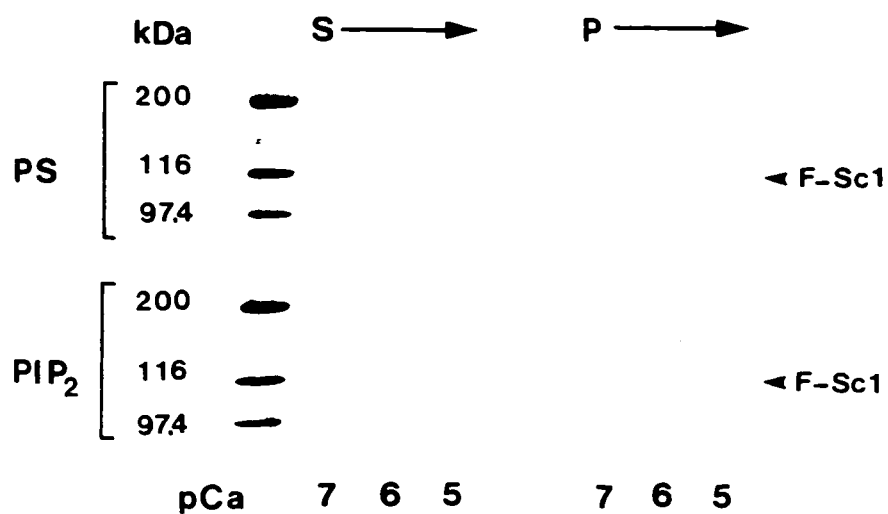


FIGURE 3.5 Binding of Fusion Protein F-Sc1 (GST-Sc) to Phosphatidylserine (PS) and Phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). Aliquots of one  $\mu\text{g}$  of fusion protein were incubated at pH 7.1 with either PS or PIP<sub>2</sub> liposomes in the presence of increasing Ca<sup>2+</sup> concentrations (10<sup>-7</sup> - 10<sup>-5</sup> M). Liposomes were separated by centrifugation and SDS-PAGE patterns of supernatants (S) and pellets (P) are shown in A and B. Fusion Sc was recovered entirely with the liposomes fractions.

substitutions in this region, respectively. The aspartate at position 109 of gelsolin corresponds to the aspartate at position 86 of scinderin. Earlier studies have shown that aspartate 109 contributes to one of the  $\text{Ca}^{2+}$ -binding sites of gelsolin (McLaughlin et al., 1993). It seems quite possible that aspartate 86 may also contribute to  $\text{Ca}^{2+}$  binding in scinderin. A sequence corresponding to the second actin-binding site of gelsolin (amino acid residues 161 to 169) is also found in scinderin (amino acid residues 138 to 146; Fig.3.6.1.B). Two amino acids in the region (corresponding to the second actin binding domain of scinderin) L140 and H141 are different from those of gelsolin. However, villin has the same amino acids in this position (Fig.3.6.1.B). Two  $\text{PIP}_2$  binding sites have been described for gelsolin and these sequences were found in the scinderin molecule (Fig.3.6.1.C). This corresponds to two amino acids sequences from positions 112 to 119 and 138 to 146. These stretches show the consensus sequence  $\text{R(X)XXXKXRR}$  typical of  $\text{PIP}_2$  binding sites of phospholipase C (Rhee et al., 1989; Yu et al., 1992). It was known since 1988 (Way et al.) that gelsolin has 6 internal repeats of three different (A, B, and C) short sequence motifs. These repeats occur six times at approximately equal distance in gelsolin and villin. Similar smaller repeats can be identified six times in scinderin from amino-terminal to carboxy terminal, Fig.3.6.2.

**FIGURE 3.6.1**

**A., B. Comparison of Amino Acid Sequences of Two Scinderin Regions with Those Sequences of Known Actin-Binding Domains of Gelsolin and Villin.**

Two stretches of amino acid sequences of scinderin from position 77 to 89 and from 138 to 146 show a high degree of homology with two (A and B) actin-binding domains previously described for human plasma gelsolin (Kwiatovski et al., 1986) and chicken intestinal brush border villin (Bazari et al., 1988). Aspartate 86 of scinderin corresponds to similar residues in positions 109 and 85 of gelsolin and villin, respectively. Aspartate 109 has been shown to be one of the  $\text{Ca}^{2+}$ -binding sites of gelsolin (McLaughlin et al., 1993). The numbers on either side indicate the position of amino acid residues.

**C. Comparison of Amino Acid Sequences of Two Scinderin Regions with Those Known  $\text{PIP}_2$  Binding Sequences.**

Two stretches of amino acid sequences of scinderin show a high degree of homology with  $\text{PIP}_2$  binding site of villin,  $\text{PIP}_2$  binding sites in domains 1 and 2 of gelsolin (Yu et al., 1992) and the  $\text{PIP}_2$  binding sequence in the conserved "X box" of rat phospholipase C family (Rhee et al., 1989). The second scinderin sequence (138-146) is identical to villin sequence from position 135 to 145. The numbers on either side indicate the position of amino acid residues.

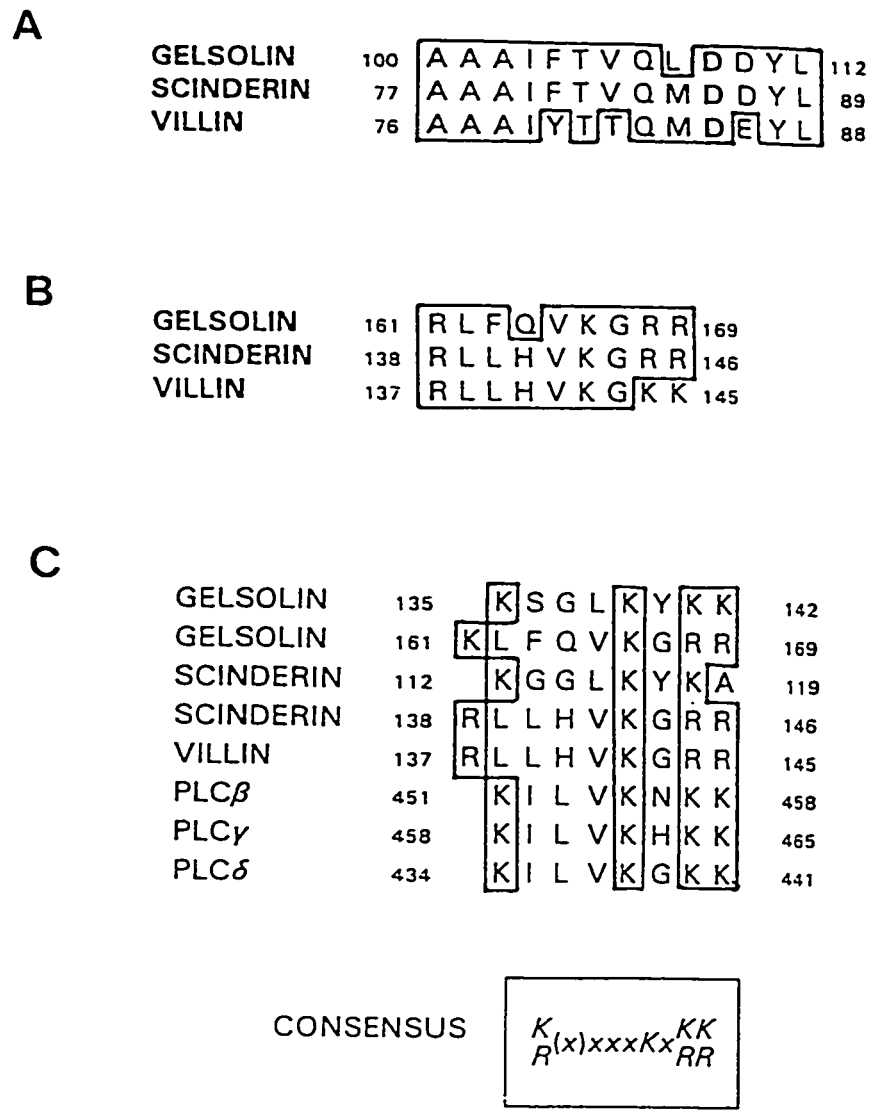


FIGURE 3.6.1

**FIGURE 3.6.2 Domain Organization of Scinderin; Segmental Alignment with Gelsolin and Villin.** Alignments are based on pairwise analysis of all combinations of sequences as performed by Way and Weeds (1988) for gelsolin and villin. Highly conserved motifs (B , A , and C) are shown in boxes. Proteins are abbreviated as follows: HGS = human plasma gelsolin; SC =bovine scinderin; VIL = chicken villin. The numbers of either end indicate the positions of amino acid residues. The large numbers at the right side indicate the domain number (1-6) for the three proteins. These domains together with motifs B, A and C are represented in the diagram at the bottom of the figure. Each motif is found once in a domain of each protein and it is repeated five additional times along the protein.  
— : Actin binding sites; ---- : PIP<sub>2</sub> binding sites.  
(Taken from Trifaró and Garcia, 1995)



### 3.7 Identification and Localization by Segmental Deletion Analysis of a Third Actin Binding Site in Scinderin.

3.7.1 Expression of Scinderin Truncations was performed using a TRX fusion system (Invitrogen, CA) as described in Materials and Methods. Different truncations of scinderin cDNA, obtained by PCR, were subcloned into expression vector pTrxFus using the restriction sites *Bam*HI and *Sal*I, or *Kpn*I and *Bam*HI. Recombinant vectors were introduced into *E coli* GI698 to express the corresponding fusion TRX-truncated-scinderin proteins. Lysates obtained from tryptophan-induced cultures showed in SDS-PAGE, a new band which corresponded to each truncated scinderin (Fig. 3.7.1.2). The molecular weights of these TRX-fusion proteins were as follows: Sc3-6 =57 KDa, Sc3-4 =43 KDa, Sc3-<sub>1/2</sub>4 =34 KDa, Sc5-6 =41 KDa, Sc<sub>2</sub>5-6 =40.6 KDa, Sc<sub>1,2</sub>5-6 =35 KDa, Sc6 =27 KDa, Sc1-4,6 =79 KDa. A schematic representation of the position of these scinderin fragments in the scinderin molecule is shown in Fig.3.7.1.1. Some of these fusion proteins (Sc3-4 to Sc<sub>2</sub>5-6 and Sc1-4,6) were extremely well expressed amounting to more than 50% among total *E.coli* proteins (Fig.3.7.1.2). Because the scinderin polyclonal antibody did not recognize several scinderin fragments (only Sc3-6, Sc3-4 and Sc1-4,6 were recognized by the antibody), Western Blots (Fig.3.7.1.2, B) were stained with a mouse TRX monoclonal antibody (Invitrogen, CA); Sc1-4,6 and Sc full length were tested with antibody against native

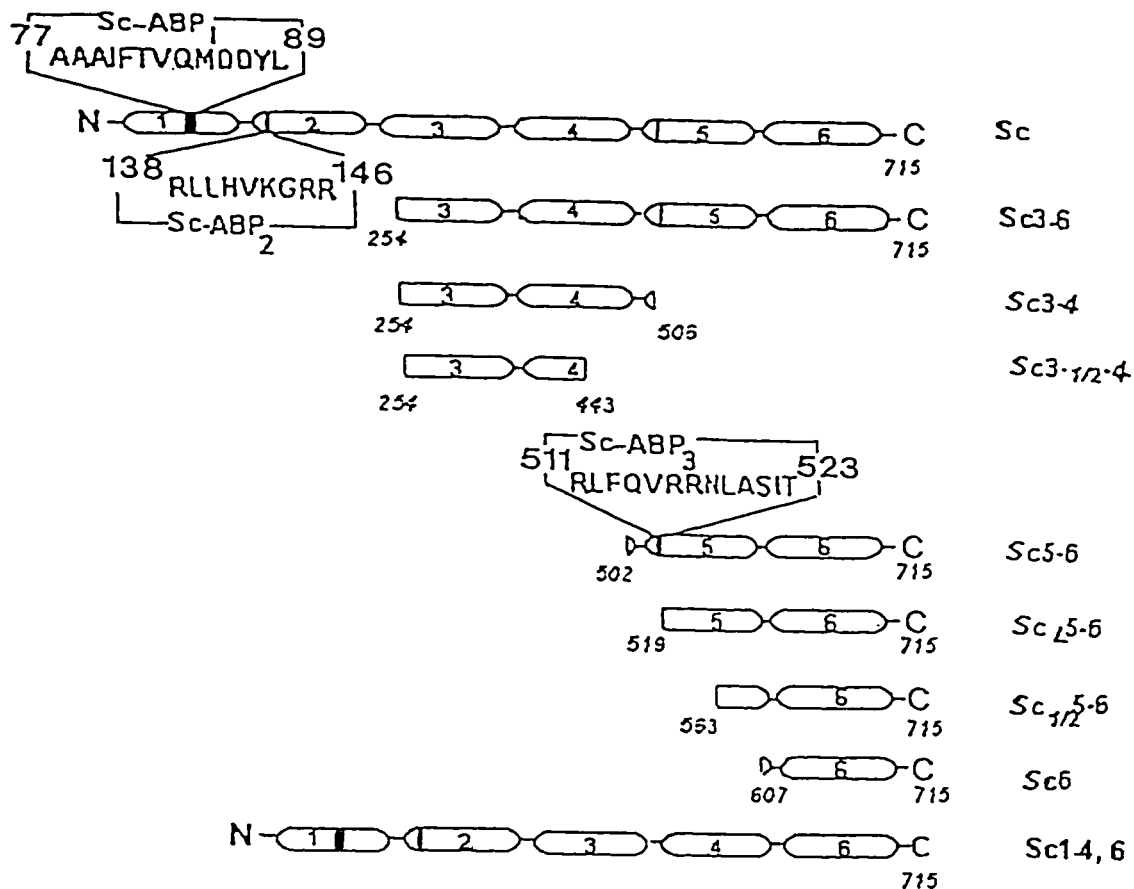


FIGURE 3.7.1.1 Schematic Representation of Scinderin Domains (Sc 1 to 6), Including Full Length and Fragments Obtained by Different Deletions. The number of the first and last amino acid of every recombinant protein is also indicated. The amino acid sequences and position of three scinderin-derived actin binding peptides (Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub>, Sc-ABP<sub>3</sub>) are also shown.

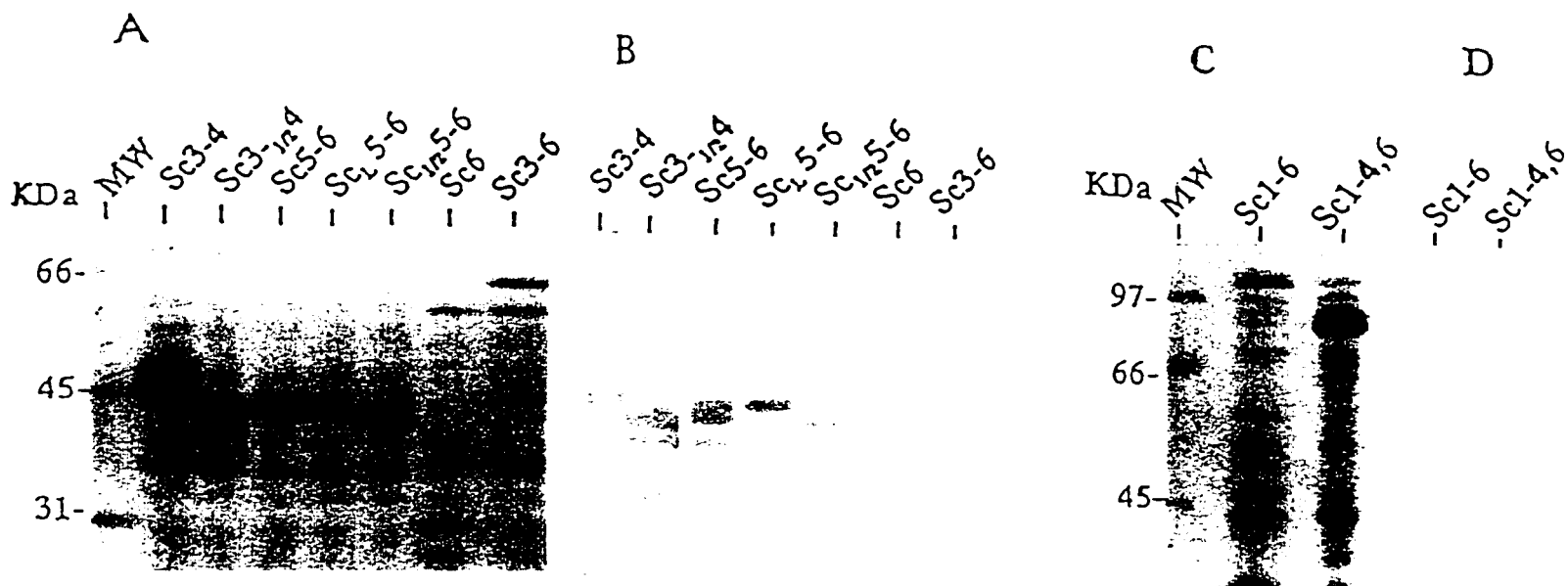


FIGURE 3.7.1.2 Expression of Different Truncated Scinderins in *E. coli* GI698. Bacteria were transfected with recombinant pTrxFus, grown and induced with tryptophan. Some truncated proteins were better expressed than others, corresponding in some cases to more than 50% of total bacterial protein. (A and C) Aliquots of bacterial lysate (30  $\mu$ g protein per well) were subjected to SDS PAGE, followed by staining with Coomassie Blue as indicated in Materials and Methods. The Western blot corresponding to A is depicted in B where TRX-fusion proteins were detected with a monoclonal antibody against TRX (Invitrogen, CA). In D r-Sc (Sc1-6) and Sc1-4,6 were detected with a polyclonal antibody against Sc.

scinderin (3.7.1.2, D.). Due to the fact that enterokinase does not efficiently cut TRX-Sc fusion proteins and because scinderin is very sensitive and undergoes fast proteolysis at room temperature, all experiments were carried on with TRX-Sc fusion proteins as previously shown, (Marcu et al., 1996; Zhang et al., 1996). TRX does not seem to interfere with the function of scinderin (Marcu et al., 1996; Zhang et al., 1996), and has no effect in *in vitro* tests, when compared to TRX-Sc.

**3.7.2 Binding of Recombinant Scinderin Truncations to Actin** The ability of scinderin fragments to bind actin was tested using two different actin-binding assays.

a) *Binding to Actin-DNAseI-Sepharose 4B beads* - Truncated scinderin fusion proteins were tested for actin binding *in vitro* as indicated in Materials and Methods. The experiments were performed first in  $\text{Ca}^{2+}$  containing buffer (buffer A). Under these conditions only two Sc-derived proteins, Sc3-6 and Sc5-6, were found to bind actin (Fig.3.7.2.1, A). Moreover, once bound in the presence of 1 mM  $\text{Ca}^{2+}$ , Sc3-6 and Sc5-6 could not be eluted from the beads by a EGTA buffer (buffer B). As a matter of fact, both Sc3-6 and Sc5-6 were found to bind to actin equally well in the presence of either 1 mM  $\text{Ca}^{2+}$  or  $\text{Ca}^{2+}$ -free:10 mM EGTA (Fig. 3.7.2.1, C). Due to the fact that the Sc3-6 and Sc5-6 could not be eluted with EGTA containing buffer, all gel wells were loaded with

**FIGURE 3.7.2.1 Binding of Recombinant Scinderin Fragments to Actin-DNase I-Sepharose 4B Beads.** The various forms of truncated scinderin were tested for binding to actin in the presence of 1 mM  $\text{Ca}^{2+}$  as indicated in Materials and Methods. Because two of the bound proteins (Sc3-6 and Sc5-6) could not be eluted with either buffer A or B ( $\text{Ca}^{2+}$ -free 10 mM EGTA), at the end of incubations, the Sepharose 4B beads carrying the protein complexes were boiled with the SDS sample buffer and the supernatants were loaded onto the gel. Therefore, actin (+) and DNase I ( $\Delta$ ) are also visible in the Coomassie stained gel (A). As noticed, only recombinant fragments Sc3-6 and Sc5-6 were found to be bound to actin under these conditions. Recombinant scinderin fragments Sc3-4, Sc<sub>L</sub>5-6, Sc<sub>M</sub>5-6 and Sc6 did not bind to actin. (B) Because domain 4 of gelsolin has been shown to contain an actin binding site (36) and the experiment described above in (A) does not indicate any binding of actin by Sc3-4, an additional experiment was carried out to further analyse the lack of binding of actin to this Sc segment using Sc5-6 as a positive control. In the absence of actin and presence of either Sepharose 4B (lanes 1,2) or Sepharose 4B-DNase I (lanes 3,4), both Sc3-4 and Sc5-6 were found not to bind to the beads. Both Sc constructs were recovered in the supernatants (lanes 1'-4'). When Sepharose 4B-DNase I-actin beads were present in the medium (lanes 5,6), Sc 3-4 did not bind to the beads and it was recovered in the supernatant (lane 5'). On the other hand, Sc5-6 was found to be bound to the beads (lane 6) and absent from the supernatant (lane 6'). Actin and DNase I bands are indicated by + and  $\Delta$  respectively. (C) Western blot of an experiment performed to test the binding of Sc3-6 and Sc5-6 in either 1mM  $\text{Ca}^{2+}$  or  $\text{Ca}^{2+}$ -free 10 mM EGTA. Both proteins bind well under these two conditions, as shown by the intensity of the bands obtained with TRX monoclonal antibody. Samples loaded in the gel were prepared as above.

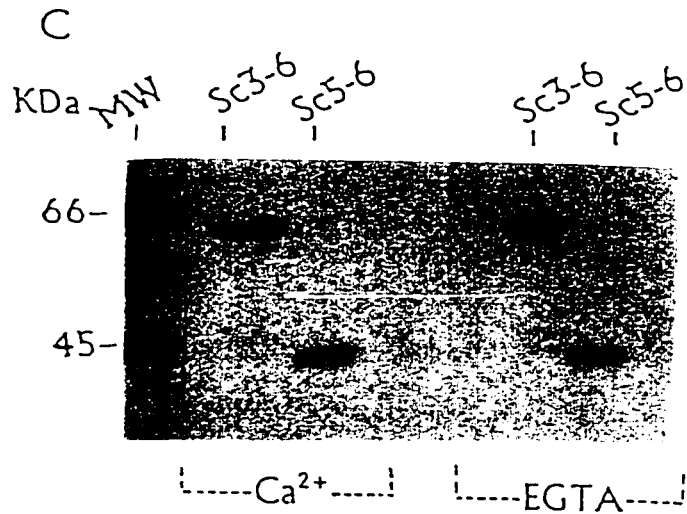
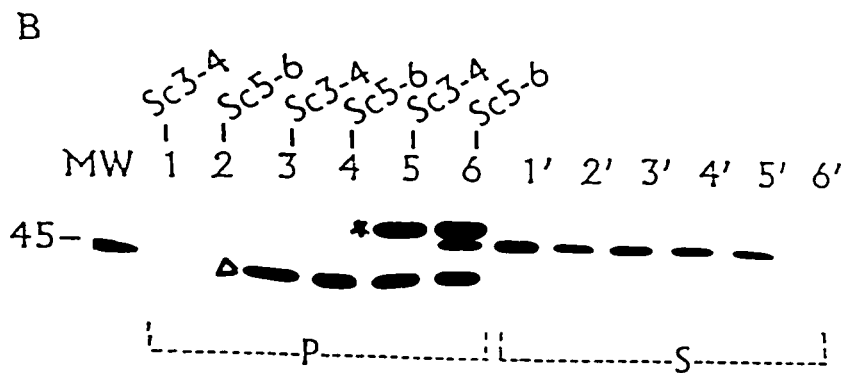
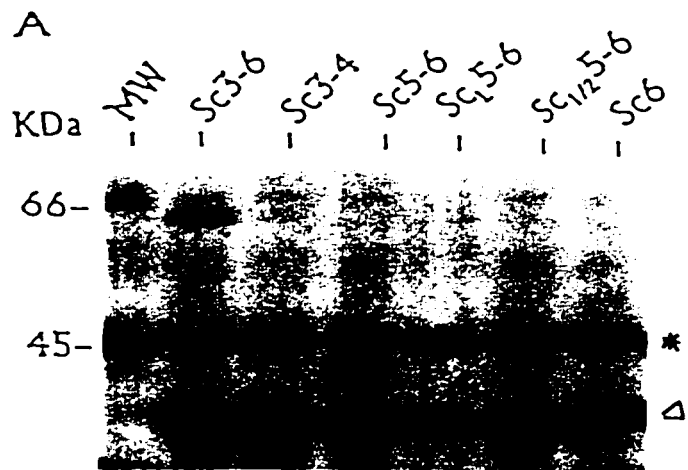


FIGURE 3.7.2.1

supernatants obtained after the Sepharose 4B beads were boiled in SDS-PAGE sample buffer. Therefore, the presence of actin and DNaseI was evident in all Coomassie-stained gels (Fig. 3.7.2.1, A, B). It should be pointed out that, when a deletion of 16 amino acids (502 to 518) from the beginning of domain 5 of scinderin was performed, the resultant recombinant scinderin fragment Sc<sub>5</sub>-6, which corresponds to amino acids 519-715 of scinderin, did not bind actin. This suggests the presence of an actin binding site at the beginning (NH<sub>2</sub>-terminal) of domain 5. Because it has been shown that domain 4 of gelsolin contains an actin binding site (Pope et al., 1995), a set of experiments tested Sc3-4 and Sc5-6 for binding under different conditions (Fig.3.7.2.1., B). These two constructs did not bind either Sepharose 4B or Sepharose 4B-DNase I beads (Fig.3.7.2.1., B, lanes 1-4). Only when actin was added to the system, Sc5-6 but not Sc3-4 bound to the beads (Fig.3.7.2.1., B, lane 6).

*b) Co-sedimentation of recombinant truncated scinderins with actin* - All constructed deletions were also tested for co-sedimentation with actin as described in Materials and Methods. Here again, with the exception of Sc3-6 and Sc5-6 all recombinant scinderin fragments failed to co-sediment with actin under these experimental conditions (Fig.3.7.2.2, A). These results and especially the observation that Sc<sub>5</sub>-6 did not co-sediment with actin whereas Sc5-6 did, also suggest the

presence of a third actin binding site at the 5' end of domain 5 (Sc5) of scinderin. To further demonstrate the presence of a third actin binding site we have constructed a 13 amino acid peptide (Sc-ABP<sub>3</sub>) with the sequence: RLFQVRRNLASIT. This peptide is 6 amino acids longer than the possible actin binding site (RLFQVRR). The length of the peptide was chosen just to be sure that the difference in binding to actin between Sc5-6 and Sc<sub>L</sub>5-6 was not due to a possible disruption of a binding site whose length remains, at this point, to be elucidated. The scinderin-derived peptide (Sc<sub>511-523</sub> or Sc-ABP<sub>3</sub>) but not a scrambled 13 amino acid peptide (AVNIRLRFSTLQR), with sequence without homology to those present in the EMBL data bank, when present in ratio to actin of 10:1, reduced by 49% (as shown by densitometry measurements) the binding of either Sc3-6 or Sc5-6 to actin when tested in the actin co-sedimentation assay (Fig.3.7.2.2, B). The smaller deletion of 16 amino acids which produced Sc<sub>L</sub>5-6 was enough to suppress the binding to actin. Consequently, the actin binding site should be localized at the beginning of the first half of domain 5 of scinderin, within a stretch of 13 amino acid starting at amino acid 511 and ending at amino acid 523 of scinderin.

**FIGURE 3.7.2.2 (A) Cosedimentation of Various Recombinant Scinderin Fragments with Actin.** The proteins were mixed with actin and incubated in the presence of 10  $\mu\text{M}$   $\text{CaCl}_2$  for 90 min at room temperature as described in the Materials and Methods. Preparations of fusion proteins and polymerized actin were centrifugated at 100,000 g for 60 min; sediments were washed, resuspended in the SDS sample buffer and then loaded onto the gel. The asterisk indicates the position of actin. Sc3-6 (data not shown) and Sc 5-6 were the only two constructs which cosediment with actin. (An additional experiment was performed in  $\text{Ca}^{2+}$ -free 10 mM EGTA, with the same result (data not shown).)

**(B) Inhibition of Sc3-6 cosedimentation with actin in the presence of a scinderin-derived actin binding peptide (Sc-ABP<sub>3</sub>).** Sc-ABP<sub>3</sub> (lane 3) and a control scramble (SCR) peptide (lane 4) were incubated together with Sc5-6 and actin, in molar ratios of 100: 1 :10. (Similar experiments were performed on Sc3-6, with the same results, data not shown). After incubation, the preparations were centrifuged and pellets (P) and supernatants (S) thus obtained were subjected to SDS-PAGE. Sc-ABP<sub>3</sub> (lane 3) reduced by 49% the binding of Sc5-6 to actin when compared to control-scramble peptide, which did not have any effect on binding (lane 4). Densitometric analysis of the gel shown in B) resulted in areas of 0.98 and 1.05 arbitrary units for the actin bands in lanes 3 and 4 respectively, whereas the areas of Sc5-6 in the same lanes were 0.058 and 0.030 arbitrary units respectively. Lanes 1 and 1' represent actin polymerized alone and lanes 2 and 2' actin polymerized in the presence of Sc5-6.

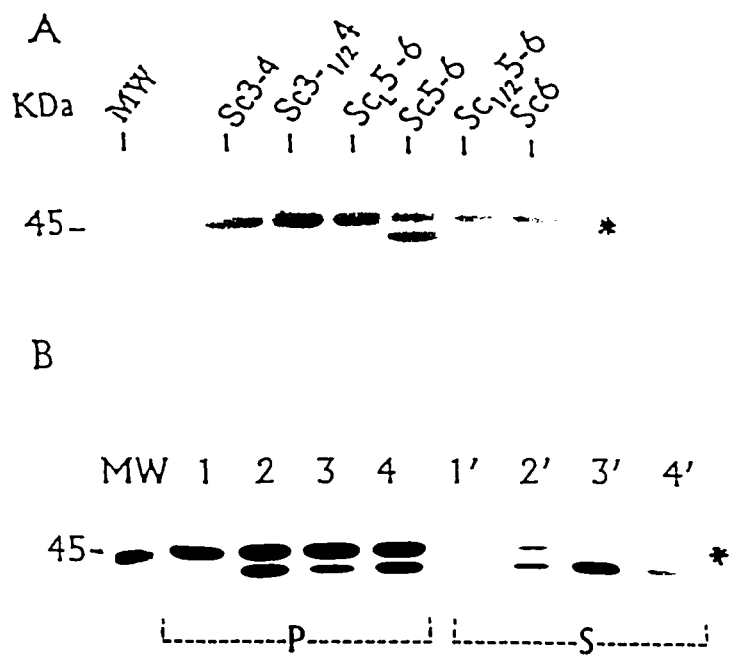


FIGURE 3.7.2.2

### 3.8 Biochemical Properties of the Third Actin Binding Site of Scinderin

3.8.1 Affinity of Sc5-6 for Actin,  $K_d$ . Actin co-sedimentation experiments were also performed with different construct: actin molar ratios to determine affinity constants ( $K_d$ ). The experiments were carried out either in the presence or absence (10 mM EGTA) of  $Ca^{2+}$  to determine whether or not  $Ca^{2+}$  would affect the affinity for actin. Moreover, in these experiments Sc3-4 was also included to rule out the possibility that the absence of binding to actin by this construct observed in the experiments described above was due to the fact that only one concentration of Sc3-4 and only one ratio of Sc3-4 to actin was tested. Here, again, the experiments clearly demonstrated that only Sc5-6 was bound to actin at all ratios tested (Fig.3.8.1, B). Scatchard analysis of the data (Fig.3.8.1, A) revealed a  $K_d$  for Sc5-6 of  $0.30 \mu M$  when tested in the presence of  $Ca^{2+}$  and  $0.33 \mu M$  in  $Ca^{2+}$ -free environment. This indicates that Sc5-6 binds actin with equal affinity either in the presence or absence of  $Ca^{2+}$ .

FIGURE 3.8.1. Determination of Dissociation Constants ( $K_d$ ) by Co-sedimentation of Actin with Either Sc3-4 or Sc5-6. Construct Sc3-4 or Sc5-6 was co-sedimented as indicated in Materials and Methods after incubation in the presence of  $10 \mu\text{M Ca}^{2+}$  at the following construct: actin molar ratios: 0.15:1 (lane 2), 0.35:1 (lane 3), 0.5:1 (lane 4), 0.75:1 (lane 5), 0.95:1 (lane 6) and 1.2:1 (lane 7). Preparations were centrifuged as indicated in Materials and Methods and the content of pellets (P) and supernatants (S) were separated by SDS-PAGE. The Coomassie blue stained gels are shown in (B). Lane 1 represents actin alone. As with the experiment described in Fig.3B, only Sc5-6 was found to co-sediment with actin. Protein bands were scanned densitometrically and ratios between free and bound Sc5-6 and between bound Sc5-6 and actin were determined and then plotted using the Scatchard method (A). The Sc5-6  $K_d$  obtained from this analysis was  $0.30 \mu\text{M}$ . In a similar experiment, this time performed in  $\text{Ca}^{2+}$ -free  $10 \text{ mM EGTA}$ , the  $K_d$  obtained was  $0.33 \mu\text{M}$ .

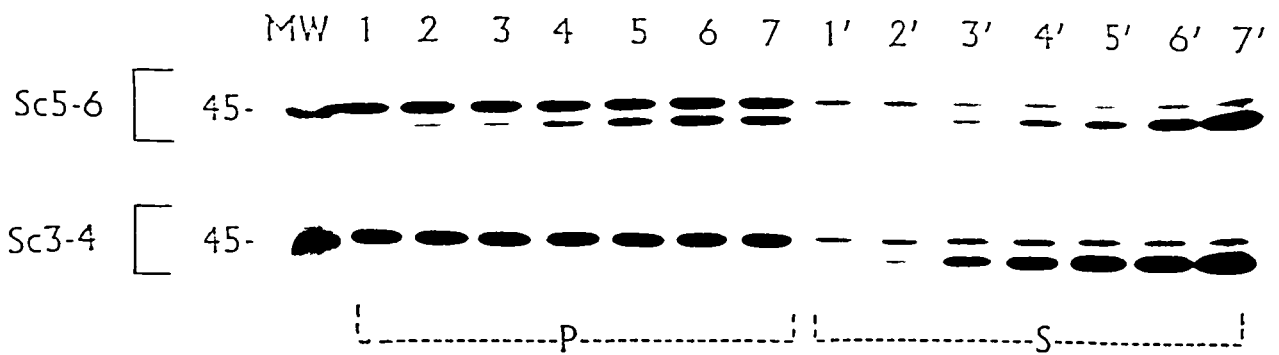
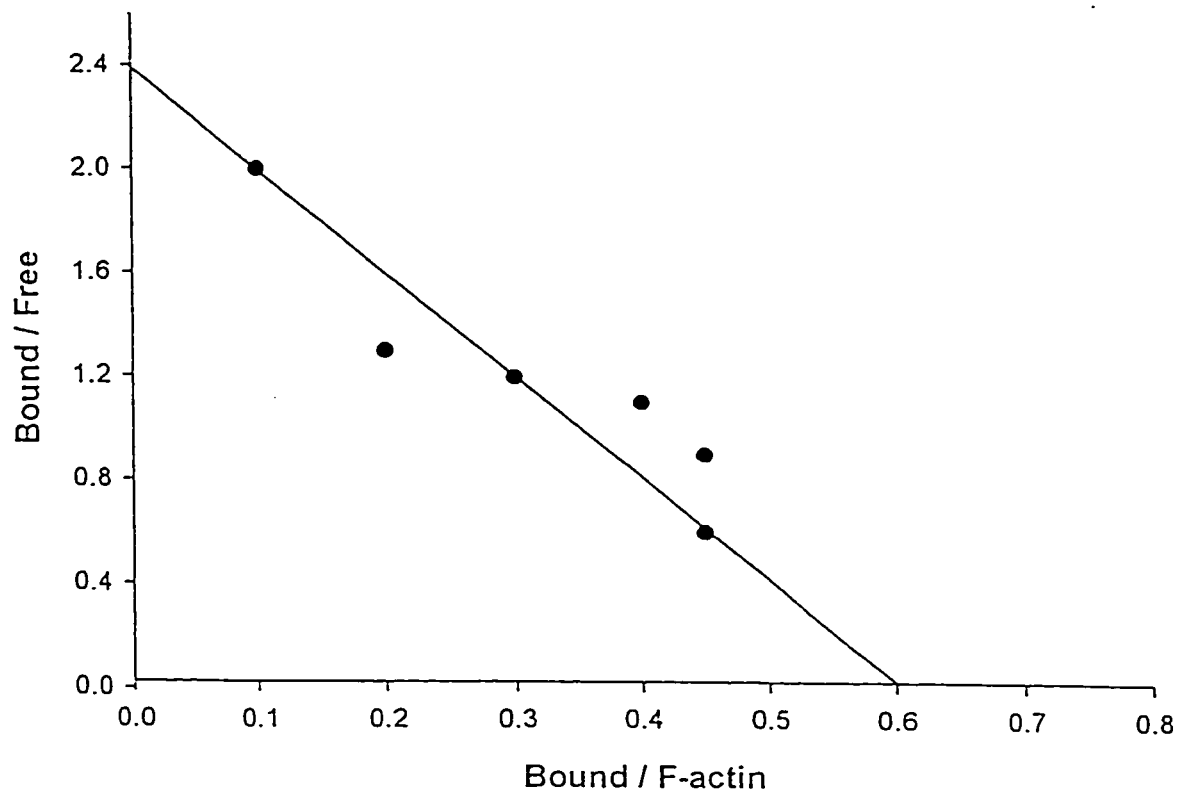


FIGURE 3.8.1

**3.8.2 Severing Activity of Recombinant Full Length Scinderin and its Fragments.** The severing activity of recombinant scinderins was determined by measuring F-actin gel apparent viscosity. The low-shear viscosity of the samples was measured using a falling ball viscometer. This technique measures apparent viscosity because cross-linked actin filaments form a non-Newtonian fluid, as described by MacLean-Fletcher and Pollard (1980). Therefore, the assay is regarded as semiquantitative. Previous experiments from our laboratory have shown that contrary to full length scinderin, Sc3-6 does not increase the secretory response of chromaffin cells (Zhang et al., 1996). Measurement of viscosity of actin gels in the presence of recombinant Sc, Sc3-6, Sc5-6, Sc1-4,6 and a scinderin-derived acting binding peptide (Sc-ABP<sub>3</sub>) showed that Sc3-6 (data not shown) and Sc5-6 do not seem to have any effect on viscosity of actin gels (Fig.3.8.2, A). However, and as expected, recombinant full length scinderin (r-Sc) was very effective in decreasing viscosity (Fig.3.8.2, A). Mixtures of r-Sc with actin in the presence of either Sc5-6 or Sc-ABP<sub>3</sub> in different molar ratios were tested in the presence of 10  $\mu$ M Ca<sup>2+</sup>. Under these conditions r-Sc alone decreases the viscosity of actin gels (Fig.3.8.2, B). However, in the presence of either Sc5-6 or Sc3-6 (data not shown), r-Sc ability to decrease viscosity of actin gels was reduced (Fig.3.8.2, B). Moreover, when r-Sc was tested in the presence of Sc-ABP<sub>3</sub>, a similar inhibition of r-Sc effect on

**FIGURE 3.8.2 Effect of Recombinant Full Length (r-Sc), Sc1-4,6 and Sc5-6 Scinderins and Peptide Sc-ABP<sub>3</sub> on the Apparent Viscosity of F-actin.** A) Effect of various molar ratios of r-Sc or Sc5-6 to actin. G-actin was induced to polymerize for 1 h at 22°C and then it was diluted to 0.22  $\mu$ M in the PIPES-based buffer, mixed with various concentrations of either r-Sc or Sc5-6 on ice, then drawn into 100  $\mu$ l glass capillaries, sealed at one end and held horizontally at room temperature for 2 h before determination of the apparent viscosity. The tube was mounted at a 45° angle and viscosity was determined by the falling ball technique, as indicated in Materials and Methods. B) Effect of Sc5-6 and Sc-ABP<sub>3</sub> on the fall in apparent viscosity of actin produced by r-Sc. Conditions for measurement of the apparent viscosity were as above. The molar ratios used in the test are specified in the figure. Both Sc5-6 and Sc-ABP<sub>3</sub> inhibited the fall in apparent viscosity produced by recombinant full length scinderin (r-Sc). C) Effect of Sc1-4,6 on the apparent viscosity of actin. Conditions were as above. Sc1-4,6 was as effective as r-Sc in decreasing the viscosity of actin and when added together it increased the fall in viscosity produced by r-Sc. Contrary to its effects on r-Sc, Sc-ABP<sub>3</sub> did not inhibit the effect of Sc1-4,6 on actin viscosity.

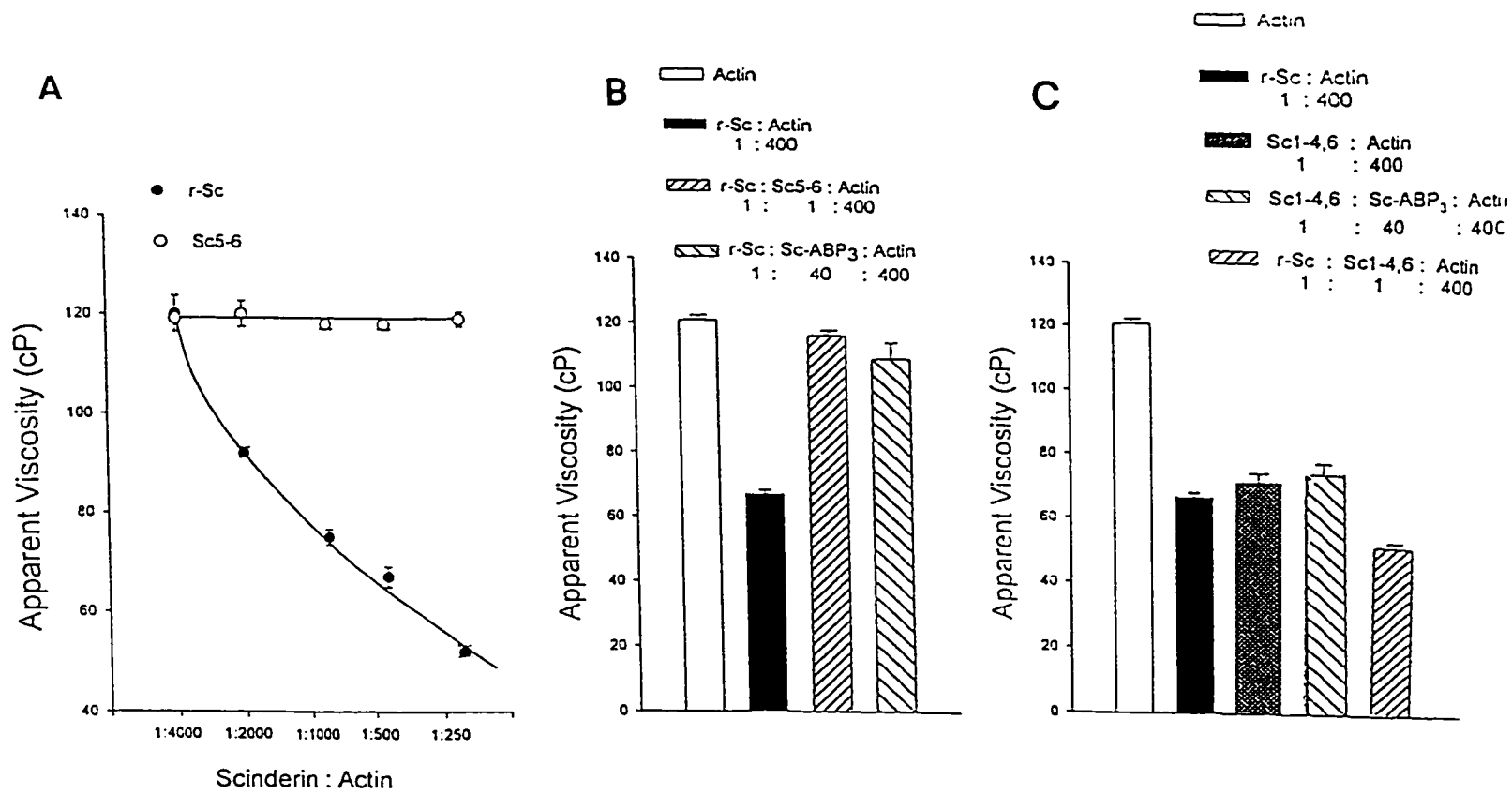
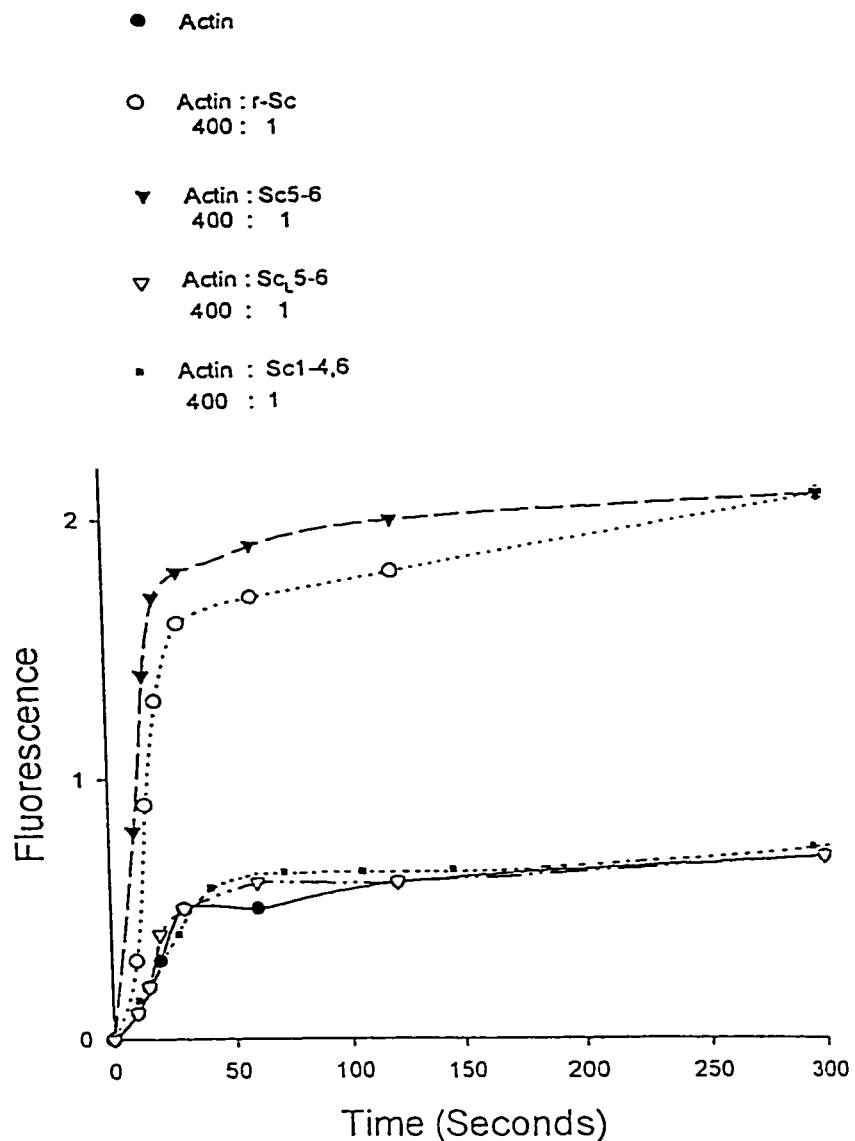


FIGURE 3.8.2

actin gels was observed (Fig.3.8.2, B). In view of these results, which indicate that the binding to actin by either domain 5 or Sc-ABP<sub>3</sub> interferes with the binding of r-Sc(Sc1-6) or its activity, Sc1-4,6 a construct with a deletion of domain 5, was tested. Sc1-4,6 was as effective as r-Sc in reducing the apparent viscosity of actin and in this case, Sc-ABP<sub>3</sub> was ineffective in blocking the actin severing activity of the construct (Fig.3.8.2., C). Moreover, when r-Sc and Sc1-4,6 were combined, there was a summation of their actin severing activities (Fig.3.8.2., C).

**3.8.3 Nucleation Activity** Actin polymerization was evaluated by measuring the increase in fluorescence of pyrene-labelled actin as indicated in Materials and Methods. In the presence of either r-Sc or Sc5-6 and after a lag period of approximately 10 and 15 s respectively, the fluorescence intensity increased exponentially reaching a maximum at 110 s (Fig.3.8.3). In the absence of r-Sc or Sc5-6, actin polymerized slowly reaching after 300 s 33% and after 120 min 30% of the fluorescence intensity reached in the presence of either recombinant scinderin, thus suggesting that spontaneous nucleation was very slow. Sc<sub>5</sub>-6, which corresponds to amino acids 519-715 of scinderin, did not nucleate actin assembly nor did Sc1-4,6, a construct in which domain 5 was deleted (Fig.3.8.3). The results indicate that Sc5-6, even when tested at a small molar ratio to actin (1:400) is a very



**FIGURE 3.8.3 Fluorescence Monitoring of Actin Nucleation.** Actin alone ( $6 \mu\text{M}$ ), and in combination with r-Sc, Sc5-6, Sc<sub>5</sub>-6, or Sc1-4,6 at the indicated molar ratios was polymerized in the presence of  $0.2 \text{ mM Ca}^{2+}$ . All samples contained 5% pyrene iodoacetamide-labeled actin. The fluorescence intensity was measured at  $407 \text{ nm}$  (emission) after excitation at  $365 \text{ nm}$ .

powerful inducer of actin polymerization and that this activity is localized at the NH<sub>2</sub>-terminal and of domain 5 (Sc<sub>502-513</sub>).

## FUNCTIONAL STUDIES:

### RECOMBINANT SCINDERIN EFFECT ON CHROMAFFIN CELL EXOCYTOSIS

#### 3.9 Preparation and Purification of Recombinant Scinderins (TRX-Sc). Actin-DNaseI-Sepharose 4B Purification of Sc1-6 (r-Sc).

The GST-Sc fusion protein prepared as indicated in Materials and Methods was found to be mostly in inclusion bodies which were very difficult to solubilize without strong denaturants. As previously mentioned, in order to avoid this solubility problem, as well as to obtain larger amounts of active fusion scinderin, another expression system - Thioredoxin ThioFusion - was used. Full-length and different truncated scinderin forms were obtained using expression vector pTrxFus as described in Chapter 2.10.2. This plasmid uses a strong, tightly regulated promoter ( $P_{trp}$ ), inducible by tryptophan. The thioredoxin (MW = 11.7 KDa) moiety appears to confer solubility to formerly insoluble heterologous proteins. Immunoblots from SDS-PAGE of E.coli GI698 lysates obtained from one of the tryptophan induced preparations showed a new protein band of approximately 98 kDa which cross-reacted with an antibody against native scinderin (Fig.3.9.B, lane 2'). Tryptophan induction of parental vectors failed to show the 98 kDa band. Aliquots of preparations shown in lanes 1 and 2 (Fig.3.9.A) were applied in the presence of  $Ca^{2+}$  to actin-DNaseI-Sepharose 4B columns. SDS-PAGE and immunoblots with scinderin antibody of  $Ca^{2+}$ -free (EGTA) eluates from the columns

showed that only the same 98 kDa protein was retained by the affinity column and subsequently eluted with the  $\text{Ca}^{2+}$ -free buffer (Fig.3.9.A, lane 4). The cross-reactivity with scinderin antibody indicates that the protein retained by the actin-affinity column was indeed scinderin (Fig.3.9.B, lane 4'). In conclusion, a full-length recombinant scinderin was obtained and this protein interacts with actin in a similar manner to native scinderin.

The "truncated" scinderin (Tr-Sc or Sc3-6) was named this way for being the first truncation obtained with the purpose of studying the activity of scinderin devoid of the known actin and  $\text{PIP}_2$  binding sites. The Tr-Sc produced started at valine 254 and had a molecular weight of 64 kDa (Fig.3.9.C). Tr-Sc contains the entire last three domains of scinderin ( $S_4$ ,  $S_5$  and  $S_6$ ) and two thirds of the carboxy-terminal of domain  $S_3$ . In Western blots, Tr-Sc also showed cross-reactivity with the native scinderin antibody (Fig.3.9.C, lane 1').

Enterokinase cleavage between TRX and scinderin could not be efficiently achieved; in all experiments TRX-Sc (or any other deleted Sc) fusion protein was employed instead of scinderin alone.

### **3.10 Effect of Recombinant Scinderin on $\text{Ca}^{2+}$ -Induced Exocytosis in Chromaffin Cells**

Digitonin permeabilization of chromaffin cells allows study of the secretory machine, bypassing channels and receptor activation. The cytoskeleton remains unaffected by the

**FIGURE 3.9 Expression in E coli GI698 of Recombinant Scinderin and its Binding to Actin.**

(A) Fifty micrograms of protein per well were used in the SDS-PAGE. Lanes 1 and 2 contain proteins from E.coli GI698 transfected with recombinant vector pTrxFus incubated in the absence (lane 1) or presence (lane 2) of tryptophan.

(B) The presence of a 98 kD thioredoxin-scinderin fusion protein in lane 2 is suggested by the cross-reactivity of this protein with an antibody against native scinderin as shown in the Western blot (lane 2'). Aliquots of lysates of preparations shown in A (lanes 1 and 2) were applied in presence of  $Ca^{2+}$  to actin-DNase I Sepharose 4B affinity columns. These were eluted with  $Ca^{2+}$ -free buffer (EGTA) and collected fractions were subjected to SDS-PAGE. Lanes 3 and 4 in (A) correspond to aliquots from protein preparations shown in lanes 1 and 2, respectively. Only the 98 kD thioredoxin-scinderin fusion protein depicted in lane 4 was retained by the affinity column. This protein cross-reacted with a scinderin antibody as shown in the Western blot (B, lane 4').

(C) Lane 1 shows an SDS-PAGE pattern of 50  $\mu$ g of proteins from E coli GI698 transfected with vector pTrxFus carrying an insert of truncated scinderin DNA. After incubation with tryptophan, the presence of a 64kD thioredoxin-truncated scinderin fusion protein in lane 1 is suggested by the cross-reactivity of this protein with the scinderin antibody as shown in the Western blot ( lane 1').

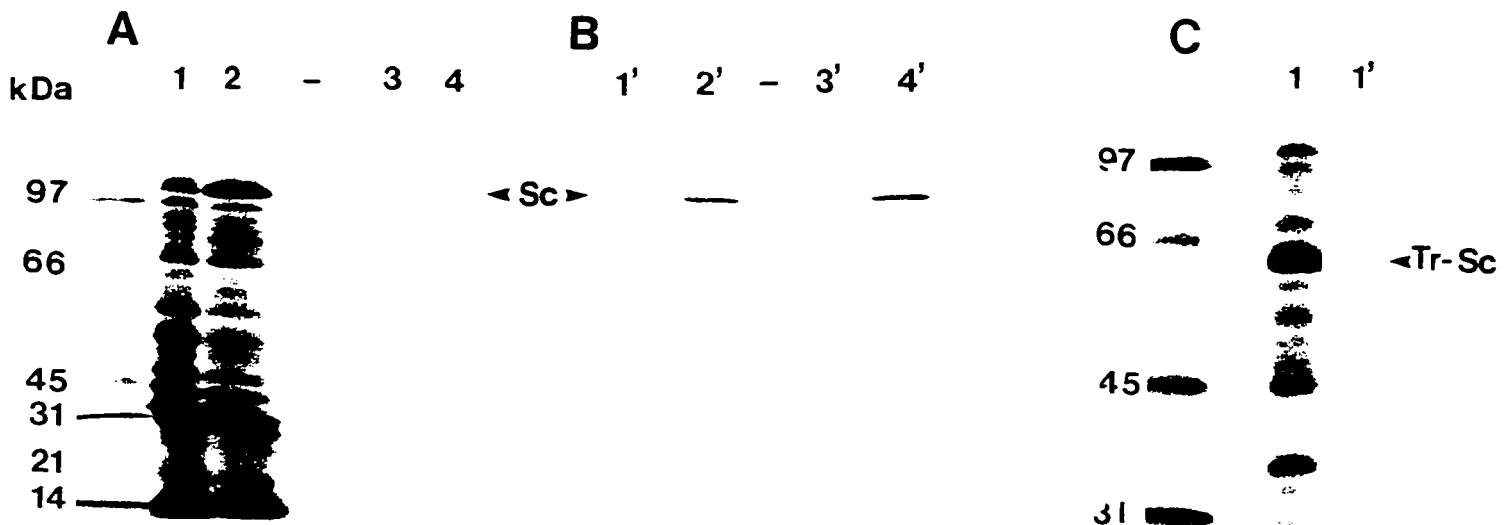


FIGURE 3.9

permeabilization (Dunn et al., 1983). Digitonin permeabilized chromaffin cells previously loaded with [<sup>3</sup>H] noradrenaline ([<sup>3</sup>H]NA) were incubated with increasing concentrations (0.02 to 0.2 μM) of recombinant scinderin. [<sup>3</sup>H]NA release was induced by 2 min exposure to 10 μM Ca<sup>2+</sup>. Under these conditions, Ca<sup>2+</sup> evoked [<sup>3</sup>H]NA release augmented with the increase in the concentration of scinderin in the medium (p<0.01; n=15; Fig.3.10.A). At the concentration of 0.1 μM recombinant scinderin, the Ca<sup>2+</sup>-induced [<sup>3</sup>H]NA output levelled off and no further increase of NA release was observed despite raising the recombinant scinderin concentrations (Fig.3.10., A). The rate of basal [<sup>3</sup>H]NA release from digitonin permeabilized cells was 0.80±0.07%/min (n=11), measured in the absence of Ca<sup>2+</sup>. The [<sup>3</sup>H]NA output under these conditions was not modified in the presence of scinderin (0.78±0.05%/min; n=11). In the presence of increasing Ca<sup>2+</sup> concentration, the response to 0.1 μM recombinant Sc was then tested. Under these conditions, 0.1 μM recombinant scinderin increased the response to Ca<sup>2+</sup>, especially at Ca<sup>2+</sup> concentrations of 1 μM (p<0.01; n=11) and above (Fig.3.10., B). The concentration-response curves obtained in these conditions suggests that scinderin treatment does not significantly increase the sensitivity of the exocytotic machinery to Ca<sup>2+</sup> (p>0.1; n=13; at 0.25 μM Ca<sup>2+</sup>), but it increases the **maximum response** to the cation. Due to the fact that enterokinase did not efficiently cut TRX out of the fusion protein (advanced Sc proteolysis was

**FIGURE 3.10 A, B - Effect of Recombinant Scinderin on Ca<sup>2+</sup>-induced Release of [<sup>3</sup>H]NA from Digitonin Permeabilized Chromaffin Cells.**

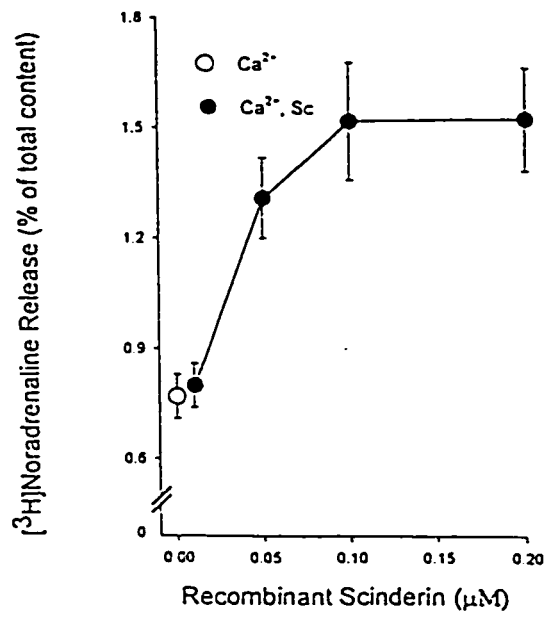
(A) Multi-well culture dishes containing each 5 x 10<sup>5</sup> cells per well were incubated in 250 μl K<sup>+</sup>-glutamate buffer (pH 6.6) for 2 min with 10 μM Ca<sup>2+</sup> alone or in the presence of increasing concentrations of recombinant scinderin (p<0.01, n=15).

(B) Conditions were similar to those described in (A), except that cells were incubated with increasing concentrations of Ca<sup>2+</sup> either in absence or presence of 0.1 μM recombinant scinderin (p<0.01, n=11). The dots (mean ± SEM, n=8) represent [<sup>3</sup>H]NA outputs after subtraction of spontaneous release.

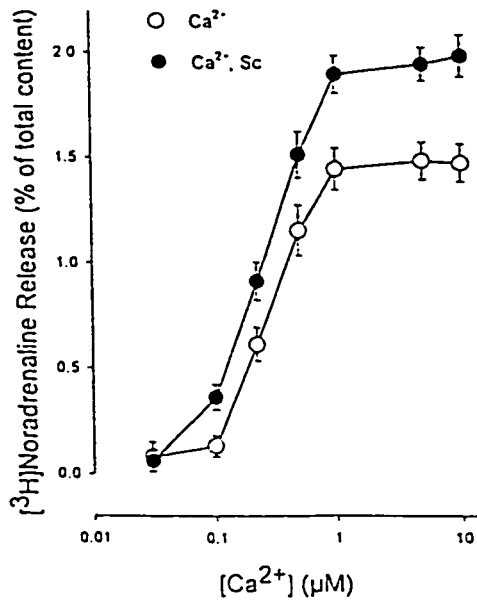
**C - Effects of Thioredoxin Alone or in Combination with Recombinant Scinderin on Ca<sup>2+</sup>-induced release of [<sup>3</sup>H]NA from Digitonin Permeabilized Chromaffin Cells.**

Ten micromolar BSA, 0.5 μM thioredoxin (TRX) or 0.1 μM recombinant scinderin (Sc) with or without 0.5 μM TRX were present in the incubation medium. Stimulation time and Ca<sup>2+</sup> concentrations were as indicated in A above. The bars (mean ± SEM, n=8) represent [<sup>3</sup>H]NA outputs after subtraction of spontaneous release.

A



B



C

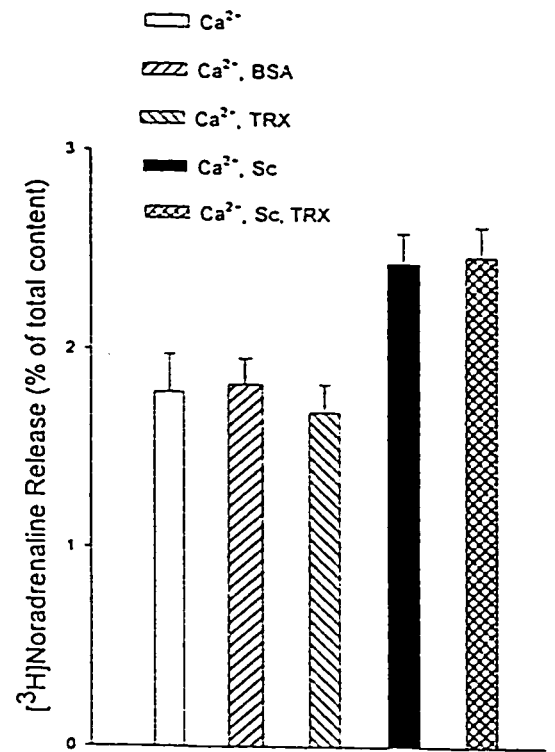


FIGURE 3.10

also evident in the required conditions) all the experiments were done using the whole fusion TRX-Sc protein. It was therefore necessary to rule out the participation of thioredoxin in the observed effects of the fusion scinderin.

E.coli GI698 were transfected with pTrx encoding TRX. TRX was isolated (by osmotic shocking the transformed E.coli) and tested on  $\text{Ca}^{2+}$ -induced [ $^3\text{H}$ ]NA release and on the potentiating effects of recombinant scinderin. TRX at the concentration of  $0.5 \mu\text{M}$  had no effect on either  $\text{Ca}^{2+}$ -evoked [ $^3\text{H}$ ]NA or on the potentiating effect of recombinant scinderin (Fig.3.10.C).

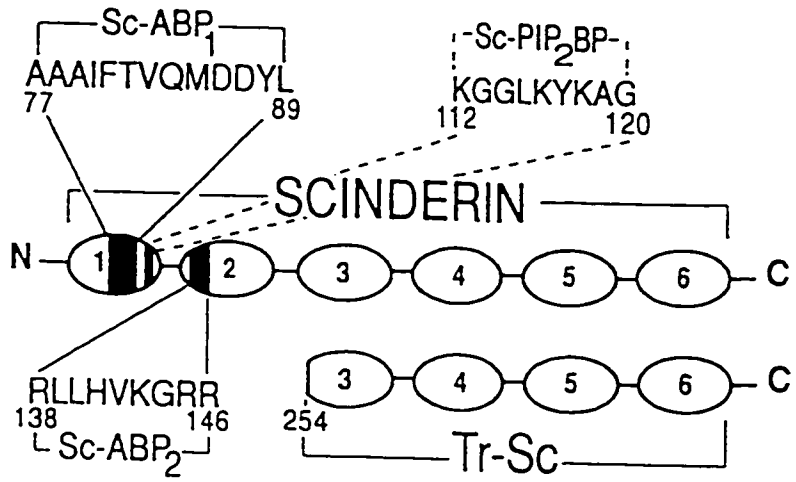
### 3.11. Effect of Scinderin-Derived Actin-Binding Peptides and of $\gamma$ -Actin on $\text{Ca}^{2+}$ -Evoked Exocytosis.

Two scinderin-derived peptides corresponding to the presumed active actin binding sites were designed : Sc-ABP<sub>1</sub> : AAAIFTVQMDDYL, and Sc-ABP<sub>2</sub> : RLLHVKGRR. If potentiation of  $\text{Ca}^{2+}$ -evoked [ $^3\text{H}$ ]NA by recombinant scinderin is due to an increased F-actin severing activity of scinderin, then the ability of scinderin to enhance release might be inhibited in the presence of exogenous  $\gamma$ -actin and/or one or both peptides (Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub>) (Fig.3.11.1.A). Fig.3.11.1.B shows the inhibition of scinderin potentiating effects by  $10 \mu\text{M}$  Sc-ABP<sub>1</sub> ( $p < 0.001$ ;  $n = 18$ ),  $10 \mu\text{M}$  Sc-ABP<sub>2</sub> ( $p < 0.001$ ;  $n = 17$ ), or  $10 \mu\text{M}$  chicken gizzard  $\gamma$ -actin ( $p < 0.001$ ;  $n = 18$ ). Moreover, when  $5 \mu\text{M}$  Sc-ABP<sub>1</sub> and  $5 \mu\text{M}$  Sc-ABP<sub>2</sub> were present together in the

**FIGURE 3.11.1 Effect of Scinderin-derived Actin-binding Peptides and of Exogenous Actin on the Potentiation of Ca<sup>2+</sup>-induced [<sup>3</sup>H]NA Release from Permeabilized Chromaffin Cells by Recombinant Scinderin**

Peptides Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub> and Sc-PIP<sub>2</sub>BP were prepared as indicated in Chapter 2. The sequence of these peptides and their positions within the domains (S<sub>1</sub> to S<sub>ε</sub>) of the scinderin molecule are shown in (A). In addition, a scrambled (SCR) peptide (YAALMFDIDATQV) to be used as control was prepared. No homology for the SCR sequence was found in the in the EMBL data library. Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub> and SCR, each at the concentration of 10 μM were present during both the 5 min digitonin permeabilization and the 2 min stimulation with 10 μM Ca<sup>2+</sup> (p<0.001, n=18 for Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub> ; p<0.05, n=12 when peptides are added together). When indicated, 0.1 μM of either recombinant scinderin or recombinant truncated scinderin (Tr-Sc) was also present during permeabilization and stimulation (B). Tr-Sc corresponds to scinderin 254-715 (A). Under the same experimental conditions, the effect of 10 μM chicken gizzard γ-actin was also tested (p<0.001, n=18)(B). The effects of the above mentioned treatments on [<sup>3</sup>H]NA outputs are shown in (B). Each bar represents the mean ± SEM of at least eight chromaffin cell preparations.

**A**



**B**

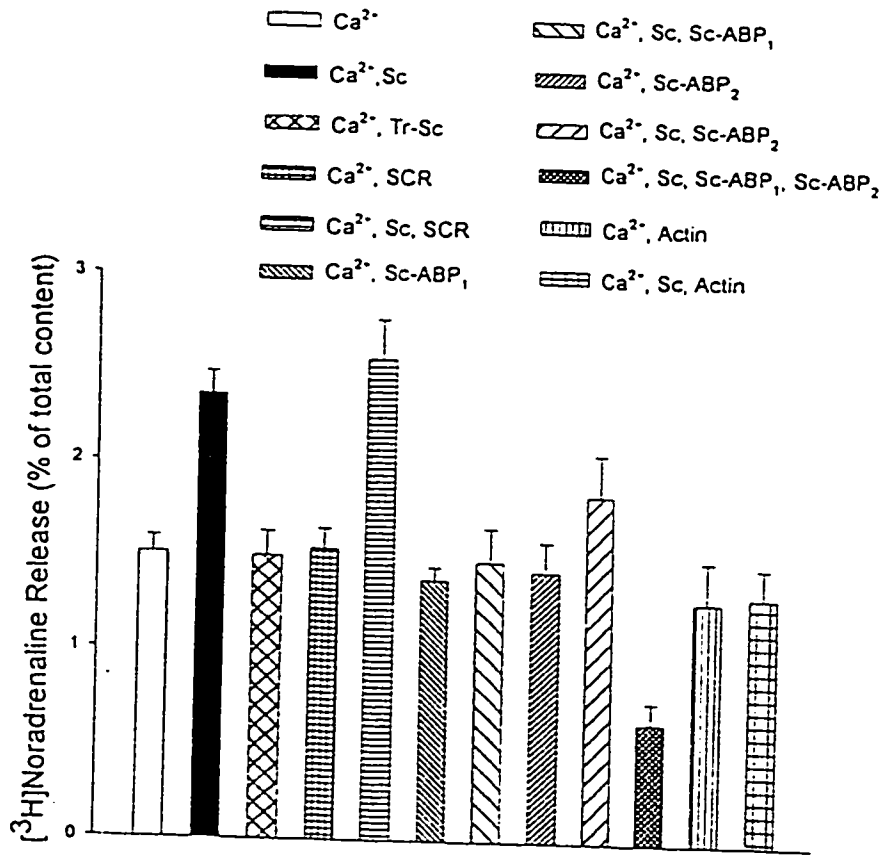
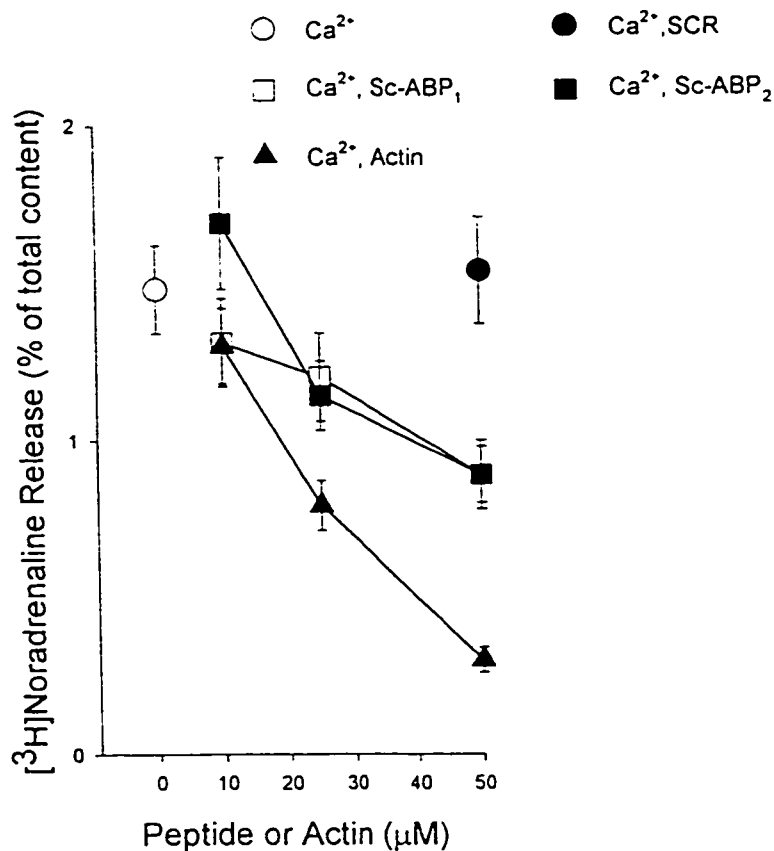


FIGURE 3.11.1

incubation medium (10  $\mu\text{M}$  peptide final concentration), not only the potentiating effect of scinderin on release was blocked ( $p < 0.001$ ;  $n = 19$ ) but this inhibition of release was larger than that produced by either peptide alone ( $p < 0.05$ ;  $n = 12$ ). In addition, the release response was 50% smaller than when induced by  $\text{Ca}^{2+}$  in the absence of scinderin (Fig.3.11.1.B). A stronger inhibition of scinderin activity was possible by blocking both Sc-binding sites of actin simultaneously. The scrambled (SCR) peptide (YAALMFDIDATQV), a mismatch of the Sc-ABP<sub>1</sub>, was used as a negative control, as well as bovine serum albumine (BSA). The presence of 10  $\mu\text{M}$  BSA or 10  $\mu\text{M}$  scrambled (SCR) peptide in the incubation media did not modify  $\text{Ca}^{2+}$ -induced [<sup>3</sup>H]NA release either in the presence or absence of recombinant scinderin (Figs.3.10.1.C and 3.11.1.B). Another scrambled peptide - LVRGKRRRLH - designed this time on the basis of the sequence of peptide Sc-ABP<sub>2</sub> also produced similar results (data not shown) to those obtained with SCR. As depicted in Fig.3.11.1., A, two of the actin-binding sites of scinderin are in domains S<sub>1</sub> and S<sub>2</sub> of the molecule. These are the putative F-actin severing domains of Sc and should be the domains responsible for the scinderin potentiation of  $\text{Ca}^{2+}$ -induced release of catecholamines. A truncated form of Sc (Tr-Sc) devoid of the first two domains should not potentiate the catecholamine release. Indeed, Tr-Sc (Fig.3.11.1.A) (amino acid 254-715) when used at the same concentrations (0.1  $\mu\text{M}$ ) as recombinant full-length scinderin



**FIGURE 3.11.2** Effect of Sc-Derived-Actin-Binding Peptides and of Exogenous Actin on  $\text{Ca}^{2+}$ -Evoked  $[^3\text{H}]\text{NA}$  Release  
 Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub>, SCR and chicken gizzard  $\gamma$ -actin, each at concentrations indicated in the graph, were present during both the 5 min digitonin permeabilization and the 2 min stimulation with 10  $\mu\text{M}$   $\text{Ca}^{2+}$ .  $[^3\text{H}]\text{NA}$  output was measured as described in Materials and Methods. Each symbol represents the mean  $\pm$  SEM of at least eight chromaffin cell preparations.

did not potentiate nor inhibit  $\text{Ca}^{2+}$ -induced catecholamine release (Fig.3.11.1.B). Moreover, in the absence of recombinant scinderin, exogenous  $\gamma$ -actin ( $p < 0.01$ ;  $n=16$  and  $p < 0.001$ ;  $n=16$  for 25 and 50  $\mu\text{M}$  actin respectively), Sc-ABP<sub>1</sub> ( $p < 0.001$ ;  $n=15$ ) and Sc-ABP<sub>2</sub> ( $p < 0.02$ ;  $n=15$ ), but not SCR, also produced a concentration -dependent inhibition of  $\text{Ca}^{3+}$ -evoked release of [<sup>3</sup>H]NA (Fig.3.11.2), therefore suggesting inhibition of endogenous scinderin activity. The above mentioned peptides bind to the actin substrate (a very abundant protein, 5-10% of total proteins in chromaffin cells); in order to be effective inhibitors, they should be present in high concentrations (micromolar range).

### 3.12 Effect of Phosphatidylinositol 4,5-Bisphosphate (PIP<sub>2</sub>) on the Potentiation of $\text{Ca}^{2+}$ -Evoked Exocytosis by Recombinant Scinderin.

In resting cells, scinderin binds to the phospholipids of the plasma membrane. PIP<sub>2</sub> modulates the severing activity of scinderin (Rodríguez Del Castillo et al, 1992; Marcu et al., 1994), therefore we tested the effect of PIP<sub>2</sub> on the potentiation of [<sup>3</sup>H]NA release induced by recombinant Sc. The ability of scinderin to increase  $\text{Ca}^{2+}$ -induced [<sup>3</sup>H]NA release was inhibited by 10  $\mu\text{M}$  PIP<sub>2</sub> ( $p < 0.001$ ,  $n=8$ ). The inhibition was greater when, before  $\text{Ca}^{2+}$  stimulation, permeabilized chromaffin cells were pre-incubated for 5 min with PIP<sub>2</sub> in a  $\text{Ca}^{2+}$ -free medium containing recombinant scinderin (Fig.3.12.A).

**FIGURE 3.12. Effect of PIP<sub>2</sub> on the Potentiation of Ca<sup>2+</sup>-evoked [<sup>3</sup>H]NA Release by Recombinant Scinderin.**

(A). PIP<sub>2</sub> at the concentration of 10 μM was present during the 5 min digitonin permeabilization, 5 min pre-incubation and 2 min stimulation with 10 μM Ca<sup>2+</sup> (p<0.001, n=25). In some experiments both PIP<sub>2</sub> and scinderin-derived PIP<sub>2</sub> binding peptide (Sc-PIP<sub>2</sub>BP), each at the concentration of 10 μM were present during these 3 periods. The sequence of Sc-PIP<sub>2</sub>BP and its position in the scinderin molecule is depicted in Fig.3.10.1 A. When indicated, 0.1 μM recombinant scinderin was present during the described periods. The bars (mean ± SEM, n=8) represent [<sup>3</sup>H] outputs after subtraction of spontaneous release.

(B) PIP<sub>2</sub> liposomes were prepared as described in Chapter 2. Recombinant scinderin (0.1 μM) was incubated alone (column 1) or with 200 μl PIP<sub>2</sub> liposomes (columns 2 and 3) in the absence (column 2) or presence (column 3) of 10 μM Sc-PIP<sub>2</sub>BP. Liposomes were collected by centrifugation and supernatants (S) and pellets (P) were subjected to SDS-PAGE followed by Western blotting with a scinderin antibody. When recombinant scinderin was incubated in the absence of liposomes, the protein was recovered in the supernatant (column 1, S) whereas in the presence of liposomes scinderin was recovered mainly with the pellets (column 2, P). It is clear from the experiments that Sc-PIP<sub>2</sub>BP blocked the binding of scinderin to PIP<sub>2</sub> liposomes since the protein was recovered with the supernatants (column 3,S).

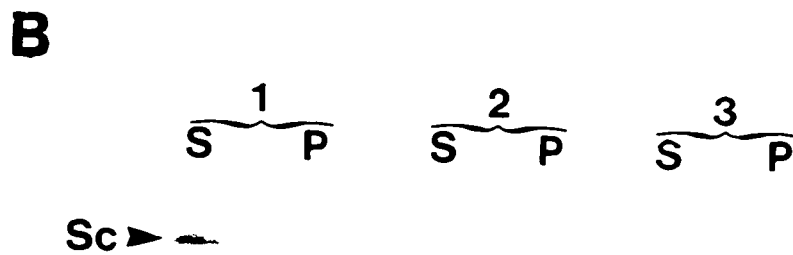
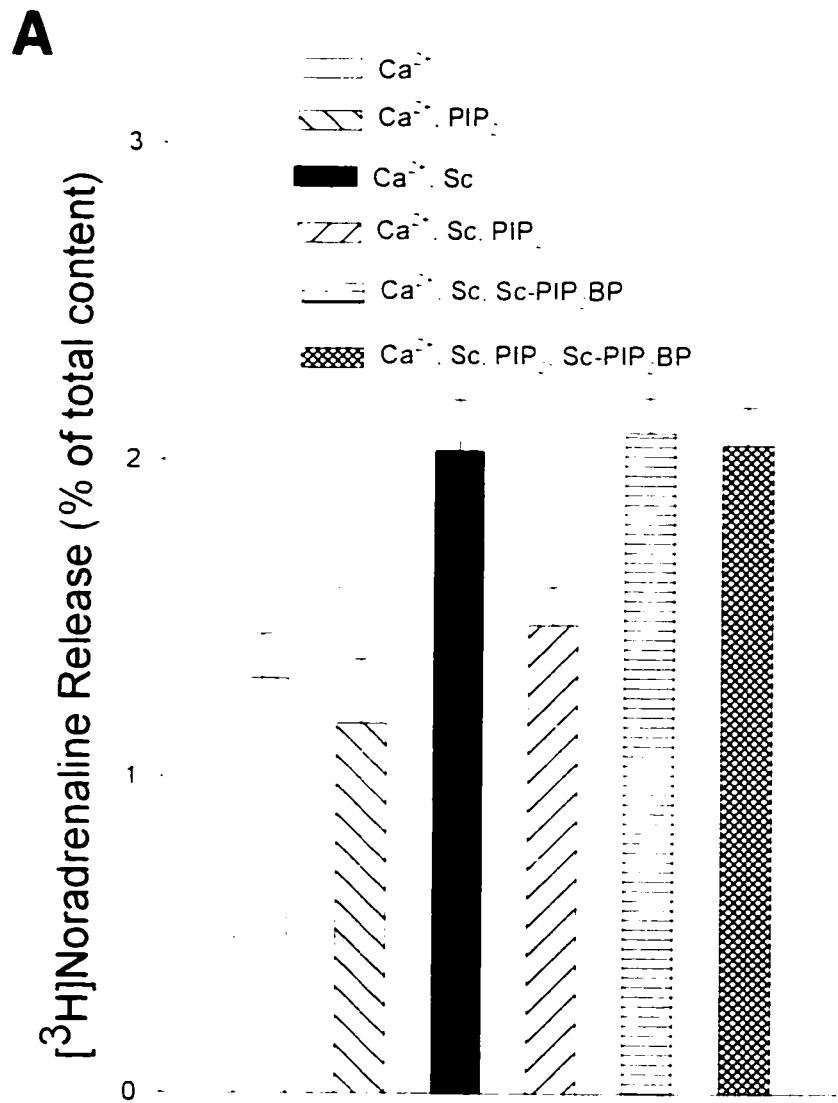


FIGURE 3.12

A scinderin-derived PIP<sub>2</sub>-binding peptide with the following sequence: (Fig.3.11.1.A) KGGLKYKAG was synthesized. When this peptide was present in the incubation medium, the inhibitory effect of PIP<sub>2</sub> was completely blocked (Fig.3.12.A). Sc-PIP<sub>2</sub>BP has a sequence corresponding to the PIP<sub>2</sub>-binding site present in domain S<sub>1</sub> of scinderin (Fig.3.11.1.A). Sc-PIP<sub>2</sub>BP effect was due to the blockade of PIP<sub>2</sub> binding to scinderin as suggested by the results obtained with PIP<sub>2</sub> liposomes (Fig.3.12. A, B). TRX-recombinant scinderin was incubated with PIP<sub>2</sub> liposomes in the presence or in the absence of Sc-PIP<sub>2</sub>BP. Liposomes were then collected by centrifugation and SDS-PAGE and Western blots with scinderin antibody were performed on supernatants and sediments (Fig.3.12.B). TRX-Sc was found with the PIP<sub>2</sub> liposomes in the sediment except when Sc-PIP<sub>2</sub>BP was present in the incubation medium. These results demonstrate that PIP<sub>2</sub> binds very well to scinderin under the same conditions (i.e. pH 6.6) in which the release experiments were performed, and that Sc-PIP<sub>2</sub>BP interferes with the binding.

### **3.13. Effect of Recombinant Scinderin on Cortical F-Actin Disassembly; Fluorescence Microscopy Studies and Video Image Analysis**

In intact resting chromaffin cells, staining of F-actin with rhodamine-phalloidin revealed a diffuse, weak cytoplasmic staining and a continuous cortical ring (Cheek et al., 1986; Vitale et al., 1991). Digitonin permeabilization followed by

rhodamine phalloidin revealed similar pattern of staining (Fig.3.13. A,a). Phalloidin is an alkaloid with high affinity for F-actin. A quantitative tridimensional image analysis of the cortical distribution of rhodamine fluorescence, representing F-actin, of the cell shown in Fig.3.13. A,a,a' is depicted in Fig.3.13. A,a''. It is known that stimulation of intact chromaffin cells with either  $10^{-5}$ M nicotine or depolarization with 56 mM  $K^+$  leads to the disruption of the continuous cortical fluorescent ring (Cheek et al., 1986; Trifaró et al., 1989; Vitale et al., 1991). The disappearance of rhodamine fluorescence (phalloidin is a probe for actin filaments) indicates disassembly of F-actin at specific subplasmalemmal areas (Vitale et al., 1991). Digitonin-permeabilization of chromaffin cells followed by incubation in  $10 \mu\text{M Ca}^{2+}$  induces disruption of the cortical fluorescent ring as well (Fig.3.13. A,b,b'). Video image analysis of the discontinuous fluorescent patterns reflects characteristic peaks and valleys (Fig.3.13. A,b''). The peaks (with similar intensities as the continuous cortical pattern seen in fluorescent studies of control cells) represent the absence of F-actin disassembly. The valleys, on contrary, depicted areas of F-actin disassembly, corresponding to decreases in the intensity of normal fluorescence of the cortical rhodamine staining. Fig.3.13. B shows a quantification of cortical F-actin disassembly under different conditions. Upon stimulation with  $10 \mu\text{M Ca}^{2+}$ , there was a significant increase ( $p < 0.001$ ;

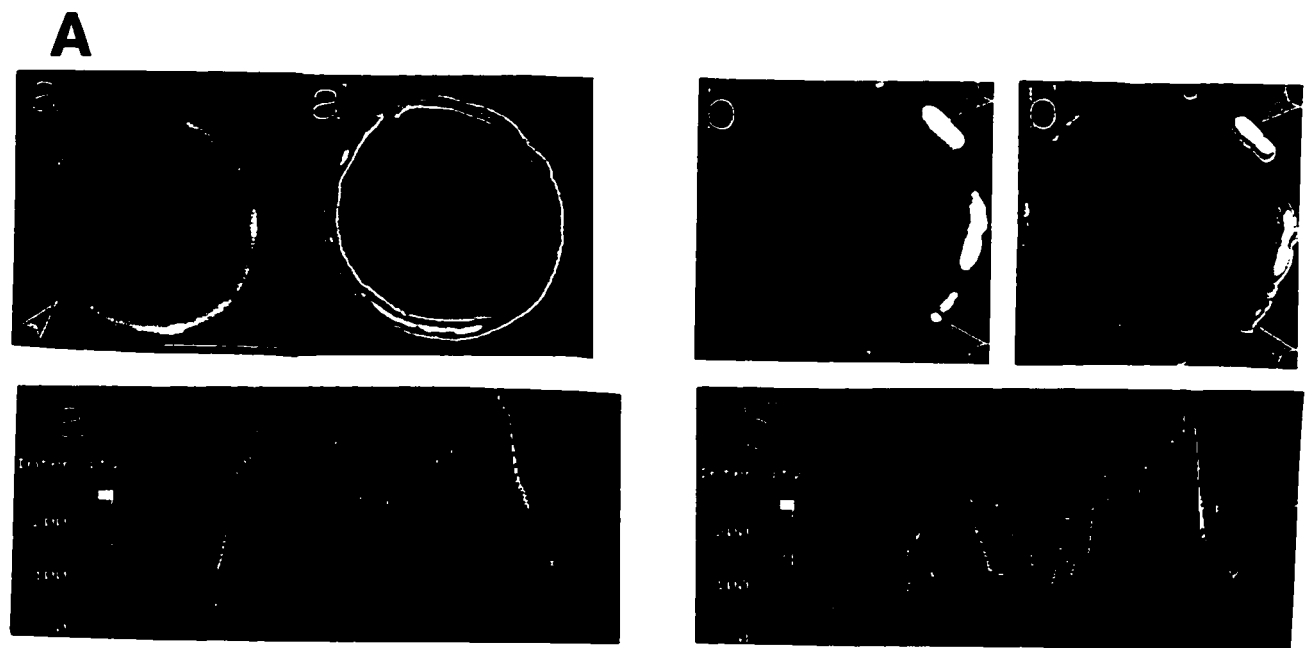
n=13) in the number of chromaffin cells displaying a disrupted cortical fluorescent ring and this was due to F-actin disassembly. When recombinant Sc (0.1  $\mu$ M) was added to the medium, the number of cells showing F-actin disassembly was even greater ( $p < 0.001$ ;  $n = 12$ ; Fig.3.13. B) and this effect was not influenced by 10  $\mu$ M SCR at all. On the other hand, 10  $\mu$ M Sc-ABP<sub>1</sub> ( $p < 0.05$ ;  $n = 13$ ; Fig.3.13. B), reduced significantly the potentiating effect of recombinant scinderin. Sc-ABP<sub>1</sub> not only blocked the effect of recombinant scinderin but it also inhibited to a certain degree the response to 10  $\mu$ M Ca<sup>2+</sup> in the absence of recombinant Sc (Fig.3.13. B), thus suggesting inhibition of **endogenous scinderin** activity. In these experiments, PIP<sub>2</sub> (similarly to its effects on Ca<sup>2+</sup>-evoked exocytosis) also reduced the increase in F-actin disassembly induced by recombinant scinderin and this inhibitory response was blocked by Sc-PIP<sub>2</sub>BP (Fig.3.13.B).

FIGURE 3.13 Effect of  $Ca^{2+}$ , Phosphatidylinositol 4,5-bisphosphate, Recombinant Scinderin and Scinderin-derived Actin- (Sc-ABP<sub>1</sub>) and PIP<sub>2</sub> (Sc-PIP<sub>2</sub>BP) Binding Peptides on Cortical F-actin.

(A) Fluorescence microscopy and video-enhanced image analysis of F-actin fluorescent profiles in single digitonin-permeabilized chromaffin cells. (B) shows the effect of different treatments on the percentage of cells displaying cortical F-actin disassembly. Two-day old chromaffin cells were permeabilized with digitonin as described in Materials and Methods and were incubated in  $K^+$ -glutamate buffer and  $10 \mu M$   $Ca^{2+}$  for 2 min in the presence or absence of  $0.1 \mu M$  of recombinant scinderin. When indicated,  $10 \mu M$  of either Sc-ABP<sub>1</sub>, SCR, Sc-PIP<sub>2</sub>BP or PIP<sub>2</sub> were present in the incubation medium. Preparations were immediately processed for fluorescence microscopy. Cells were fixed, permeabilized and stained for F-actin as described in Materials and Methods.

A, a) shows a resting digitonin permeabilized chromaffin cell after rhodamine-phalloidin staining. A weak cytoplasmic staining and a continuous and bright cortical fluorescent ring (open arrowhead) is observed. Stimulation of the cells by either  $Ca^{2+}$  or  $Ca^{2+}$  plus recombinant scinderin caused the disruption of the cortical fluorescent ring (A, b). Some fluorescent patches are shown by arrowheads (A, b, b'). Images and three-dimensional analysis of the same cells shown in (A, a, a' and b, b') are depicted in A, a'' and b''. In control cells, there is a uniform (A, a'') cortical fluorescence intensity pattern. In stimulated cells, the cortical fluorescence intensity pattern shows irregularities such as valley and peaks (A, b''). The peaks correspond to the fluorescent patches observed in the cell shown in (A, b and b'). The intensity of the fluorescent peaks is indicated by a colour scale and arbitrary units (A, a'' and b''). The value of the peaks in (A, b'') is similar to the intensity of the cortical fluorescence pattern observed in control cells (A, a''). Bar,  $10 \mu m$ .

(B) Under the fluorescence microscope, the rhodamine cortical staining was analyzed and classified as being continuous (A, a, a') or discontinuous as in (A, b, b') and the percentage of cells displaying cortical F-actin disassembly (disrupted cortical rhodamine staining) in control and treated preparations was calculated as indicated in Materials and Methods. For each of the experimental conditions, 600 cells from three different cell cultures were examined. Values shown are mean  $\pm$  SEM. ( $p < 0.001$ ,  $n = 13$  for  $Ca^{2+}$  stimulation with or without Sc;  $p < 0.05$ ,  $n = 13$  in the presence of Sc-ABP.)



**B**

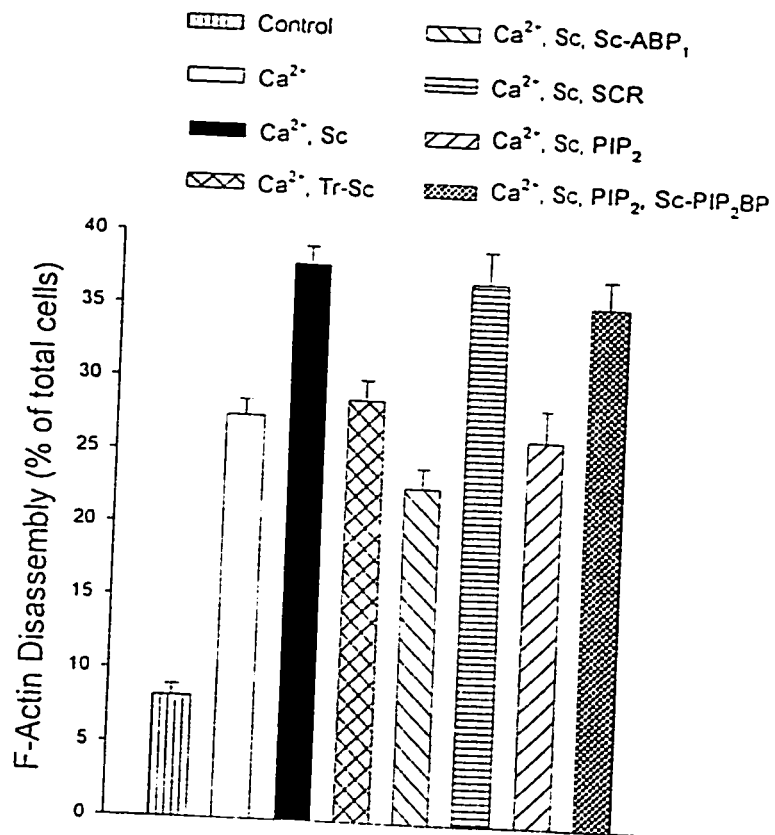


FIGURE 3.13

## EFFECT OF RECOMBINANT SCINDERIN ON SEROTONIN RELEASE FROM PERMEABILIZED PLATELETS

### 3.14 Effect of Recombinant Scinderin on Ca<sup>2+</sup>-Induced Serotonin Release.

Digitonin permeabilized platelets, previously loaded with [<sup>3</sup>H]5-hydroxytryptamine (5-HT) (see 2.19 and 2.20) were incubated with increasing concentrations of recombinant Sc. Serotonin release was induced by 45 s exposure to 10 μM Ca<sup>2+</sup>. In these conditions, recombinant Sc produced a concentration dependent increase in Ca<sup>2+</sup>-evoked 5-HT release (Fig. 3.14.1). Due to the fact that TRX could not be efficiently cut off the recombinant protein, all experiments were performed with the fusion protein TRX-Sc. Thus, in order to rule out the participation of thioredoxin (TRX) in the observed effects of the recombinant scinderin, the parental plasmid pTrx, encoding the TRX was introduced into E-coli GI698 . TRX was purified by osmotic shocking the transformed E-coli and tested on Ca<sup>2+</sup>-evoked 5-HT release. TRX at the concentration of either 0.5 or 5 μM had no effect on Ca<sup>2+</sup> induced 5-HT release or on the potentiating effect of recombinant scinderin (Fig. 3.14.2).

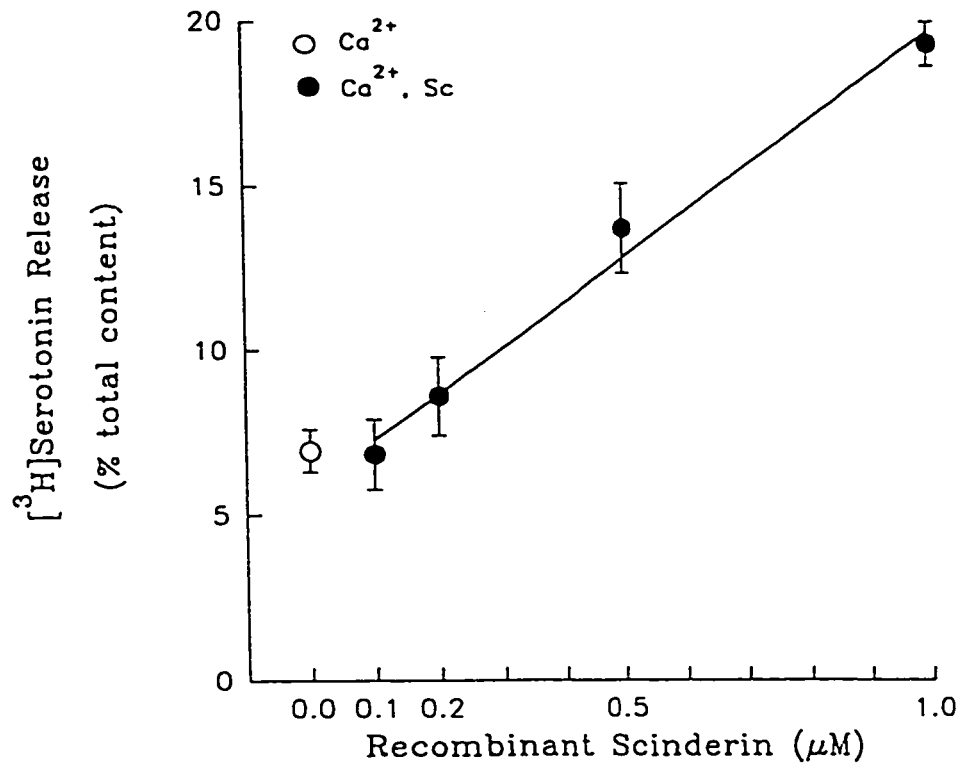


FIGURE 3.14.1 Effect of Recombinant Scinderin on  $\text{Ca}^{2+}$ -induced Release of 5-HT from Digitonin Permeabilized Platelets. Samples containing each  $7.5 \times 10^7$  platelets were incubated in  $100 \mu\text{l}$  K<sup>+</sup>-glutamate buffer (pH 7.4) for 45 s with  $10 \mu\text{M}$   $\text{Ca}^{2+}$  alone or in the presence of increasing concentrations of recombinant scinderin. The dots (mean  $\pm$  SEM, n=6) represent 5-HT outputs after subtraction of spontaneous release.

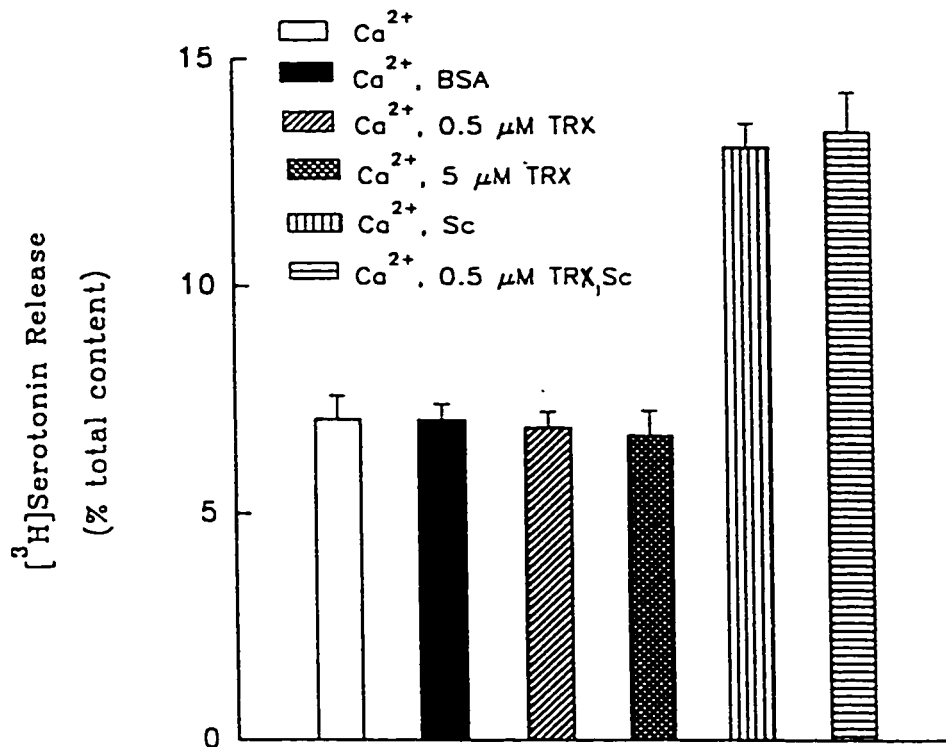


FIGURE 3.14.2 Effects of Thioredoxin Alone or in Combination with Recombinant Scinderin on Ca<sup>2+</sup>-induced Release of Serotonin from Digitonin Permeabilized Platelets Two micromolar bovine serum albumin (BSA), 0.5 and 5.0 μM thioredoxin (TRX) or 0.5 μM recombinant scinderin (Sc) with or without 0.5 μM TRX were present in the incubation medium and stimulation time and Ca<sup>2+</sup> concentrations were as indicated in legend to Fig.3.14.1. The bars (mean ± SEM, n=6) represent 5-HT outputs after subtraction of spontaneous release.

### 3.15 Effects of Scinderin-Derived Actin-Binding Peptides and of $\gamma$ -Actin on $\text{Ca}^{2+}$ -Induced Serotonin Release.

Two actin-binding peptides were designed on the basis of scinderin sequence, as described in Chapter 3.11, Sc-ABP<sub>1</sub>: AAAIFTVQMDDYL, and Sc-ABP<sub>2</sub>: RLLHVKGRR. Recombinant scinderin seems to potentiate 5-HT release by severing the actin filaments since this activity could be inhibited by 10  $\mu\text{M}$  of Sc-ABP<sub>1</sub> (90.2  $\pm$  11% inhibition), Sc-ABP<sub>2</sub> (81.4%  $\pm$  7% inhibition), (Fig.3.15.A, B) or by the addition to the incubation media of 10  $\mu\text{M}$  chicken gizzard  $\gamma$ -actin (74.4  $\pm$  9% inhibition) (Fig. 3.15.B). In the absence of TRX-Sc, exogenous actin, Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub> also decreased  $\text{Ca}^{2+}$ -induced 5-HT release (Fig. 3.15.B), therefore suggesting inhibition of endogenous scinderin activity. Two negative controls, bovine serum albumin (BSA) and a mismatched peptide (MMP): QIVIHLNSKDGSC (with no homology in the EMBL data library), added to the incubation media did not change  $\text{Ca}^{2+}$ -evoked 5-HT release either in the presence or absence of recombinant scinderin (Figs.3.14.2 and 3.15.B).

**FIGURE 3.15 Effect of Scinderin-derived Actin-binding Peptides and of Exogenous Actin on the Potentiation by Recombinant Scinderin of Ca<sup>2+</sup>-induced 5-HT Release.**

Peptides Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub> were prepared, their sequence checked (Henvick et al., 1981) and purity determined by mass spectroscopy (Edmonds et al., 1990). The sequence of these peptides and their positions within the domains (1 to 6) of the scinderin molecule are shown in (A). In addition, a mismatched peptide (MMP) (QIVIHLSKDGSC) to be used as control was prepared. No homology for the MMP sequence was found in the EMBL data library. Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub> and MMP, each at the concentration of 10 μM were present during both the 5 min digitonin permeabilization and the 45 s stimulation with 10 μM Ca<sup>2+</sup> (B). When indicated, 0.5 μM recombinant scinderin was also present during permeabilization and stimulation (B). Under the same experimental conditions, the effect of 10 μM chicken gizzard γ-actin was also tested (B). [<sup>3</sup>H]5-HT output was measured as described in Materials and Methods. The effects of the above mentioned treatments on 5-HT release are shown in (B). Each bar represents the mean ± SEM of at least 5 platelet preparations.

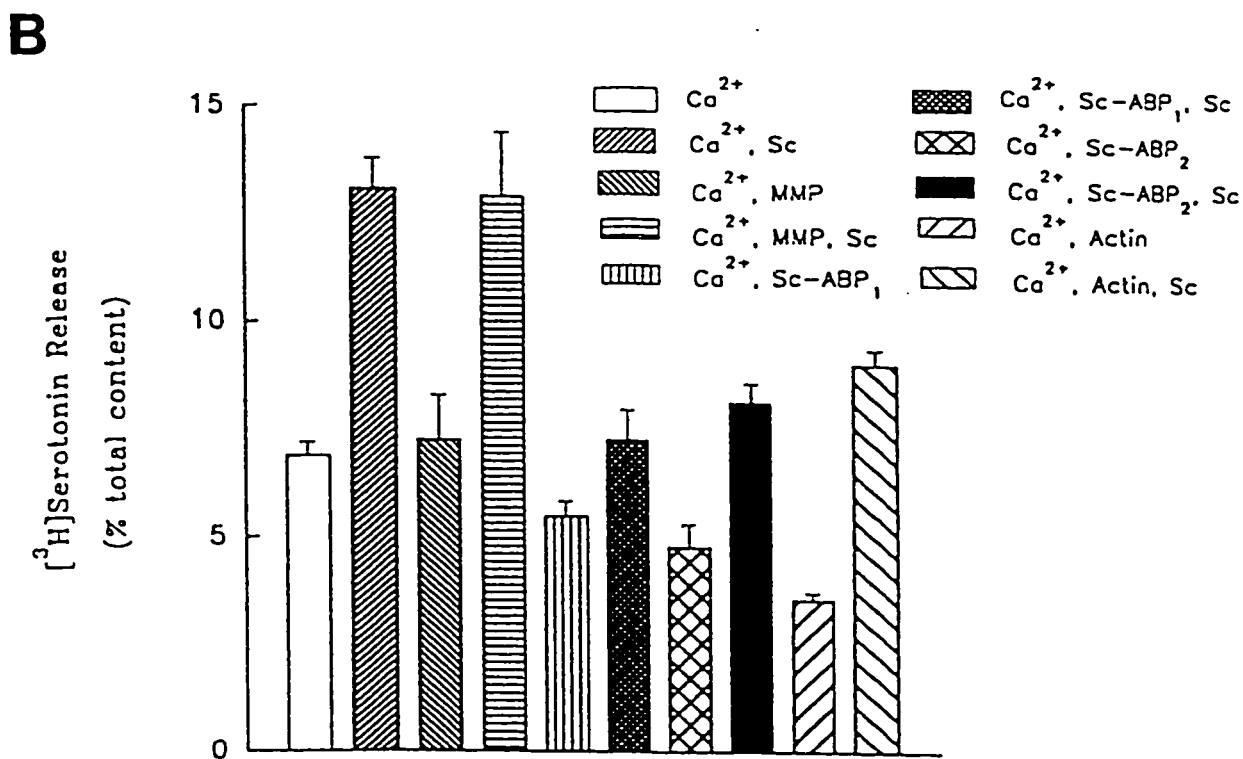
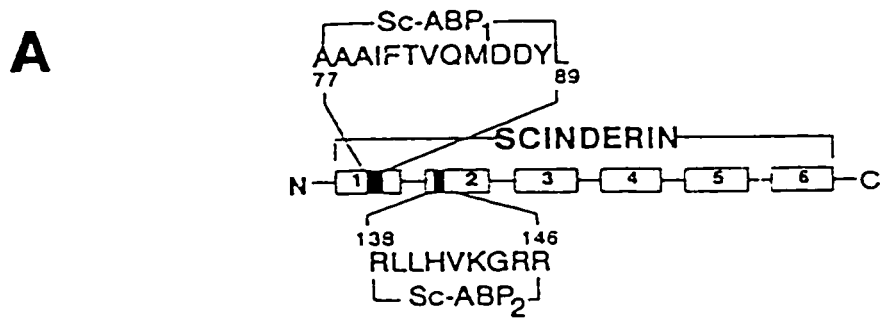


FIGURE 3.15

### 3.16 Effect of Phosphatidylinositol 4,5-Bisphosphate (PIP<sub>2</sub>) on Recombinant Scinderin Activity in Platelets

Our experiments tested the effect of PIP<sub>2</sub> on the potentiation of 5-HT release induced by recombinant scinderin. It is demonstrated that PIP<sub>2</sub> modulates the activity of scinderin (Marcu et al., 1994; Rodriguez del Castillo et al., 1992, b). The potentiating effect of scinderin on Ca<sup>2+</sup>-evoked 5-HT release was inhibited by 10 μM PIP<sub>2</sub>. The inhibitor effect of PIP<sub>2</sub> was even greater when, prior to Ca<sup>2+</sup> stimulation, the permeabilized platelets were preincubated for 5 min with PIP<sub>2</sub> in a Ca<sup>2+</sup>-free medium containing TRX-scinderin (Fig.3.16).

### 3.17 Effect of Sc5-6, Sc<sub>1</sub>5-6 and Sc1-4,6 on the Increase in Serotonin Release from Platelets Produced by Full Length Recombinant Scinderin (r-Sc).

The inhibitory effects of both Sc5-6 and Sc-ABP<sub>3</sub> on the actin severing activity of full length scinderin suggest that either the third acting binding site of scinderin is also required for full severing activity or, when occupied by the peptide does not allow the proper binding of full length scinderin to actin. Therefore, the ability of r-Sc to potentiate Ca<sup>2+</sup>-evoked release of serotonin in the presence of Sc5-6, Sc<sub>1</sub>5-6, Sc1-4,6 or Sc-ABP<sub>3</sub> was tested. Digitonin permeabilized platelets showed increased release of serotonin in response to 10 μM Ca<sup>2+</sup> (Fig.3.17, A, B,C). This secretory response is potentiated in the presence of either 0.1 μM r-Sc or Sc1-4,6 (Fig.3.17, A,C). On the other hand, the serotonin release response evoked by 10 μM Ca<sup>2+</sup> was unchanged by the presence in

the medium of 0.1  $\mu\text{M}$  Sc5-6, 0.1  $\mu\text{M}$  Sc<sub>L</sub>5-6 (Fig.3.17, A) or 10  $\mu\text{M}$  Sc-ABP<sub>3</sub> (Fig.3.17, B). However, when both r-Sc (0.1  $\mu\text{M}$ ) and Sc5-6 (0.1  $\mu\text{M}$ ) were present together in the incubation medium, the potentiating effect of r-Sc on Ca<sup>2+</sup>-induced release of serotonin was blocked by Sc5-6 (Fig.3.17, A). Similarly, the potentiating effect of r-Sc on serotonin release was blocked in the presence of Sc-ABP<sub>3</sub> (Fig.3.17, B). On the other hand, Sc<sub>L</sub>5-6 failed to block the potentiating effect of r-Sc on Ca<sup>2+</sup>-evoked serotonin release (Fig.3.17, A). Sc1-4,6 not only failed to block the effect of r-Sc on release but having an effect of its own, showed a summation effect when added together (Fig.3.17.C). Moreover, as expected Sc-ABP<sub>3</sub> failed to decrease the potentiation of Ca<sup>2+</sup>-evoked release produced by Sc1-4,6 (Fig.3.17.C). Thus the inhibitory effects of Sc5-6 and Sc-ABP<sub>3</sub> and the lack of effect of Sc<sub>L</sub>5-6 on r-Sc responses suggests, again, that the third actin binding site of scinderin may be required for the full activity of intact scinderin or, alternatively, be necessary for the proper binding of scinderin to actin.

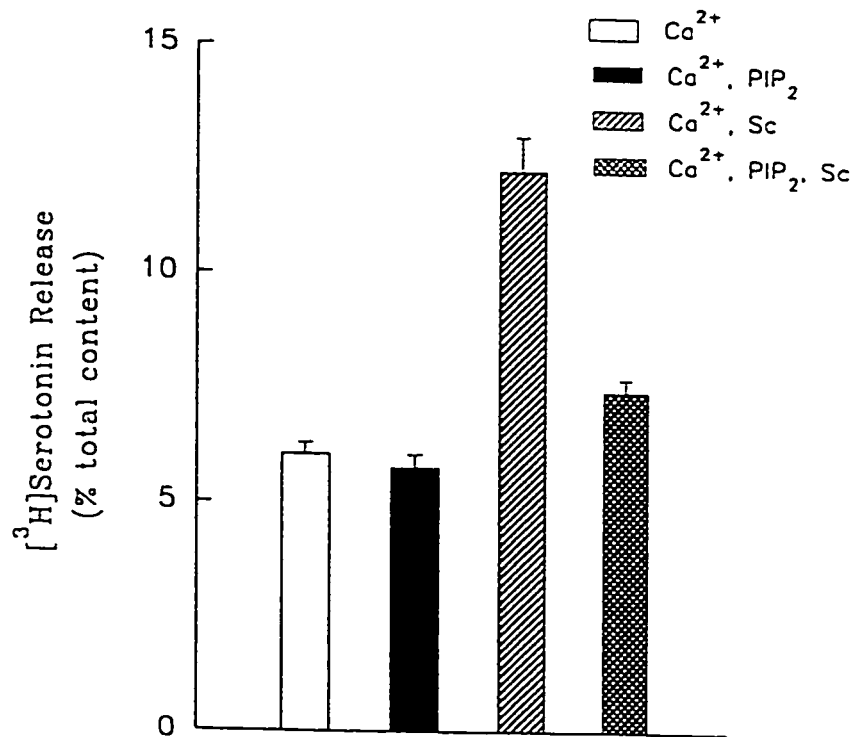


FIGURE 3.16 Effect of PIP<sub>2</sub> on the Potentiation by Recombinant Scinderin of Ca<sup>2+</sup>-induced 5-HT Release. PIP<sub>2</sub> at the concentration of 10 μM was present during the 5 min digitonin permeabilization, 5 min pre-incubation and 45 s stimulation with 10 μM Ca<sup>2+</sup>. When indicated, recombinant scinderin (0.5 μM) was present during permeabilization, pre-incubation and stimulation periods. The bars (mean ± SEM, n=8) represent 5-HT outputs after subtraction of spontaneous release.

**FIGURE 3.17** Effect of Recombinant Sc5-6, Sc<sub>L</sub>5-6, Sc1-4,6 and Peptide Sc-ABP<sub>3</sub> on the Potentiation by Recombinant Scinderin (r-Sc) of Ca<sup>2+</sup>-evoked Serotonin Release from Permeabilized Platelets. (A) Samples containing each 7.5 x 10<sup>7</sup> digitonin permeabilized platelets were incubated in 100 μl K<sup>-</sup>-glutamate buffer (pH 7.4) for 45 s with 10 μM Ca<sup>2+</sup> alone or in presence of 0.1 μM r-Sc, 0.1 μM Sc5-6, 0.1 μM Sc<sub>L</sub>5-6 or combinations of these fragments in a molar ratio 1:1. (B) Platelet samples were incubated and stimulated with Ca<sup>2+</sup> as in (A) in the presence of 0.1 μM r-Sc, 10 μM peptide Sc-ABP<sub>3</sub> (RLFQVRRNLASIT) or both. C) Samples containing platelets were incubated and stimulated with Ca<sup>2+</sup> as in (A) in the presence of 0.1 μM r-Sc, 0.1 μM Sc1-4,6, 10 μM Sc-ABP<sub>3</sub> or combinations of these peptides. [<sup>3</sup>H]serotonin (5-HT) release was measured in A, B and C as described in Materials and Methods. The bars (mean ± SEM; n=8) represents [<sup>3</sup>H]serotonin outputs after subtraction of spontaneous release.

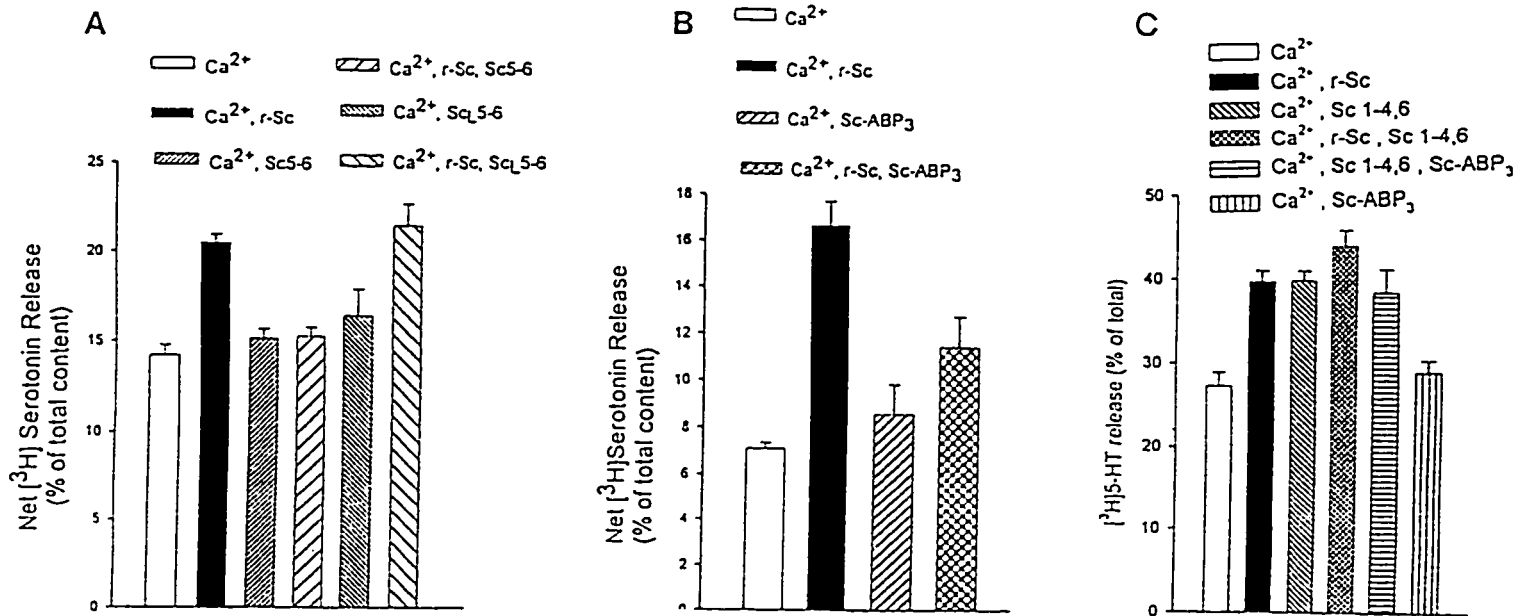


FIGURE 3.17

**CHAPTER 4**

**DISCUSSION**

#### 4.1 MOLECULAR CLONING, SEQUENCE ANALYSIS AND FUNCTIONAL EXPRESSION OF CHROMAFFIN CELL SCINDERIN

In many eukaryotic cells, actin filaments are noncovalently crosslinked in a layer between cytoplasm and cell membrane. This isotropic gel plays an important role in determining cell shape, locomotion, vesicle traffic etc, and is affected by extracellular signals (Darnell et al., 1990). The cytoplasm of eukaryotic cells contains proteins that can sever actin filaments into short fragments, promoting fast disassembly of the actin network (Stossel et al., 1985). Severing is a  $\text{Ca}^{2+}$ -activated, nonenzymatic, nonproteolytic process in which the severing protein binds to an actin subunit in the filament and induces a break with neighbouring subunits (Stossel et al., 1985).

In chromaffin cells, filamentous actin (F-actin), mainly localized in the cortical surface, acts as a barrier to the chromaffin vesicles, impeding their free movement and contact with the plasma membrane (Trifaró et al., 1982). Secretion requires, though, the movement of chromaffin vesicles toward the plasma membrane, the fusion of these vesicles with the plasmalemma and extrusion of the vesicular content to the cell exterior by exocytosis (Viveros et al., 1975; Trifaró et al., 1977). The search for factors which might regulate the dynamics of the actin cytoskeleton resulted in the discovery, in our laboratory, of scinderin (Del Castillo et al., 1990). In earlier studies, also from our laboratory, directed to the

isolation and characterization of chromaffin cell actin, scinderin was eluted together with actin from DNase I affinity column (Lee et al., 1979; Bader et al., 1986); it had an apparent MW of 80,000 Da and it was different in many ways (MW, isoelectric points, amino acid composition, etc) (Rodriguez Del Castillo et al., 1990) from gelsolin, an ubiquitous F-actin calcium dependent severing protein, and was present mainly in tissues with high secretory activity (Tchakarov et al., 1990). Some of scinderin's properties could be demonstrated after purification of small amounts from bovine chromaffin cells (Rodriguez Del Castillo et al., 1990) but the answers to many questions regarding this novel protein were to be found once the scinderin cDNA could be isolated, sequenced, expressed in large amounts followed by purification in order to perform studies on biological systems (secretory cells).

Previous data from our laboratory has demonstrated a cortical cellular localization of scinderin (Vitale et al., 1991) and its redistribution upon cell stimulation providing  $Ca^{2+}$  ions are present (Vitale et al., 1991; Rodriguez Del Castillo et al., 1992,b). Therefore it was suggested that  $Ca^{2+}$  entry during cell depolarization might modulate the activity of  $Ca^{2+}$ -dependent severing proteins such as scinderin (Vitale et al., 1991). Upon activation, these severing proteins would contribute to the disassembly of the F-actin network, removing the mechanical barrier and allowing free movement of the

secretory vesicles toward the plasma membrane, with the consequent fusion and exocytosis (Trifaró et al., 1993).

The first part of the present discussions (Chapter 4) is concerned with the molecular cloning, actin and phospholipid (PS and PIP<sub>2</sub>) binding properties and antibody specificity of the fusion protein obtained by expression of a full-length scinderin cDNA clone in a bacterial system. The amino acid sequence indicates that bovine chromaffin cell scinderin is a protein of 715 amino acids which shares 63% and 53% homology respectively with gelsolin and villin, two other F-actin severing proteins. Gelsolin (87,000 Da), the first actin-severing protein described (Yin et al., 1979) is found in almost all vertebrate cells. Intestinal and kidney absorptive epithelial cells, in addition to gelsolin contain villin (95,000 KDa) which is encoded by a different gene (Pringault et al., 1986).

The fusion protein obtained by expression of the full-length scinderin cDNA (F-Sc1, GST-Sc) not only binds to actin but also has the ability to bind, in a Ca<sup>2+</sup>-dependent manner, to PS and PIP<sub>2</sub>, thus behaving very similarly to native scinderin (Marcu et al., 1994). Furthermore, the polyclonal antibody raised in rabbits against the fusion protein (GST-Sc) has absolute specificity for native scinderin and showed identical results to those obtained with an antibody against native scinderin when used in immunofluorescence studies to demonstrate and quantitate the cellular redistribution of

scinderin in response to nicotinic receptor stimulation (Fig.3.3). Our results clearly indicated that the fusion protein expressed in *E.coli* behaved functionally as native scinderin.

Sequence analysis of scinderin and its close relatives, gelsolin and villin, revealed interesting information: scinderin, as well as the other two proteins, has six internal repeats of three short sequence motifs (A,B,C) occurring at approximative equal distance in the molecule. Two smaller actin filament severing proteins, severin from *Dictyostelium discoideum* (André et al., 1988) and fragmin from *Physarum polycephalum* (Ampé et al., 1987) also contain homologies to scinderin: they have about half of the molecular mass of scinderin and, as with gelsolin (Way et al., 1988), the homologies in sequence are high in the N-terminal half, also, the A,B,C motifs are to be found three times in severin and fragmin. After alignment of motifs A,B,C, gelsolin, villin and scinderin reveal six domains (1 to 6) with strong similarities between domains 1 and 4, 2 and 5, 3 and 6 in all these three proteins (Fig.3.6.2). This data suggested that these three proteins might have derived by gene duplication from an ancestral actin filament-severing protein (which was similar to the N-terminal half of these molecules) looking probably a lot like severin or fragmin. It has also been suggested that this family of actin filament-severing proteins may have evolved by tandem gene triplication with a predicted

14 kDa monomer unit of 120-130 amino acid residues (Way et al., 1988), such as (approximately) domain 1 of gelsolin. However, chymotrypsin digestion of gelsolin and scinderin carried out under identical conditions produced a distinct 16 kDa fragment only from gelsolin (Rodriguez Del Castillo et al., 1990). Meanwhile, the smaller fragment obtained from scinderin under these conditions had a molecular mass of 32 kDa (Rodriguez Del Castillo et al., 1990). Amino acid sequence analysis of three cyanobromide peptides of the 40 kDa Sc fragment has shown (once the whole sequence available) that they are pieces of the N-terminal half of the protein.

Earlier data from our laboratory has pointed out that two molecules of actin bind one molecule of scinderin and this interaction is  $\text{Ca}^+$ -dependent (Trifaró et al., 1992). Furthermore, the two main fragments (40 and 38 kDa) obtained by limited proteolytic digestion of Sc interacted with actin, also in a  $\text{Ca}^{2+}$ -dependent manner, yielding protein complexes of molar ratios 1:1 (Del Castillo et al., 1990). These results suggested that each Sc fragment contains at least one actin and one  $\text{Ca}^{2+}$ -binding site. Comparison of the sequences of scinderin to those of gelsolin and villin showed the presence of two actin-binding sequences with high homology, in domains 1 and 2 respectively (Fig.3.6.1, A). It is therefore possible that the type of interaction (recently described by crystallographic studies) between gelsolin (domains 1 and 2) and actin regarding the mechanisms of filament severing

(McLaughlin et al., 1993) might be similar for scinderin and villin - the filament-severing activity of scinderin might reside in the first two domains as it was demonstrated for gelsolin. In this case, in addition to domain 1 of gelsolin, another stretch of a few amino acid residues from domain 2 (second actin-binding site, Fig.3.6.1.B) was necessary for full severing activity (Way et al.,1988). It is possible, therefore, that severing activity requires interaction between more domains. Isoleucine residue 103 in gelsolin first actin-binding site also seems to be important for the interaction with subdomains 1 and 3 of actin (McLaughlin et al.,1993). Scinderin and villin have an isoleucine in the same position (residues 80 and 79 respectively) within their actin-binding sites (Fig.3.6.1.A). Previous work from our laboratory has also shown that the C-terminal half of scinderin binds monomeric actin in a  $Ca^{2+}$ -dependent manner (Trifaró et al.,1992) the same way as the C-terminal half of gelsolin (Pope et al.,1991). It was suggested recently that one  $Ca^{2+}$  ion is intermolecularly bound between D109 of gelsolin and E167 of actin, with the rest of coordination links coming from domain 1 of gelsolin (crystallographic study, McLaughlin et al.,1993). Comparison of amino acid sequence of actin-binding sites of scinderin and gelsolin indicate that D86 of scinderin corresponds to D109 of gelsolin. A second  $Ca^{2+}$  site has also been identified in domain 1 of gelsolin (McLaughlin et al., 1993). This site is an intramolecular pocket with binding

coming from G65, D66, E97 and V145 and having the seven coordination links free for interaction with a phospholipid head group, as suggested for the annexins (Huber et al., 1990). Scinderin domain 1, also, has the same conserved amino acid sequence (G44, D66, E75, V122) with the first two amino acids in motif B and the third in motif A of domain 1 as in gelsolin, making quite possible that scinderin will display the same spatial arrangement for the binding of  $\text{Ca}^{2+}$ . Published evidence indicates that binding *in vitro* of domain 1 of gelsolin to actin is  $\text{Ca}^{2+}$  independent (Chaponnier et al., 1986; Bryan et al., 1989), whereas in the case of N-terminal half of scinderin, this requires the presence of  $\text{Ca}^{2+}$  for binding to actin (Trifaró et al., 1992). From this point of view, scinderin resembles villin rather than gelsolin.

A large amount of data indicate that phospholipids of the membrane (mainly  $\text{PIP}_2$ ) modulate the activity of many actin-regulatory proteins, as gelsolin and scinderin (Janmey et al., 1987; Lassing et al., 1988; Maekawa et al., 1990; Rodriguez Del Castillo et al., 1992, b). *In vitro*,  $\text{PIP}_2$  vesicles bind the N-terminal of gelsolin and villin and inhibit actin severing activity. We showed here that GST-Sc, a fusion protein obtained by expression of scinderin cDNA in prokaryotes, binds both PS and  $\text{PIP}_2$  in a  $\text{Ca}^{2+}$ -dependent manner very much the same way as the native scinderin (Trifaró et al., 1992). Additional work from our laboratory has shown that binding of scinderin to PS and  $\text{PIP}_2$  is also pH-dependent

(Rodriguez Del Castillo et al., 1992,b). Amino acid sequence analysis revealed the presence in scinderin, in the C-terminal of domain 1 and N-terminal of domain 2, of sequences with high homology to those known to bind  $\text{PIP}_2$  in gelsolin (Fig.3.6.1.B, C), in the same positions. This fact suggests the presence in scinderin of at least two  $\text{PIP}_2$  binding sites. As shown in Figure 3.6.1.B,C, villin has only one  $\text{PIP}_2$  binding site with this homology. There is a consensus sequence for the  $\text{PIP}_2$  binding sites in all these actin filament-severing proteins (Fig.3.6.1.C) and this sequence can also be found in several phospholipase C isozymes where it is known as the "X Box" (Rhee et al., 1989; Janmey et al., 1992; Yu et al., 1992)). In scinderin consensus sequence there is a difference in one of the  $\text{PIP}_2$  binding motifs where an alanine (A) is present instead of lysine (K) at position 119 (Fig.3.6.1., C); is it possible that the replacement of the KK motif by a KA would impair the ability of scinderin to interact with  $\text{PIP}_2$ ? Most probably not since published evidence on site-directed mutagenesis of gelsolin, has shown that a mutant with a single lysine (K) in the "X Box" substituted by an alanine (A) does not show any decrease either in  $\text{PIP}_2$  binding or in its ability to modulate the severing activity (Yu et al., 1992).

An interesting observation was that the second  $\text{PIP}_2$  binding site of scinderin overlaps with the second actin binding site, the same way as in gelsolin. This could explain, probably, the inhibitory effect of  $\text{PIP}_2$  on gelsolin,

villin and scinderin F-actin severing activities.

GST-scinderin also binds PS, as the native scinderin does (Rodriguez Del Castillo et al., 1992, b); this is not true for gelsolin (Janmey et al., 1987). We do not know which scinderin domain binds PS and which is the result of this interaction. However, we suggested in earlier studies that plasma membrane PS might provide a binding site for scinderin in the inner surface of the membrane in resting cells (Rodriguez Del Castillo et al., 1992, b). This hypothesis is based on the following : 1) scinderin binding to PS liposomes in the presence of other cytosolic proteins is higher at pH 6.8 and  $10^{-8}$  M  $Ca^{2+}$ , a condition similar to that found in resting cells and 2) under resting conditions, scinderin cortical distribution does not depend on its binding to actin 3) scinderin cortical fluorescent ring recovered before the actin fluorescent ring following stimulation suggesting the binding of scinderin to a cellular component other than actin. (Vitale et al., 1991; Rodriguez Del Castillo et al., 1992, b).

Studying the expression of scinderin, we have found that it is preferentially localized in secretory cells (Tchakarov et al., 1990). Its distribution changes along with F-actin disassembly during cell stimulation (Vitale et al., 1991) and this would suggest an important role for scinderin in the secretory process. In conclusion, all these data clearly indicate that scinderin should be added to the family of gelsolin-like proteins.

#### 4.2 LOCALIZATION AND FUNCTIONAL CHARACTERIZATION OF A THIRD ACTIN-BINDING SITE IN SCINDERIN, DOMAIN 5

As discussed above, scinderin shares six internal repeats of short sequence motifs (A,B,C) with gelsolin and villin (Marcu et al., 1994; Trifaró et al., 1995). Previous published work has demonstrated the presence and functional properties of two actin-binding sequences in domains 1 and 2 of scinderin (Marcu et al., 1994, 1996; Zhang et al., 1996). The high homology of these two actin-binding sites in gelsolin, villin and scinderin would suggest that the type of interaction described for domains 1 and 2 of gelsolin with actin (McLaughlin et al., 1993) might be similar for scinderin and villin. However, it should be pointed out that published evidence indicates that the *in vitro* binding of domain 1 of gelsolin to actin seems to be  $Ca^{2+}$ -independent (Chaponnier et al., 1986; Bryan, 1988), whereas in the case of scinderin, this binding requires the presence of  $Ca^{2+}$  (Trifaró et al., 1992). Therefore, because of this property scinderin resembles villin rather than gelsolin. The results of our experiments lead to the conclusion that a third actin-binding site in scinderin is localized to the  $NH_2$ -terminal half of segment 5 of scinderin since the construct Sc5-6 but not the Sc<sub>1</sub>5-6 was able to bind actin. The first 5 amino acids in the sequence (RLFQVRRNL) of this site which correspond to amino acids in position 511-519 of scinderin are the same as those present in positions 161-165 which correspond to the second actin-binding site

(RLFQVKGRR) of gelsolin segment 2. The site (aa511-519) present in Sc5-6 is able to bind actin monomers, a property similar to gelsolin S4-6 (Way et al., 1989). However, the actin binding site in this part of gelsolin has been localized to segment 4 (Pope et al., 1995). The results from different types of experiments indicate that segment 4 of scinderin has no actin binding properties. Moreover, and opposite to what has been described for gelsolin S4-6 and similar to gelsolin S-2 (Way et al., 1989), the binding of actin to Sc5-6 is  $\text{Ca}^{2+}$ -independent, since the present experiments clearly demonstrate that Sc5-6 was bound to actin DNase-I-Sepharose 4B beads quite effectively in the presence of 10 mM EGTA. The affinity of Sc5-6 for actin was similar in  $\text{Ca}^{2+}$  ( $K_d=0.3 \mu\text{M}$ ) or  $\text{Ca}^{2+}$ -free conditions ( $K_d=0.33 \mu\text{M}$ ) as indicated by similar  $K_d$ s obtained by Scatchard plot analysis of the data obtained in these two buffer solutions. These values are within the range of those published for gelsolin domain 4 (Pope et al., 1995) and domains 2-3 (Yu et al., 1991). Previous experiments from our laboratory have demonstrated that intact native scinderin interacts with actin in a  $\text{Ca}^{2+}$ -dependent manner and is eluted from actin Sepharose 4B affinity columns with EGTA buffers (Marcu et al., 1994; Trifaró et al., 1989;). Therefore, it is quite possible that the third actin binding site of scinderin is hidden and it is only exposed upon binding of  $\text{Ca}^{2+}$  and/or actin to other scinderin sites with the consequent changes in the configuration of the protein. The observed binding of

actin to Sc3-6 or Sc5-6 in a  $\text{Ca}^{2+}$ -free environment could be explained by the fact that these fusion proteins are, perhaps, not folded as the native one and, therefore, the third actin binding site is permanently exposed. Alternatively, either one-third or two-thirds of the  $\text{NH}_2$ -terminal part of the scinderin is missing in the Sc3-6 and Sc5-6 constructs respectively, and this might produce a more "open" configuration in these fusion proteins permitting the access and binding of actin to the third site in a  $\text{Ca}^{2+}$ -free environment. The Sc5-6 fragment of scinderin not only binds monomers of actin but also, and similar to gelsolin S4-6 (Way et al., 1989), is capable of nucleating actin assembly in a polymerization assay. In this case Sc5-6 was as effective as recombinant full-length scinderin (r-Sc). However, Sc<sub>1</sub>5-6 and Sc1-4,6 were ineffective in promoting nucleation, suggesting again the presence of an actin-binding site with actin nucleation properties at the  $\text{NH}_2$ -terminal half of domain 5 of scinderin.

More about the third actin-binding site of scinderin was learned from the viscometry analysis (and platelet serotonin release experiments which are described in 4.4.2). Viscometry provides a very sensitive measuring of severing because viscosity is most strongly influenced by the longest actin filaments and these have the highest probability of being severed. The decrease in viscosity produced by r-Sc was completely blocked in the presence of either Sc5-6 or peptide

Sc-ABP<sub>3</sub> (with amino acid sequence corresponding to the third actin-binding site). The inhibitory effect of Sc-ABP<sub>3</sub> was observed at micromolar concentrations. These concentrations were necessary since the peptide binds to actin (the scinderin substrate), an abundant cellular protein. Therefore, to see the inhibitory effect of the peptide, it became necessary to occupy a large number of scinderin-binding sites of actin.

In summary, our experiments demonstrated the presence of the third actin-binding site which needs to be occupied by actin in order to position scinderin in a way that would allow the protein to exert its full severing activity. The experiments do not discard the possibility that, in addition to the two actin-binding sites present at the NH<sub>2</sub>-terminal half of scinderin, other actin-binding sites may also be present in this half of the molecule, since a systematic study of the NH<sub>2</sub>-terminal half of gelsolin recently published indicated the presence of an additional actin-binding sequence at the COOH-terminal of segment 2 of gelsolin (Van Troys et al., 1996).

#### 4.3 RECOMBINANT SCINDERIN ENHANCES EXOCYTOSIS IN CHROMAFFIN CELLS

Adrenal medullary chromaffin cells are an excellent system to study the mechanism of secretion; these cells can be maintained in primary culture and the influences on catecholamine secretion can be rigidly controlled (Trifaró et al., 1980; Kumakura et al., 1986). All cell types are capable of transporting proteins to the extracellular space via a vesicle-mediated constitutive secretory pathway (Tartakoff, 1978). Neural, endocrine and exocrine cells additionally possess a **regulated** secretory export pathway of hormones, neurotransmitters and enzymes (Tartakoff, 1978). For these systems, the main intracellular signal which triggers secretion is an increase in cytoplasmic  $Ca^{2+}$  (Douglas et al., 1961, 1982). The proteins that participate in  $Ca^{2+}$ -dependent exocytosis are still to be fully identified. Vesicle movement and fusion in the early steps of the secretion seem to involve a multitude of cytosolic and extrinsic membrane proteins; exocytosis itself might be even more complex (Rothman et al., 1992).

$Ca^{2+}$ -dependent secretion is studied in preparations of cells whose plasma membrane was permeabilized by different procedures. These procedures result in various molecular-size cutoffs. Digitonin permeabilization of chromaffin cells leads to the loss of soluble cytosolic and extrinsic membrane

proteins - consequently, the secretory response of these cells to a  $Ca^{2+}$  challenge is diminished until the leaked proteins (if important in exocytosis) are added to the system (Dunn et al., 1983; Wilson et al., 1983).

After obtaining the scinderin clone, the protein was expressed and purified from prokaryotic cells. This was followed by the application of recombinant scinderin to digitonin-permeabilized chromaffin cells in culture. The purpose of these experiments was to understand the role of scinderin in secretion.

Stimulation of chromaffin cells produces disassembly of cortical actin network and removal of the barrier which might impede the free movement of secretory vesicles (Trifaró et al., 1982, 1984, 1989; Cheek and Burgoyne., 1986, 1987). Cytochemical experiments with rhodamine-labelled phalloidin and actin antibodies demonstrated that in resting cells, a filamentous actin network is visualized as a strong cortical fluorescent ring (Lee et al., 1981; Cheek et al., 1986, 1987; Sontag et al., 1988; Trifaró et al., 1989). Upon stimulation there is a fragmentation of the cortical fluorescent ring and areas devoid of fluorescence can be noticed (Cheek and Burgoyne, 1986, 1987; Trifaró et al., 1989). The modifications are accompanied by a decrease in F-actin associated with the concomitant increase in G-actin as evaluated by the DNase I inhibition assay (Cheek et al., 1986; Trifaró et al., 1989). F-actin network disassembly has also

been observed in depolarized synaptosomes (Bernstein et al., 1985). The cortical F-actin network is not only a barrier to secretion but it also controls the access of chromaffin vesicles to the subplasmalemmal area (release-ready vesicle pool) and consequently, the initial rate of exocytosis (Vitale et al., 1995).

Digitonin permeabilization of chromaffin cells did not affect the integrity of cortical F-actin network since fluorescence microscopy and image analysis showed a similar pattern of F-actin fluorescence for digitonin-treated or intact cells (Fig.3.13. A,a',a"). Upon stimulation of permeabilized cells with increasing concentrations of  $Ca^{2+}$  in the medium, F-actin disassembly is evident since the fluorescence patterns are similar to those obtained with intact cells stimulated by either nicotine or a depolarizing concentration of  $K^+$ . Therefore we concluded that, in this system, the cortical F-actin network dynamics is preserved by the digitonin treatment.

Most of the dynamic changes in the F-actin network during exocytosis are thought to be the result of activation of F-actin severing proteins (Burgoyne et al., 1987; Cheek et al., 1991; Trifaró et al., 1985). In chromaffin cells, at least two  $Ca^{2+}$ -dependent actin-binding proteins are present: gelsolin and scinderin (Trifaró et al., 1985; Bader et al., 1986; Rodríguez Del Castillo et al., 1990; Vitale et al., 1991). What role do these proteins play (together, complementing each

other, or, on contrary, competing for the required factors) in the reorganization of cortical actin network brought about by cell stimulation ? Comparison between their tissue distribution and apparent redistribution following stimulation seems to favor scinderin as a more important player in chromaffin cell secretion:

1) Gelsolin is a widely distributed  $\text{Ca}^{2+}$ -dependent actin filament capping and severing protein (Yin et al., 1979; Yin et al., 1981; Stossel et al., 1985). Scinderin, on the other hand, is expressed only in tissues with high secretory activity such as brain, pituitary, platelets, etc. (Rodríguez Del Castillo et al., 1990; Tchakarov et al., 1990).

2) Nicotine receptor stimulation or  $\text{K}^+$ -evoked depolarization of chromaffin cells induces simultaneously cortical F-actin disassembly and redistribution of subplasmalemmal scinderin (Vitale et al., 1991). Fodrin, another actin-binding protein present in the cortical region of the chromaffin cell, is also re-distributed during cell stimulation (Perrin et al., 1985). Scinderin redistribution is  $\text{Ca}^{2+}$ -dependent and precedes exocytosis (Vitale et al., 1991). Moreover, exocytotic sites are preferentially localized to cortical areas showing F-actin disassembly (Vitale et al., 1991). The distribution of gelsolin, on the other hand, is not affected by either nicotine receptor stimulation or  $\text{K}^+$ -evoked depolarization (Vitale et al., 1991). All these observations suggest that cell stimulation and  $\text{Ca}^{2+}$  entry activate scinderin which

contributes to the disassembly of cortical F-actin networks. This hypothesis is strengthened by the experiments with recombinant scinderin presented in this thesis.

The fusion protein (TRX-Sc) potentiates  $\text{Ca}^{2+}$ -evoked exocytosis in digitonin-permeabilized chromaffin cells. When tested at different  $\text{Ca}^{2+}$  concentrations, recombinant scinderin did not increase the affinity of the elements of the exocytotic machinery for  $\text{Ca}^{2+}$  but it increased the maximal response to  $\text{Ca}^{2+}$ , thus suggesting an increase in the number of secretory vesicles available for release (Fig.3.10, B). Fig.3.13 shows that, concomitantly with the potentiation of release, TRX-Sc increased the number of cells displaying F-actin cortical disassembly, suggesting that the potentiation of release by scinderin was due to the removal of the F-actin barrier, allowing the movement of chromaffin vesicles toward the release sites. By comparison, recombinant truncated scinderin (scinderin 254-715), a fusion protein devoid of (active) severing actin-binding sites, failed to induce F-actin disassembly or potentiate  $\text{Ca}^{2+}$ -evoked exocytosis. Two scinderin-derived actin binding peptides, Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub>, designed to correspond to the two known actin binding sites of scinderin, inhibited the disassembly of cortical actin provoked by scinderin. Each of the two peptides also blocked the potentiation by recombinant scinderin of  $\text{Ca}^{2+}$ -evoked exocytosis (Fig.3.11.1) and the inhibition was more effective when both peptides were present, probably due to the

simultaneous blocking of the two scinderin-binding sites of actin . When exogenous chicken gizzard  $\gamma$ -actin was present in the incubation medium, the potentiation effect on release of recombinant scinderin was also blocked, most probably due to the fact that chicken gizzard  $\gamma$ -actin competed with **endogenous** chromaffin cell actin for recombinant scinderin. In the absence of recombinant scinderin, Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub> and chicken gizzard  $\gamma$ -actin also inhibit Ca<sup>2+</sup>-evoked release, thus suggesting the involvement of **endogenous** scinderin effect of F-actin disassembly.

The inhibitory effects of Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub> were observed at relatively large concentrations (micromolar range), but these were necessary since the peptides bind to actin (scinderin-binding sites) which is an abundant cellular component (10-20% among cytosolic proteins). Our previous molecular cloning experiments (Marcu et al., 1994) and sequence analysis have described two PIP<sub>2</sub> binding sites in scinderin and additional experiments have demonstrated the ability of PIP<sub>2</sub> liposomes to bind scinderin in a Ca<sup>2+</sup>- and pH-dependent manner (Rodríguez Del Castillo et al., 1992,b ; Marcu et al., 1994). The two PIP<sub>2</sub> binding sites of scinderin show the consensus sequence R(X)XXXXXRR, characteristic of PIP<sub>2</sub> binding sites of phospholipase C (Marcu et al., 1994). Furthermore, one of the PIP<sub>2</sub> binding sites of scinderin overlaps with its second actin binding site (Marcu et al., 1994) and this might account for the inhibitory

effect of PIP<sub>2</sub> on scinderin F-actin severing activity (Maekawa and Sakai., 1990) and the inhibition of the recombinant scinderin effects on catecholamine release. On the other hand, Sc-PIP<sub>2</sub>BP, a scinderin-derived PIP<sub>2</sub>-binding peptide, blocked both the binding of PIP<sub>2</sub> liposomes to scinderin and the PIP<sub>2</sub> inhibition of scinderin effects. Our results strongly suggest that the effects of PIP<sub>2</sub> on F-actin disassembly and Ca<sup>2+</sup>-induced exocytosis are mediated through the interaction between scinderin and the intact phospholipids of the membrane. In the presence of 10 μM Ca<sup>2+</sup> scinderin has greater affinity for actin than PIP<sub>2</sub> (Rodríguez Del Castillo et al., 1992, b), therefore it was necessary to pre-incubate the permeabilized chromaffin cells with recombinant scinderin and PIP<sub>2</sub> in a Ca<sup>2+</sup>-free medium before stimulation in order to observe the PIP<sub>2</sub> inhibition upon a Ca<sup>2+</sup> challenge. Was the PIP<sub>2</sub> inhibitory effects due to the activation of a specific PIP<sub>2</sub> transduction pathway ? It seems unlikely, since the plasma membrane PIP<sub>2</sub> binds scinderin under resting (low Ca<sup>2+</sup> levels) conditions (Rodríguez Del Castillo et al., 1992, b), and the inhibitory effect of the phospholipids on scinderin F-actin severing activity (Maekawa and Sakai., 1990) would indicate that activation of phospholipase C-PIP<sub>2</sub> pathway might release scinderin from binding sites, thus removing the inhibitory effect of PIP<sub>2</sub>.

In conclusion, our data suggest an important role for scinderin in the exocytotic machinery: the dynamics of

cortical F-actin network and the availability of secretory vesicles to be released seem to be controlled by scinderin in a  $Ca^{2+}$ -dependent manner.

#### 4.4 POTENTIATION OF SEROTONIN RELEASE FROM PERMEABILIZED PLATELETS BY RECOMBINANT SCINDERINS

##### 4.4.1 Full-Length Scinderin Activity

Biochemical and morphological evidence suggest that actin - the predominant protein of blood platelets - polymerizes in response to various external stimuli which can activate these cells. Collagen, thrombin, ADP and other agents activate these unicellular secretory systems, altering their structure as well as their metabolism. Upon activation, platelets undergo changes in shape (from disks to spheres covered with spines), acquire pseudopodia, aggregate and secrete. These changes are accompanied by a significant increase in actin polymerization. In resting platelets, a major part of actin is unpolymerized; the estimated actin concentration in platelets is 0.2-1 mM, at least 1000 times above the critical spontaneous polymerization concentration (Fox et al., 1986; Pollard et al., 1986). The possible candidate proteins to help maintain this control over actin depolymerization are profilin (Carlsson et al., 1976), gelsolin (Lind et al., 1982) and scinderin (Rodriguez Del Castillo et al., 1992, a). Gelsolin can sever actin filaments remaining then bound to the fast growing ends (barbed ends), preventing monomer addition. Previous work from our laboratory has identified the presence

of scinderin in platelets in concentration of about 75 fmol/mg total protein (Rodriguez Del Castillo et al., 1992,a). Present data point to gelsolin as the major actin filament-severing activity in platelets, with scinderin being responsible for 10-11% of the severing activity, if both proteins are equally potent. Actin polymerization-depolymerization cycles take place in different areas (i.e. pseudopodia and central contractile gel) during platelet activation (Carroll et al., 1982). It is quite possible that scinderin and gelsolin are separately involved in controlling actin filament length in platelets. Work from our laboratory has shown that during chromaffin cell stimulation, a  $Ca^{2+}$ -dependent redistribution of cortical scinderin together with disassembly of the cortical F-actin are observed (Vitale et al., 1991), and these events, which precede exocytosis, seem to be necessary requirements for secretion to occur (Trifaró et al., 1992). What is the role of scinderin in blood platelets secretion? It has been suggested that during platelet aggregation, actin polymerizes and the content of the secretory granules is released to the cell exterior (Siess et al., 1989; Carroll et al., 1982). The experiments described here involved the incubation of recombinant scinderin (TRX-Sc) with permeabilized platelets and the study of the effects of this protein and its derived actin-binding peptides on the 5-HT secretory process (Fig 3.14.1, 3.14.2). Thioredoxin alone was used as control due to the fact that scinderin could not be

separated from the TRX protein. The experiments with Sc-ABP<sub>1</sub> and Sc-ABP<sub>2</sub> suggest that when the peptides are bound to actin occupying either one of the binding sites for scinderin, this protein fails to produce both, the severing of F-actin and the potentiation of 5-HT release. The two known actin binding sites which are present in domains 1 and 2 of scinderin (Fig.3.15.A) are necessary for its severing activity. Furthermore, Sc-ABP<sub>1</sub>, Sc-ABP<sub>2</sub> and  $\gamma$ -actin inhibited Ca<sup>2+</sup>-evoked serotonin release in the absence of recombinant scinderin suggesting an inhibition of platelet **endogenous** scinderin. These observations together with the potentiation of 5-HT release by recombinant scinderin in the absence of Sc-ABP<sub>1</sub> or Sc-ABP<sub>2</sub>, suggest, as in the case of chromaffin cells, that F-actin disassembly, perhaps at a specific site, is required for platelet secretion and that scinderin might be an important component of the exocytotic machinery in platelets, as well. In the presence of recombinant scinderin, the increase in 5-HT release in response to Ca<sup>2+</sup> stimulation might be the result, as in the case of chromaffin cells (Vitale et al., 1995), of an increase in the number of secretory vesicles available at release sites, as a result of F-actin network disassembly by scinderin. Published data shows that PIP<sub>2</sub> regulates the activity of many actin binding proteins including gelsolin and scinderin (Janmey et al., 1987; Marcu et al., 1994). Scinderin can bind and interact with both PIP<sub>2</sub> and phosphatidylserine in a Ca<sup>2+</sup>-dependent manner (Rodriguez

Del Castillo et al., 1992, b). In the presence of 10  $\mu\text{M}$   $\text{Ca}^{2+}$  scinderin has greater affinity for actin than for phosphatidylserine or  $\text{PIP}_2$  (Rodriguez Del Castillo et al., 1992, b) and this would explain the lack of inhibition by  $\text{PIP}_2$  of  $\text{Ca}^{2+}$ -induced release of serotonin in the absence of recombinant scinderin. Therefore, it was necessary to preincubate recombinant scinderin with  $\text{PIP}_2$  in a  $\text{Ca}^{2+}$ -free solution (Fig. 3.16) for the inhibition to occur. As discussed above, sequence analysis of scinderin has indicated the presence of two  $\text{PIP}_2$  binding sites with the consensus sequence of the  $\text{PIP}_2$  binding site of several phospholipase C isozymes (Marcu et al., 1994). Phospholipase C-induced inositol phospholipid hydrolysis is one of the major signal transduction pathways in platelets (Siess et al., 1989; Peterson et al., 1994). The present results showing inhibition by  $\text{PIP}_2$  of scinderin potentiation of  $\text{Ca}^{2+}$ -induced serotonin release suggest, therefore, that activation of the  $\text{PIP}_2$  pathway during platelet stimulation might release scinderin from binding sites, thus removing its inhibition by  $\text{PIP}_2$ .

In conclusion, the molecular biology studies together with the functional studies performed on two secretory systems (chromaffin cells and platelets) strongly suggest that scinderin is an important member of the gelsolin  $\text{Ca}^{2+}$ -dependent actin-severing proteins. Scinderin binds to actin and phospholipids and exerts its functions probably through cooperation between the multiple sites located in the

different domains of the molecule. For the regulation of secretion, scinderin seems to be more important than gelsolin: it is scinderin which is specifically present in tissues with high secretory activity, whereas gelsolin is a ubiquitous protein. Scinderin is mainly present in the subplasmalemmal region of chromaffin cells and it is rapidly redistributed together with the F-actin disassembly. Moreover, the exocytosis sites are preferentially localized to cortical areas devoid of F-actin (filaments previously severed by scinderin). In both secretory systems studied by us, recombinant scinderin potentiated the release of secretory products from permeabilized cells, and this effect was blocked by the scinderin-derived actin-binding peptides and  $PIP_2$ .

#### 4.4.2 Effect of Different Sc Truncations

The functional importance of the third actin-binding site of scinderin (domain 5) was learned from platelet serotonin release experiments. We have previously demonstrated that recombinant full-length scinderin (r-Sc) potentiates  $Ca^{2+}$ -evoked release of serotonin from permeabilized platelets, an effect blocked by  $PIP_2$  or peptides with sequences corresponding to the two actin binding sites present in domains 1 and 2 of scinderin (Marcu et al., 1996). Opposite to r-Sc and Sc1-4,6, Sc5-6 did not increase  $Ca^{2+}$ -evoked release of serotonin from platelets. However, the fact that

in the presence of Sc5-6 or Sc-ABP<sub>3</sub>, r-Sc was completely ineffective, but it was quite effective in the presence of Sc<sub>5</sub>-6, in potentiating Ca<sup>2+</sup>-evoked serotonin release, suggests that the third actin-binding site of scinderin should be occupied by actin for scinderin to display full activity. Furthermore, Sc1-4,6 in addition to increase Ca<sup>2+</sup>-evoked release of serotonin, it was able to show a summation of effects when combined with r-Sc. This idea gained more support when the severing activity of scinderin was evaluated by viscometry of actin gels (see 4.2).

## SUMMARY OF CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Bovine scinderin cDNA was cloned and its nucleotide and amino acid sequences were determined and analyzed. Therefore, it was possible to clearly establish the identity, molecular weight, the presence of actin- and PIP<sub>2</sub> -binding sites, and other properties of this novel protein. These observations allowed a better understanding of scinderin function and its relation to other Ca<sup>2+</sup>-dependent actin-severing proteins such as gelsolin and villin.

2. Large amounts of recombinant scinderin were expressed and purified using two different bacterial expression systems, the best conditions for expression and purification were selected among a variety of chromatographic procedures. Recombinant scinderin was functioning as well as native protein, in vitro systems. A new and more sensitive antibody was obtained against recombinant scinderin. This antibody was used in several experiments described in this thesis.

3. Applied to two different biological systems (permeabilized chromaffin cells and platelets), recombinant scinderin enhanced Ca<sup>2+</sup>-dependent exocytosis, thus availability of recombinant scinderin permitted the study of its mechanism of action and its relevance in the neurosecretory process. The experiments discussed here clearly demonstrate that scinderin is an important component of the

exocytotic machinery.

4. A large number of truncations and deletions of scinderin were also obtained, expressed and purified. This work lead to the localization and identification of a third, previously unknown, actin- binding site in the molecule. The biochemical properties (binding,  $K_d$ , actin nucleation and severing, etc) of this actin-binding site were elucidated and its properties were compared to those of other two actin binding sites of scinderin.

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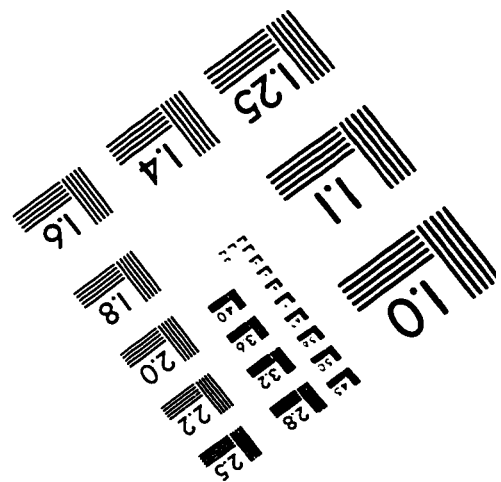
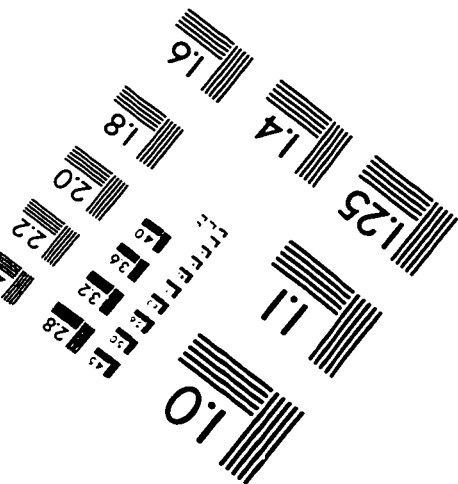
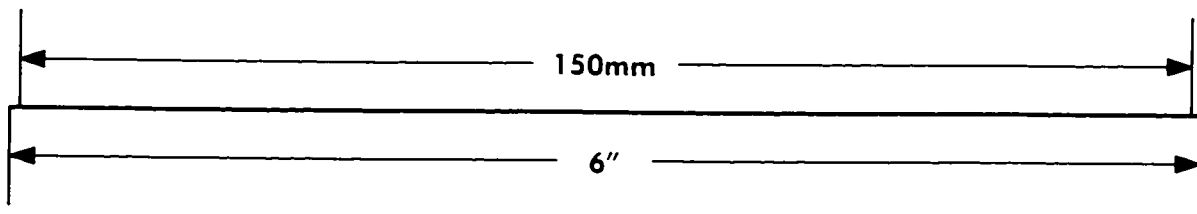
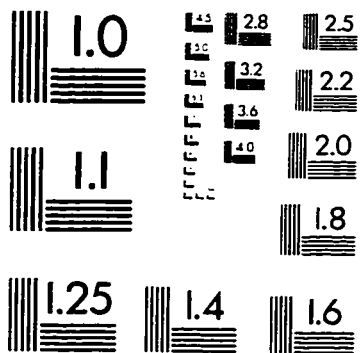
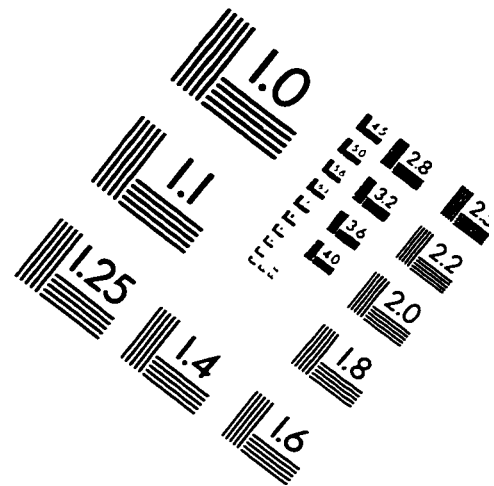
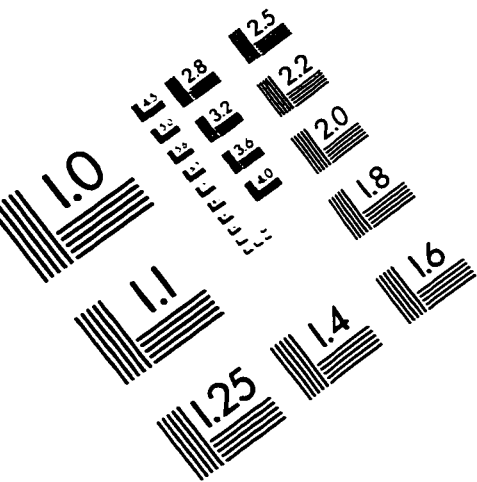
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