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The Annexins and Exocytosis

Carl E. Creutz

The annexins are a group of homologous proteins that bind phospholipids in the presence of calcium. They may provide a major pathway for communication between cellular membranes and their cytoplasmic environment. Annexins have a characteristic "bivalent" activity in the sense that they can draw two membranes together when activated by calcium. This has led to the hypothesis that certain members of this protein family may initiate contact and fusion between a secretory vesicle membrane and the plasma membrane during the process of exocytosis.

Introduction

Membranes compartmentalize cells and isolate them from their immediate environment. The process of exocytosis is one of the major routes by which this isolation is broken. Secretory products, including small molecules as well as complex proteins, are sequestered in membrane-bound secretory vesicles. The membranes of these vesicles may then fuse with the cell surface membrane, releasing the contents of the vesicles, which may be messengers, such as insulin or epinephrine, or laborers, such as invertase or trypsin, or building blocks, such as collagen or proteoglycan.

A complete understanding of the process of exocytosis requires knowledge of the molecular events comprising vesicle formation, vesicle translocation, vesicle fusion, and membrane recovery by endocytosis. As this knowledge is gained, our general understanding of membrane structure and regulation will advance in parallel. The study of exocytosis may be the beneficiary of advances in other areas of molecular cytology, or it may be a guiding beacon for other fields of exploration. Most probably, it will be both.

In the late 1970s, application of the traditional "grind and find" approach of the biochemist to the problem of membrane fusion in exocytosis led to the identification of synexin (*1*), a protein that catalyzes secretory vesicle membrane contact and fusion in vitro. It was hypothesized that synexin might act at the point of fusion of secretory vesicles with the plasma membrane, as well as between fusing vesicles in compound exocytosis. Subsequently, it was found that synexin is but a single representative of a class of homologous proteins, the annexins (*2*), that bind to lipid membranes in a calcium-dependent manner. Members of this group of proteins may be involved more widely in intracellular membrane trafficking, as well as in the regulation of a diverse array of calcium-dependent events on membrane surfaces (*2*). Because of the newly recognized breadth of the annexin family and the explosion of hypotheses for annexin functions, their involvement in exocytosis has recently received less singular attention. However, the apparent versatility of this protein family should not detract from recognition of its potential importance in exocytosis. In this article, the "annexin hypothesis" for the process of exocytosis will be re-evaluated in the context of recent advances in the study of membranes, annexins, and exocytosis.

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Membrane Fusion Mediated by the Annexins

In common with the prototype, synexin (now also referred to as annexin VII), most of the annexins are able to promote the calcium-dependent aggregation of isolated secretory vesicles. Conversely, all proteins that have been found to exhibit a similar activity have proven, on the basis of primary structure, to be members of the annexin family. Synexin was originally isolated as the active principle that promoted adrenal medullary chromaffin granule aggregation when the granules were incubated with crude cytosolic extracts (*1*). Synexin appeared to act as a glue in this process, rather than as an enzyme that catalyzed changes in the membrane surface, as the synexin was found to bind to the chromaffin granules at 5 to 10 μM calcium (*3*). However, half-maximal amounts of chromaffin granule aggregation by synexin required

more calcium, on the order of 200 μM (*1*). Therefore, an additional event, beyond initial binding to the membrane surface, appeared to be essential for membrane aggregation. It was then found that isolated synexin underwent self-association in the presence of calcium, and that this event had the same calcium dependence as membrane aggregation (*4*). This led to the hypothesis that in order to bring membranes into contact, synexin molecules on one membrane must interact with synexin molecules on a second membrane. Studies employing fluorescence energy transfer techniques have confirmed that such intermembrane annexin contacts occur when chromaffin granules are aggregated by synexin or two other annexins (annexins IV and VI) at intermediate concentrations of calcium (*5*). At very high concentrations of calcium (about 1 mM), the proteins may act monomerically to promote membrane contacts (*5*).

An important exception to this mechanism of annexin-induced membrane aggregation may be exhibited by the annexin II (calpactin) tetramer. In this complex, two annexin molecules are joined together as each binds to one subunit of a dimer of molecules, called p10, which are from the S-100 family of proteins (*6, 7*). This tetramer may be able to bind simultaneously to two membranes without further need to self-associate. Indeed, the annexin II tetramer is able to promote chromaffin granule aggregation at lower concentrations of calcium (about 1 μM) than any other annexin (*8*). The p10 subunit cannot bind calcium itself, so this subunit is not directly responsible for the high sensitivity to calcium. Apparently the removal of the calcium requirement allows this tetrameric complex to aggregate membranes at concentrations of calcium similar to those needed to promote exocytosis from permeabilized chromaffin cells (*9*).

When chromaffin granules are aggregated by synexin or another annexin in vitro, the aggregates are fairly stable (*1*), and membrane fusion occurs only at a very slow rate (*10*). Thus, the annexins do not appear to be fusogenic proteins such as found, for example, in viral envelopes. Instead, their function in overall membrane fusion appears to be primarily to promote membrane contact. Kinetic studies with liposomes that undergo fusion in the presence of synexin have localized synexin action to this initial step of membrane aggregation (*11*). In the case of chromaffin granules, the rate of fusion (Fig. 1) is dramatically increased by the addition of small (1 to 2% by weight) amounts of free, *cis*-unsaturated fatty acids (*12*). The polyunsaturated fatty acids are the most effective in promoting fusion (*12*). This may be physiologically relevant, as arachidonic acid is released from membranes in stimulated secretory cells at the right time and in appropriate amounts to act as an annexin cofactor in membrane fusion. The fusion induced by this biological detergent between chromaffin granules is "semi-conservative" in the sense that, at physiological concentrations, the fatty acid promotes fusion but not general membrane lysis. Only the contact point between chromaffin granules formed by the annexin is affected, and the macromolecular contents of the granules are retained in the vesicle resulting from fusion (*12*). This surprising degree of specificity may be due to the ability of inverted phospholipid micelles to form more readily at locations where two membranes are held in close contact rather than locations where an aqueous layer bounds both sides of a single lipid bilayer. The fusion of isolated chromaffin granules aggregated by an annexin also appears to be accelerated by osmotic changes in the granule core, as the rate of fusion can be suppressed by increasing the external osmotic strength (*10*).

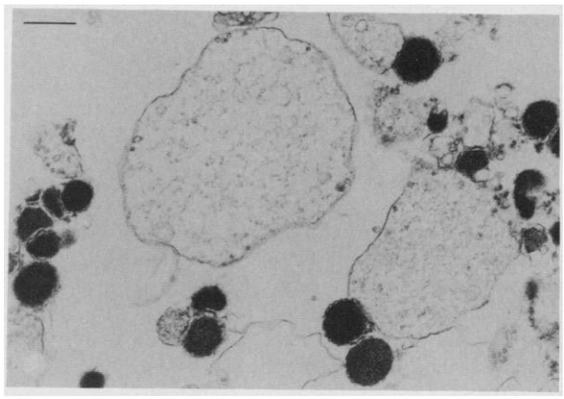

Fig. 1. Electron micrograph illustrating the in vitro fusion of chromaffin granules (secretory vesicles of the adrenal medulla) when incubated with synexin (annexin VII), calcium, and arachidonic acid. The dense core vesicles form contacts with one another and then fuse, resulting in the formation of the large vesicles that retain the diluted core proteins. This event provides a model for the fusion that occurs between secretory vesicles during compound exocytosis in the chromaffin cell. Bar represents 0.3 μm. [Reproduced from (*12*)]

Control of Exocytosis

The requirement for an aggregating agent, the annexin, as well as a destabilizing agent, the fatty acid, in the process of membrane fusion in vitro suggests that exocytosis may be subject to control by two signaling pathways. One pathway would control the availability of calcium, the other would control the breakdown of lipid to generate the fatty acid cofactor. The second pathway may be controlled by calcium also, but could alternatively be activated by a distinct signaling pathway involving the interaction of a guanosine triphosphate (GTP)–binding protein with a lipase. Because the calcium requirement of the annexin is lowered by the presence of fatty acid (*10, 13*), it is possible the second pathway might activate the annexin even without a change in the ambient calcium concentration. Therefore, the model system predicts that, in some circumstances, GTP-binding proteins may control exocytosis, and that exocytosis might be initiated without a rise in the concentration of intracellular calcium.

Calcium Sensitivities of Annexins

The hypothesis that annexins function as proposed is frequently challenged by the observation that they appear to require supra-physiological concentrations of calcium to promote membrane contacts. Although this challenge is effectively answered in the case of calpactin by the high sensitivity of this protein to calcium (*8*), the properties of the other annexins remain a concern. One message is clear from structural studies on annexins: some of the most highly conserved features of the annexin family are amino acid side chains involved in binding calcium (*14, 15*). We can therefore conclude that the binding of calcium is indeed an important aspect of the biology of these proteins.

Under intracellular conditions, the affinity of annexins for calcium may be different than observed in cell-free systems. Annexins may interact with other protein cofactors, as illustrated by annexin II (calpactin), which, after association with the p10 light chain, has an increased sensitivity to calcium (*8*). Annexin XI (synexin II) associates tightly with calcyclin (also a member of the S-100 family of calcium-binding proteins) at the annexin XI amino terminus (*16*), although it is not yet known how this may influence the calcium sensitivity of annexin XI. The specific lipid composition of membranes also influences the calcium requirements of the annexins (*17*). Because the binding of calcium and lipid is linked, one cannot specify a representative calcium-binding constant for a given annexin without also specifying the lipid present. For example, annexin II binds 100% phosphatidylserine vesicles at

less than 10^{-8} M calcium (*18*). Therefore, specific domains of lipid composition particularly amenable for annexin binding at low calcium concentrations might exist, perhaps transiently, on relevant membranes in stimulated cells.

Consideration of the kinetics of a binding reaction where three components (lipid, calcium, and protein) are involved implies that the apparent sensitivity of a given annexin to calcium may vary widely. If the three components are treated as individual particles combining to form a tripartite complex, then the apparent dissociation constant (K_d) for any one is inversely related to the concentrations of the other two. For example, a calcium titration curve for the formation of the complex from a constant amount of protein and lipid would have an apparent K_d that is inversely proportional to the lipid concentration.

Because experiments done in vitro typically use lipid and protein concentrations two to three orders of magnitude lower than those that occur in the cell, the potential exists for the annexins to bind to membranes at far lower concentrations of calcium in the cell than seen in vitro. Little experimental work has yet been done to validate this extrapolation. Data obtained with annexin IV (endonexin) indicate that the calcium sensitivity of this annexin is not as strongly affected by protein or lipid concentrations as predicted (*19*).

Modeling studies indicate that this may be because multiple calcium-binding sites are present on the annexin, and because lipid membranes appear to some extent as two-dimensional surfaces, independent of lipid concentration (*20*). Nonetheless, a theoretical analysis of data for the binding of annexin VI to lipid vesicles (*21*), which considered the binding of a population of annexin molecules to a vesicle as a sequence of individual binding steps, indicated that the binding of the first few annexin molecules, which see a high lipid concentration, may occur at calcium concentrations two orders of magnitude lower than required by the last few annexin molecules, which see a greatly reduced lipid concentration because of sequestering of lipid by the annexin molecules bound earlier.

The properties of the annexins in vitro may in fact be accurate indicators of the calcium concentrations to which they are exposed in vivo. The proteins may be exposed to three very different environments. Some annexins, like the annexin II (calpactin) tetramer, may experience general cytoplasmic calcium concentrations in stimulated cells of no greater than 1 or 2 μM. Such a protein may be responsible for the phenomena underlying exocytosis as observed in permeabilized secretory cells. Other annexins, like annexin IV (endonexin) or annexin I (lipocortin), may be activated only near the plasma membrane or near internal stores of calcium where the ions may stream into the cytoplasm in a stimulated cell and produce transient, high calcium concentrations (*22*). Such proteins might be active in the microdomains of high calcium concentration that have been detected at sites of neurotransmitter release (*23*). Alternatively, some annexins may indeed be intended to function at extracellular concentrations of calcium, although not in the process of exocytosis. Annexin I and annexin V (endonexin II), for example, may function extracellularly as phospholipase inhibitors or inhibitors of blood coagulation, respectively, through their ability to bind to acidic lipids in the presence of calcium (*24, 25*). Finally, some annexins may be designed to function intracellularly only when calcium homeostasis has failed and lipid membranes require protection from destructive hydrolysis.

The annexins may therefore participate in a broad range of cellular activities, at a broad range of calcium concentrations. Indeed, it is possible that the calcium sensitivity of some annexins in the intracellular environment is so great that they are not regulated by calcium, but rather by changes in lipid composition or availability of free fatty acids. Such annexins might even contribute to membrane fusion steps occurring early in the secretory pathway, as transport vesicles move between the endoplasmic reticulum and the Golgi. Such fusion steps require only ambient (resting) calcium concentrations (*26*).

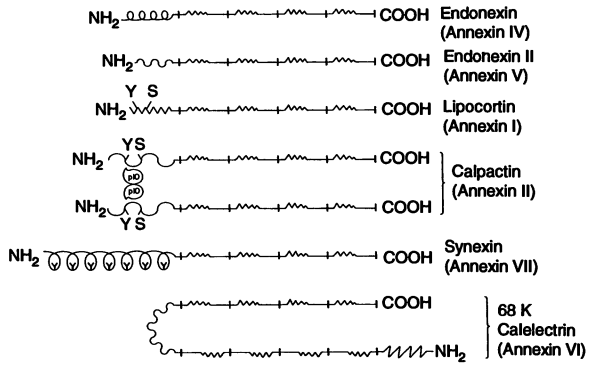

Fig. 2. Primary structures of six annexins. The four (or eight) homologous domains, represented by a saw-tooth line, each contain the 17–amino acid endonexin fold sequence [KGhGTDExxLIpILApR; h, hydrophobic residue; p, polar residue; and x, variable residue (*35, 80*)]. The structures near the amino termini (NH_2) are unique. Y (tyrosine) and S (serine) represent phosphorylation sites in the tails of calpactin and lipocortin. The calpactin (annexin II) tetramer is drawn showing the association of the amino-termini of the heavy chains with the light chain (p10) dimer. The Y's (tyrosines) inside the loops in the tail of synexin represent a pro-beta helix (*81*). Names used for the annexins in this article are given on the right, as well as a reference nomenclature that has been adopted for the protein family (*82*). [Figure adapted from (*83*)]

Annexin Structure

Five to 6 years ago the annexin field emerged from the coalescence of research in diverse areas of membrane biology as the sequences of a number of membrane-binding proteins were found to be related. These included chromobindins (chromaffin granule-binding proteins such as synexin) (*27*), lipocortins (*28*), calpactins (*29*), calelectrins (*30*), endonexins (*31*), calcimedins (*32*), certain inhibitors of blood coagulation (*33*), and collagen-binding proteins (*34*). The common body plan of the annexins consists of four or eight repeats of 70 amino acids in length. The repeats are 40 to 60% identical in sequence (Fig. 2). A highly conserved 17–amino acid consensus sequence in each repeat has been termed the "endonexin fold" because of its initial identification in peptides derived from bovine endonexin (annexin IV) (*35*). Conspicuously absent from the annexin sequences is any region homologous to the "EF-hand" domain of intracellular calcium-binding proteins of the calmodulin, troponin C, and parvalbumin family.

The annexins have divergent sequences at their amino termini. These termini are regulatory regions in that they provide the attachment site for additional subunits (p10 or calcyclin) (*7, 16*) and sites for phosphorylation by protein kinase C (*36*) or tyrosine-specific protein kinases (*37*). In addition, proteolytic cleavage at these termini alters the calcium sensitivities of the proteins (*8, 38*).

Diffraction quality crystals have been prepared from several annexins (*15, 39*), and high resolution structures obtained for two conformations of annexin V (*15*) (Fig. 3). Because of the high degree of sequence similarity among the annexins in the core domains containing the four 70–amino acid repeats, the annexin V structure may be a valuable guide to the structures of the cores of the other annexins. Each 70–amino acid domain forms a bundle of five, almost parallel (or anti-parallel), alpha helices wound into a right-handed superhelix. The path of the polypeptide chain between these helices serves to stitch them into a tight protein domain. The four domains are arrayed about a central hydrophilic channel in an approximately planar manner.

All of the calcium-binding sites are on one side of the planar annexin molecule (*15*) (Fig. 3), which also is the membrane-binding face as determined by analysis of two-dimensional crystals that form on a lipid monolayer (*40*). Three high-affinity sites are apparent, corresponding to three of the four repeats. Two lower affinity ion-binding sites were also identified on the basis of lanthanum binding, but these are not homologous to the high-affinity sites (*15*). Calcium does not appear to bind to the third repeat because of sequence and structure deviations in this repeat of annexin V. Each high-affinity calcium-binding site involves the first few residues of the endonexin fold and, unexpectedly, an acidic residue in a second loop, 39 residues downstream. Although no high resolution structure is available that incorporates a bound phospholipid, there are appropriate basic residues near the calcium-binding site that might interact with the negative charges of the lipid headgroup, and indeed the phosphate of the lipid might participate in coordination of the calcium. The overall conformation of the annexin calcium-binding site is remarkably similar to that of the calcium-binding site found in secreted phospholipase A2 (*15, 41*), even though extended sequence similarity between these protein families is not apparent.

The regulatory amino-terminal tail, which is particularly short in annexin V, is present on the slightly concave face (the "cytoplasmic face"), opposite the membrane-binding face. It seems to form a cinch holding the first domain to the fourth domain and thus maintaining the circular arrangement of the 70–amino acid domains.

Implications of Annexin Structure

Although the individual 70–amino acid repeats of any given annexin are highly similar to one another, they are not identical. Therefore the individual domains may have differential specificities for particular lipid headgroups. As four or eight repeats are present in each annexin, each protein may be able to integrate information about the lipid composition of membranes. This may explain the ability of annexins to distinguish different organelle membranes (*42*), providing specificity of their actions, and may enable the annexins to signal underlying changes in membrane lipid composition through changes in conformation or localization. Each annexin also differs significantly from its siblings in sequence; thus the presence of multiple annexins in a single cell might provide a complex system for sensing, or modulating, local membrane lipid compositions.

Localization of all the calcium- and phospholipid-binding sites on one face of the annexin molecule (*15*) raises the question of how these proteins can express a "bivalent" character and pull two membranes together. In the case of the annexin II (calpactin) tetramer, in which the p10 binding site near the amino terminus of the heavy chain presumably faces the cytoplasm, as does the annexin V amino terminus, it is possible the two heavy chains have opposite orientations and can therefore bind two approaching membranes simultaneously. For other annexins, a protein self-association event seems the most likely explanation. If this occurs at the cytoplasmic or lateral faces of membrane-bound annexins, then alterations in the tail structure might dramatically affect the ability of the annexins to promote membrane contacts. Indeed, this may be the significance of phosphorylation of annexin I (lipocortin) by protein kinase C: The membrane-aggregating activity of annexin I is strongly inhibited by phosphorylation, even though its membrane-binding activity is slightly enhanced (*43*). Annexin I becomes rapidly phosphorylated in the chromaffin cell in response to secretogogues (*44*). This may be a mechanism for down-regulating the secretory apparatus; a corresponding dephos-

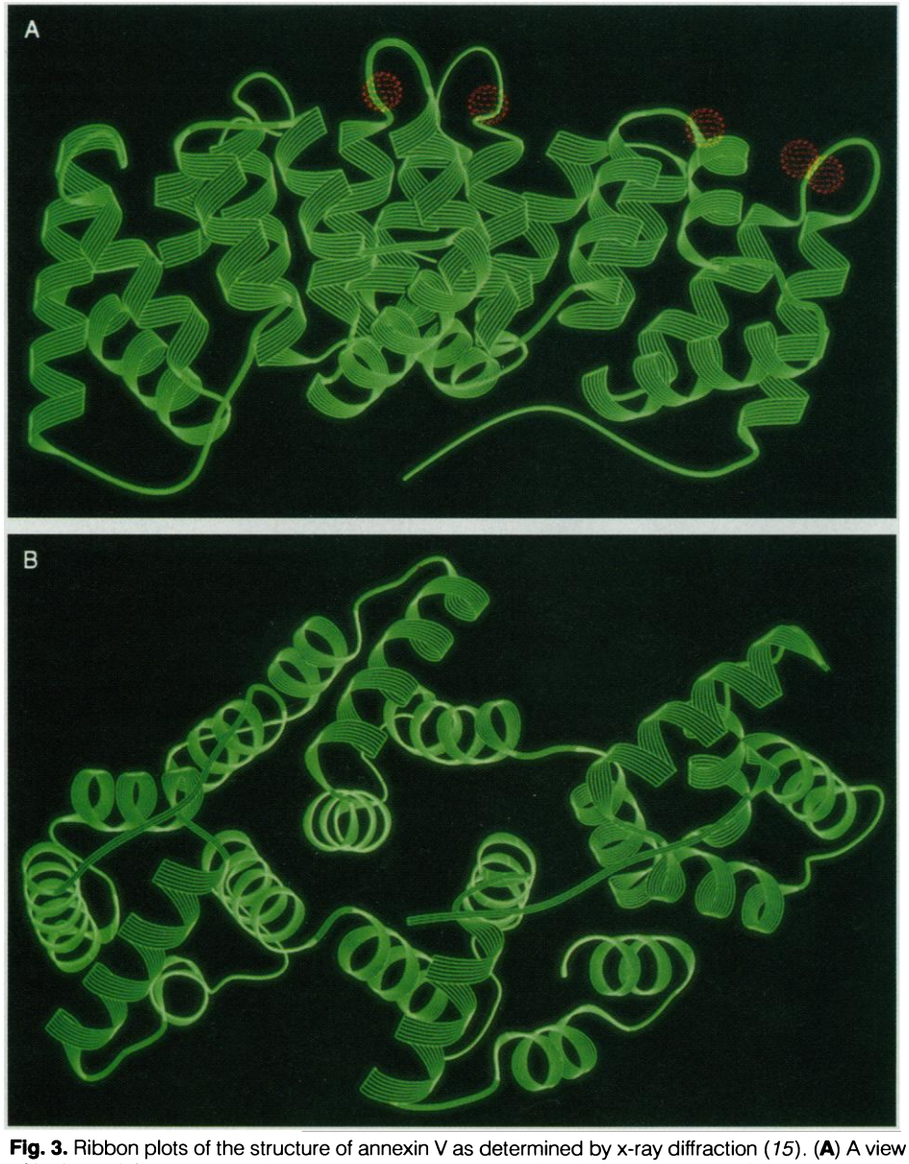

Fig. 3. Ribbon plots of the structure of annexin V as determined by x-ray diffraction (*15*). (**A**) A view with the calcium-binding sites and the membrane-binding face at the top. Calcium ions are represented by the red spheres. The high-affinity sites are the first, second, and fifth from left to right; the third and fourth sites are low-affinity ion-binding sites that were identified by lanthanum binding (*15*). The extended amino-terminus is at the bottom. (**B**) A view of the "cytoplasmic" side of the molecule and the amino terminus. The calcium-binding sites are on the far side. The center of the molecule is relatively open and may serve as an ion channel.

phorylation might be necessary to initiate exocytosis.

The striking hydrophilic central channel of annexin V, as well as the bundles of alpha helices oriented perpendicular to the membrane surface, are reminiscent of the predicted structure of transmembrane ion channels. Indeed, annexin V and synexin form voltage-dependent ion channels across synthetic lipid bilayers on the tip of a patch pipet (*45, 46*). As the external surface of the annexin molecule is hydrophilic, it has been suggested that this channel activity may result either from insertion of the molecule in the bilayer after extensive structural rearrangement of the protein to form external faces compatible with the hydrophobic portion of the bilayer (*45*), or from disruption of the membrane structure by the electrostatic effects of the protein when bound to the membrane surface (*46*). In either model, a high degree of ion selectivity would be provided by a pathway for ion permeation through the hydrophilic central channel of the protein. The channel structure determined by crystallography is not actually "open," but would require rearrangements of side chains and salt-bridges to allow an ion to pass (*46*). Such rearrangements may constitute the voltage sensor or selectivity gate. In view of the potential importance of the amino-terminal domain of the annexin in holding the four domains together, phosphorylation of the tail might regulate the conformation of this molecular cinch and thus alter the conductance properties of the channel.

The Exocytotic Pore

The channel-forming properties of the annexins might be responsible for the formation of the initial exocytotic pore that develops when the secretory vesicle first fuses with the plasma membrane. During exocytosis, the area of the plasma membrane of a secretory cell expands as secretory vesicle membrane is transiently incorporated in the plasma membrane. This can be documented by measurement of the capacitance of the cell surface membrane through the use of a patch pipet in the whole cell configuration (*47*). Coincident with detection of a vesicle fusion event, the conductivity through the opening of the vesicle can be measured to give an estimate of pore size and lifetime (*48*). The pore becomes undetectable as it enlarges indefinitely.

The annexins might form such an expanding pore if the amino-terminal cinch of one annexin could be released from one fourth domain and form a new attachment to the fourth domain of an adjacent annexin molecule. This would result in the formation of a larger channel surrounded by eight domains. The process could then be repeated without limit as the channel expanded.

Unfortunately, such pore expansion has not been observed in measurements of the single-channel activity of annexins in artificial bilayers, and the conductivity of the annexin channels is an order of magnitude less than that of the initially detectable exocytotic pore (10 to 30 pS, as compared to 200 to 300 pS). Because annexin-mediated chromaffin granule fusion does not proceed rapidly unless a *cis*-unsaturated fatty acid is added (*12*), such a cofactor might be required to initiate the molecular rearrangements necessary for channel expansion. A test of these speculations might be performed with the use of a patch pipette to measure the capacitance changes and pore conductivities that should be detectable as cell-sized vesicles are formed by the annexin-mediated fusion of isolated chromaffin granules in vitro (*12*).

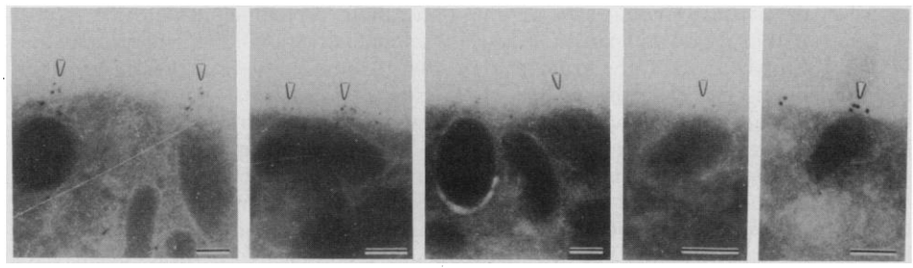

Fig. 4. Localization of annexin II (calpactin) on ultrathin sections of secretogogue-activated chromaffin cells at the electron microscopic level. The 5-nm gold particles marking the location of annexin II (indicated by the arrowheads) are closely associated with the chromaffin granule attachment sites at the plasma membrane. Bars represent 0.1 μm. [Reproduced from (*50*)]

Lessons from Cells

Exocytosis, by its very nature, is a cellular phenomenon. Therefore, concepts developed through the analysis of subcellular components must ultimately be tested in whole cells. The locations of annexins in cells may provide clues to their biology. Although they are generally assumed to be soluble proteins at low concentrations of calcium, some annexins, like annexin II (calpactin) in the chromaffin cell, are closely associated with the plasma membrane (*49, 50*). Indeed, electron microscopy and immunocytochemistry places annexin II directly between chromaffin granules and the plasma membrane in stimulated chromaffin cells (*50*) (Fig. 4). Translocation of annexins might also be expected when cells are stimulated; annexin III appears to move to the periphery of phagosomes when neutrophils dine on yeast (*51*). Other annexins have been found in other cells to be distributed in the cytoplasm where they might interact with internal membrane systems (*52*).

A preparation that bridges the gap between the in vitro systems and the intact cell is the permeabilized secretory cell. Permeabilization may be performed by electric discharge (*9*), selective detergents (*53*), pore-forming toxins (*54*), or with a micropipette (*47*). The digitonin- or toxin-permeabilized chromaffin cell has been studied particularly extensively. The permeabilized cell carries out exocytosis when challenged with concentrations of calcium that activate, among other things, membrane aggregation by the annexin II (calpactin) complex. If the permeabilized cells are preincubated with the calcium chelator EGTA, they slowly lose the ability to carry out exocytosis. However, simultaneous incubation of the cells with EGTA and with crude cytosol or annexin II tends to slow the rate of this decline in activity (*55*). Although this might suggest a critical function for annexin II in exocytosis, the experiment is not as satisfying as a true reconstitution experiment because addition of annexin II to already depleted cells does not reactivate the cells. Therefore, in these experiments, the annexin II might serve only to postpone the loss of some other critical component by mechanisms unrelated to the physiological mechanism of exocytosis.

Certain fractions of brain cytosol do have a slight reactivating effect on the EGTA-extracted chromaffin cell (*56*). One of these fractions, called EXO 1, is comprised of a protein or proteins closely related to the 14-3-3 family of proteins (*57*). Although the functions of these proteins have been a puzzle, no easier to solve than that of annexin function, one member of the 14-3-3 family exhibits calcium-stimulated phospholipase A2 activity (*58*). Therefore, the EXO 1 protein might be working to provide a fatty acid cofactor for annexin-mediated membrane fusion. If so, EXO 1 and one or more annexins might act synergistically when tested in the permeabilized chromaffin cell.

Measurements of the production of free fatty acids in permeabilized chromaffin cells appear to contradict the importance of the

fatty acid as an essential cofactor for exocytosis. Measurable calcium-stimulated release of arachidonic acid can be blocked by specific inhibitors without blocking secretion (59). If the fatty acid is indeed an essential cofactor, then resting levels must be adequate, perhaps after stimulus-dependent redistribution, or the increase in free fatty acid concentration must occur only in an extremely limited locale. Alternatively, other factors in the cell, including possibly proteins that modulate lipid structure in a stimulus-dependent manner, may serve the same function as the fatty acid.

The permeabilized secretory cell might provide a testing ground for specific annexin inhibitors. As the structure and mechanism of action of the annexins is revealed, increasingly specific inhibitors may be designed based on synthetic peptides that block protein interactions. For example, a synthetic peptide that can compete for the binding of the annexin II (calpactin) heavy chain to the light chain (60) may help elucidate the function of the annexin II tetramer in exocytosis or other processes. In one study such a peptide was found not to influence secretion from permeabilized chromaffin cells (61). However, experiments have not been done to determine the kinetics or efficacy of annexin II tetramer disruption by this peptide in situ. The tail domain of annexin XI (synexin II) might similarly be used to judge the significance of the association between annexin XI and calcyclin in the cell (16).

The importance of annexins in exocytosis might be assessed by genetic means in simple organisms such as hydra, slime molds, or yeast. Annexins have been isolated from hydra (62) and *Dictyostelium* (63, 64), and a gene deletion experiment has been performed on *Dictyostelium* synexin (64). Without synexin, slime mold amoebae take longer to re-initiate growth when transferred to a new medium. It has been suggested that this may be related to a deficiency in the ability to secrete autocrine growth factors (64). A group of calcium-dependent membrane-binding proteins has been isolated from *Saccharomyces cerevisiae* (65), but it will remain unclear whether these proteins can be classified as annexins until their sequences are obtained. A concern with genetic "knock-out" experiments in these organisms is that the multiplicity of annexins, as seen in most organisms, may reflect some degree of redundancy of functions. Therefore, deletion of a single annexin might result in little or no change in phenotype. Alternatively, a membrane trafficking event mediated by a specific annexin might be essential to cell viability; the "knockout" could be too successful, necessitating more subtle genetic modifications.

Interactions with *sec* Mutants

Although it is not yet clear whether yeast cells express endogenous annexins, this versatile organism may be used to test certain activities of the mammalian annexins in a more natural environment than that of the test tube. Mammalian annexins can be expressed in high amounts (0.2 to 1% of total protein) in wild-type yeast without apparent ill effects on the physiology of the yeast cell (66). However, when five mammalian annexins (I, IV, V, VI, and VII) were expressed individually in a number of yeast secretory (*sec*) mutants, specific interactions were apparent with three "late" *sec* mutants, *sec2*, *sec4*, and *sec15* (66). The products of these three *SEC* genes are required for the final step in the secretory pathway in yeast, the fusion of the secretory vesicle with the plasma membrane (67). These three *SEC* genes interact strongly with one another, as overexpression of one can suppress specific mutant alleles of the others (68). The activities of the complex presumably formed by the products of these three genes may be controlled by the product of the *SEC4* gene which is a small GTP-binding protein (68).

Expression of human synexin (annexin VII) has a dominant negative effect on the growth of *sec2*, *sec4*, and *sec15* mutants and on the movement of proteins through the secretory pathway of these mutants (66). Bovine annexin IV (endonexin) weakly suppresses the *sec2* mutant. No other interactions are seen between the five annexins (I, IV, V, VI, and VII) and the seven other late (exocytosis) *sec* mutants, or with the "early" (Golgi or endoplasmic reticulum) mutants, *sec17*, or *sec18*, nor with the phospholipid transfer mutant, *sec14*. Therefore annexins IV and VII have highly specific effects on a particular event in constitutive exocytosis in this system. In addition, three annexins (I, VI, and VII) promote the more rapid adaptation of *sec2* mutants to galactose-containing medium when transferred from glucose-containing medium (66). Part of the adaptation process involves expression and incorporation of the galactose transport protein into the plasma membrane. In *sec2* mutants, the ability to accomplish this translocation of the transport protein is defective (69). Expression of human annexin I (lipocortin) accelerates the appearance of galactose transport activity in the plasma membrane of the *sec2* mutant (70). These data suggest that the annexins may be involved in the process of incorporation of transport proteins into the plasma membranes of cells by an exocytosis-like fusion of precursor vesicles with the plasma membrane.

Although these genetic interactions evoke the interpretation that the annexins can influence membrane trafficking in cells, it is difficult in these hybrid experiments to be certain of the mechanisms involved. Indeed, one can envision mechanisms whereby any one or several of the proposed functions of the annexins could cause these effects, including promotion of membrane fusion, inhibition of membrane fusion, alteration in lipid organization or metabolism, or changes in ion fluxes. Some insight into mechanism should be gleaned if mutations of the annexin structure or specific inhibitors of the annexins can be demonstrated to have similar consequences for a given in vitro activity of the annexin and the effects of the annexin on yeast cell physiology.

The Broader Context

The annexins are abundant proteins, in some cells comprising on the order of 1% of the total protein. They interact with universal elements of the cellular environment, phospholipids and calcium. They are part of the fabric of the cell, not just the buttons and zippers. It is therefore likely their importance in cell and membrane biology extends well beyond the process of exocytosis. The annexins may participate in membrane fusion events elsewhere in the cell, as suggested by the cytoplasmic distributions of some family members. However, certain essential components of the fusion machinery at early points of the secretory pathway have already been identified and it is not clear how their functions would be integrated into a model for annexin function. Perhaps additional components, including small GTP-binding proteins and the multimeric "fusion" protein, NSF (*N*-ethylmaleimide Sensitive Factor) (71), enhance membrane specificity or membrane fusion activity, features that may be partially deficient in the annexins if they are specialized for the initiation of membrane-to-membrane contacts. Additional proteins characteristic of synaptic or secretory vesicle membranes, such as synaptophysin or synaptotagmin (72), might similarly assist the annexins during exocytosis at the plasma membrane.

Annexin VI may underlie a process which is actually the reverse of exocytosis: Endocytosis of surface membrane through the formation and "pinching off" of clathrin-coated vesicles. Annexin VI appears to be essential for pinching off membrane when endocytosis is reconstituted in vitro with isolated plasma membranes and cytosolic fractions (73). One of the contexts in which annexin VI was discovered was as an inhibitor of synexin (74); it may be that annexin VI regulates membrane aggregation by other annexins, both in vitro and in vivo, by promoting the reverse reaction.

Beyond promotion of membrane contacts, some of the most general functions suggested for the annexins are the organization or metabolism of lipids, including metabolism of lipid-derived inositol-phosphates (*75*), formation (*45*) or modulation (*76*) of ion channels, and the organization or membrane attachment of cytoskeletal elements (*29, 77*). Although the annexin hypothesis for exocytosis holds that the annexins are directly responsible for drawing two membranes together to initiate membrane fusion, some of these other functions of annexins may in fact turn out to be their only contribution to the process. More highly specialized functions for the annexins are likely to have arisen also, such as regulation of blood coagulation (*33*) and bone mineralization (*78*), although such functions unique to animals would not explain the existence of these proteins in green plants (*79*) and slime molds (*63, 64*). Evolution has likely favored the property of binding to membranes in response to changes in calcium concentration or lipid composition to regulate a number of processes that may seem superficially unrelated. Analysis of the possible functions of annexins in exocytosis cannot help but broaden our understanding of cellular membrane biology in general.

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80. Abbreviations for the amino acid residues are: A, Ala; D, Asp; E, Glu; G, Gly; I, Ile; K, Lys; L, Leu; R, Arg; S, Ser; T, Thr; and Y, Tyr.
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Transport of Proteins Across the Endoplasmic Reticulum Membrane

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The biosynthesis of many eukaryotic proteins requires their transport across the endoplasmic reticulum (ER) membrane. The process can be divided into two phases: (i) a targeting cycle, during which, by virtue of their signal sequences, nascent polypeptides are directed to translocation sites in the ER and (ii) the actual transfer of proteins across the membrane. The first phase has been well characterized, whereas the latter until recently was completely unresolved. Key components of the translocation apparatus have now been identified and it seems likely that they form a protein-conducting channel in the ER membrane. The transport process is similar to the process of protein export in bacteria.

A great number of proteins are transported across the ER membrane as they are synthesized. These include secretory proteins and proteins of the plasma membrane, lysosomes, endosomes, and all organelles of the secretory pathway. Synthesis of these proteins begins in the cytoplasm, but they are then targeted to the ER membrane by signal sequences, which are characterized by a continuous stretch of 6 to 20 apolar amino acids and are often located at the NH_2-terminus of precursor molecules. Recognition of the signal sequence and targeting of the nascent chain generally requires the combined function of the signal recognition particle (SRP) and of its membrane receptor, but alternative targeting pathways exist. This review summarizes briefly our knowledge of the targeting process (for previous reviews, see *1*, *2*).

The main focus of this review is the translocation process that succeeds the targeting phase. Proposed mechanisms of translocation have ranged from the idea that the transport of a polypeptide chain occurs directly through the phospholipid bilayer without participation of membrane proteins to models in which polypeptides are transported through a hydrophilic or amphiphilic channel formed from transmembrane proteins (*1*). It now seems that a protein-conducting channel does exist. The evidence comes from electrophysiological data and from the identification of membrane proteins as putative channel constituents. Three powerful approaches have contributed to the recent progress—genetic screening for translocation components, identification of membrane proteins adjacent to translocating polypeptides by chemical crosslinking, and reconstitution of the translocation components into proteoliposomes after their solubilization and purification. This review summarizes our knowledge of the various components of the translocation site.

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The Targeting Cycle: Role of the SRP

In eukaryotes, most proteins are targeted to the ER membrane by the SRP. The SRP is a ribonucleoprotein particle consisting of a 7S RNA molecule and six polypeptide subunits of 9, 14, 19, 54, 68, and 72 kD (*2*). In vitro experiments with the mammalian SRP have suggested a scheme for the function of the SRP (Fig. 1). As soon as the signal sequence of a growing polypeptide chain has emerged from the ribosome, it is bound by the SRP (step 1). Next, the complex containing the nascent chain, ribosome, and SRP is specifically targeted to the ER membrane by an interaction with a membrane-bound receptor, the SRP receptor or docking protein (*3*), which consists of α and β subunits (*4*) (step 2). Guanosine triphosphate (GTP) is required for the next step, during which the SRP is released from both the ribosome and the signal sequence (*5*) (steps 3 and 4). The nascent chain is transferred into the membrane and the ribosome becomes membrane bound through its attachment to a ribosome receptor. Finally, GTP hydrolysis leads to the dissociation of the SRP from its receptor, and a new targeting cycle can begin (*6*) (step 5). The actual transfer of the polypeptide through the membrane does not require the SRP or its receptor and commences only after their disengagement (after step 4). According to this scheme, the SRP has two basic functions: First, it targets the polypeptide chain to the ER membrane by interacting both with the signal sequence and with the translocation apparatus. Second, it keeps the bound signal sequence segregated from the rest of the polypeptide chain and thereby prevents aberrant, premature folding.

The signal sequence is recognized by the 54-kD polypeptide of the SRP (SRP54) (*7*). This subunit contains a methionine-rich M domain and a GTP-binding G domain (*8*, *9*). The former domain interacts with signal sequences (*10*). The methionines in the M domain are assumed to be located on one side of three α helices and could form or contribute to the formation of a hydrophobic pocket into which the hydrophobic cores of signal sequences could be buried (*9*). The flexible side chains of methionines appear to be particularly well suited to accommodate signal sequences of different structure. The G domain, which is not needed for signal sequence binding, seems to take part in targeting (*11*). GTP hydrolysis at this site may result in the release of erroneously bound signal sequences from the M domain; it would thus be required for a proofreading mechanism during signal sequence recognition. SRP54 can bind to signal sequences in the absence of any other component of the SRP (*12*).

GTP binds to both subunits of the SRP receptor. The α subunit interacts with the SRP, and the GTP binding site of the α subunit appears to be important for the targeting reaction (*13*). It seems likely that a guanine nucleotide exchange reaction is induced by the contact of the SRP with the α subunit of the SRP receptor (step 3 in Fig. 1). Occupation of the site by GTP initiates the release of the signal sequence from the SRP (step 4). The function of the GTP-binding site of the β subunit of the SRP receptor is unknown.

The SRP and its membrane receptor are found in all organisms that have been examined. Homologs to the mammalian components have been detected in plants, yeast, and even bacteria. Depletion of *Saccharomyces cerevisiae* cells of SRP components or of the SRP receptor leads to defective translocation of many exported