THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 43, Issue of October 27, pp. 33329–33335, 2000

Printed in U.S.A.

Selective Degradation of Annexins by Chaperone-mediated Autophagy*

Received for publication, June 27, 2000, and in revised form, August 7, 2000
Published, JBC Papers in Press, August 9, 2000, DOI 10.1074/jbc.M005655200

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Annexins are a family of proteins that bind phospholipids in a calcium-dependent manner. Analysis of the sequences of the different members of the annexin family revealed the presence of a pentapeptide biochemically related to KFERQ in some annexins but not in others. Such sequences have been proposed to be a targeting sequence for chaperone-mediated autophagy, a lysosomal pathway of protein degradation that is activated in confluent cells in response to removal of serum growth factors. We demonstrate that annexins II and VI, which contain KFERQ-like sequences, are degraded more rapidly in response to serum withdrawal, while annexins V and XI, without such sequences, are degraded at the same rate in the presence and absence of serum. Using isolated lysosomes, only the annexins containing KFERQ-like sequences are degraded by chaperone mediated-autophagy. Annexins V and XI could associate with lysosomes but did not enter the lysosomes and were not proteolytic substrates. Furthermore, four annexins containing KFERQ-like sequences, annexins I, II, IV, and VI, are enriched in lysosomes with high chaperone-mediated autophagy activity as expected for substrate proteins. These results provide striking evidence for the importance of KFERQ motifs in substrates of chaperone-mediated autophagy.

Annexins constitute a protein family of more than 12 different members in humans, each of which is able to bind calcium and phospholipds (1, 2). A conserved 70-amino acid sequence is repeated either four or eight times in the sequences of annexin family members forming the core domain of the protein responsible for calcium and phospholipid binding (2, 3). Each of the annexins has a unique amino-terminal region susceptible to secondary posttranslational modifications believed to be responsible for the specific annexin functions (4). In addition, differences in their tissue and intracellular distribution also determine the different functions for the 40–50% identical and 70–80% similar proteins (5).

Although the physiological relevance of annexins is still unclear, different intracellular and extracellular functions have been proposed (1). Modulation of intracellular calcium homeostasis, regulation of inflammatory processes, binding of extracellular matrix molecules, and activation of plasminogen are some of the proposed functions (1, 2). In addition to those specific functions, many authors have proposed the regulation of intracellular vesicular transport such as endocytosis and exocytosis as the primary function of annexins (6, 7). Annexins have been implicated as membrane fusogens, calcium sensors, mediators of actin binding to membranes, and signal transducers (7, 8). The role of the different annexins in vesicular transport may also differ depending on the cell type and the metabolic state of cells.

Annexins are mainly located in the cytosol, but they can shift to different subcellular locations in a calcium-dependent manner. Annexins have been localized to the plasma membrane (9), early endosomes (10, 11), late endosomes, multivesicular bodies (11, 12), phagosomes (10, 12), and Golgi (11). In addition to the vesicular system, annexins have also been detected in mitochondria and the nucleus (5).

Interestingly, rates of synthesis of annexins are not acutely regulated (13). Mechanisms other than changes in annexin mRNA levels might contribute to altered protein expression levels or activities under different conditions. Different secondary modifications such as phosphorylation and alkylation have been described for some of the annexins (1, 2). Little is known about changes in annexin degradation rates or even the pathways by which annexins are normally degraded.

Analysis of the sequences of the 12 human annexins revealed the presence in nine of them of a motif biochemically related with the pentapeptide KFERQ (14) (Table I diagrams the annexins analyzed in this work). That motif has been implicated in targeting of mammalian proteins for degradation in lysosomes by chaperone-mediated autophagy (15). The substrates for this pathway constitute a heterogeneous group of proteins that includes some glycolytic enzymes, transcription factors and their inhibitors, subunits of some cytosolic proteases, and the cytosolic form of a secretory protein (16, 17). The only common feature in all of the substrates for this pathway is that they contain the KFERQ-related motif. This motif, present in about 30% of cytosolic proteins (15, 18), is specifically recognized by a chaperone, the heat shock cognate protein of 73 kDa (hsc73)[1] when chaperone-mediated autophagy is activated (19). The complex of substrate protein/chaperone then binds to the

* This work was supported by National Institutes of Health Grants AG00829 (to A. M. C.) and AG06116 (to J. F. D.), an American Federation for Aging Research research grant (to A. M. C.), and a grant from the University of the West Indies Campus Research Fund (to J. A. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: hsc73, heat shock cognate protein of 73 kDa; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propanesulfonic acid, pH 7.2.

lysosomal membrane through a receptor protein (20), and the substrate is then transported into the lysosomal matrix for degradation. The transport of substrate requires the presence of a second chaperone, the lysosomal hsc73, in the lysosomal lumen (21, 22). Chaperone-mediated autophagy is mainly activated under conditions of nutrient deprivation in a tissue-dependent manner (18, 23) and after exposure to specific toxin compounds (24).

The KFERQ-related motif seems to be phylogenetically conserved. Recently a pathway similar in many respects to chaperone-mediated autophagy has been described in yeast (25). Isolated yeast vacuoles could selectively import and degrade glyceraldehyde-3-phosphate dehydrogenase type 1 in an ATP/hsc73-dependent manner. The yeast protein also contains a KFERQ motif near its amino terminus (QRKDI, residues 21–25).

In this work we have directly analyzed the effect that changes in the activity of this lysosomal pathway under conditions such as nutrient deprivation have on the half-life of four annexins, two of which contain a KFERQ motif and two of which do not. We also analyze whether or not the annexins can be taken up and degraded in lysosomes by chaperone-mediated autophagy *in vitro*.

EXPERIMENTAL PROCEDURES

Animals and Cells—Male Wistar rats (200–250 g) were starved for 20 h to deplete liver glycogen. Rats had free access to water. In some experiments fasting was prolonged to 64 or 88 h as indicated. Primary human lung fibroblasts (IMR-90) from the Coriell Cell Repositories (Camden, NJ) were maintained in Dulbecco's modified Eagle's medium (Sigma) in the presence of 10% newborn calf serum. To deprive cells of serum, plates were extensively washed with Hanks' balanced salts solution (Life Technologies, Inc.), and medium without serum was added.

Chemicals—Sources of chemicals and antibodies were as described previously (20, 23, 26, 27). For immunoblot analysis, the monoclonal antibodies against annexins I, II, IV, and VI were obtained from ICN Biomedicals (Aurora, OH), and against annexin V from Dr. Y. Imai (National Institute of Neuroscience, Tokyo, Japan). For immunoprecipitation purposes the polyclonal antibodies against annexins II and VI were from Santa Cruz Biotechnology (Santa Cruz, CA), against annexin V from Dr. A. Hofman (Max-Planck-Institut für Biochemie, Martinsried, Germany), and against annexin XI from Dr. W. J. van Venrooij (University of Nijmegen, Nijmegen, The Netherlands). The cDNAs for annexins II, V and VI were from Dr. V. Gerke (University of Münster, Münster, Germany), Dr. A. Hofman (Max-Planck-Institut für Biochemie), and Dr. S. Moss (University College, London, United Kingdom), respectively.

Measurement of Annexin Half-lives in Cultured Cells—The half-lives of different annexins were measured in human fibroblasts in culture as described (28). Briefly, fibroblasts at 60–70% confluence were radiolabeled with the [^{35}S]methionine/cysteine mixture (0.2 mCi/ml) for 48 h in methionine/cysteine-free medium (Sigma) supplemented with 10% serum. After extensive washing, cells were maintained in the presence or absence of 10% serum, and supplemented with a 2 mM mixture of nonradioactive methionine and cysteine. At increasing times, cells were recovered and lysed in 50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% Nonidet P-40 (lysis buffer). Lysates were cleared by centrifugation, and supernatants were incubated with the specific antibodies against the different annexins previously conjugated to protein A/protein G-Sepharose beads (Oncogene Research Products, Calbiochem, La Jolla, CA). After extensive washing with lysis buffer, the immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and dried gels were exposed to a PhosphorImager screen. The half-lives of the different annexins for cells maintained in the presence or absence of serum were calculated after quantification in the PhosphorImager system from the formula $t_{1/2} = ln2$/degradation rate.

Isolation of Lysosomal Fractions—Lysosomes were isolated from a light mitochondrial fraction in a discontinuous metrizamide density gradient (29) by the shorter method reported previously (27). In some studies two groups of lysosomes with different activities for chaperone-mediated autophagy were isolated as described (22). The integrity of the lysosomal membrane after isolation was measured by β-hexosaminidase latency as described previously (26). Only preparations with more than 95% intact lysosomes were used.

Lysosomal membranes and matrices were separated after hypotonic shock of isolated lysosomes as reported (30). The lysosomal membranes recovered by centrifugation were then washed with 20 volumes of 10 mM MOPS, pH 7.2, without additions or with 1 mM $CaCl_2$, 3 mM EDTA, or 3 mM EGTA.

In Vitro Synthesis of Annexins—The cDNA of annexins II, V and VI cloned in the pRC-CMV vector (Invitrogen, San Diego, CA) were transcribed/translated in a nuclease-treated rabbit reticulocyte lysate system (T7TNT® Coupled Reticulocyte Lysate System, Promega, Madison, WI) using a [^{35}S]methionine/cysteine mixture (Easy Tag-Express [^{35}S]; PerkinElmer Life Sciences). The reaction mixtures were incubated at 30 °C for 1.5 h, and the total radioactivity incorporated was determined by precipitation with 10% trichloroacetic acid. All synthesis reactions were adjusted to 0.3 M sucrose before adding into the lysosomal transport assay to maintain the osmolarity of the medium.

Uptake of Annexins by Isolated Rat Liver Lysosomes—Isolated lysosomes (100 μg of protein) were incubated with 100 μM chymostatin for 10 min at 0 °C and then diluted three times with 10 mM MOPS, pH 7.2, 0.3 M sucrose (MOPS buffer) (23, 27). *In vitro* synthesized annexins (approximately 150,000 dpm) were added and samples were incubated for 20 min at 37 °C. Samples were then incubated for 10 min at 0 °C with proteinase K at a final concentration previously determined to be sufficient to completely degrade the amount of annexins synthesized. Lysosomes recovered by centrifugation were subjected to SDS-PAGE. The amount of annexins associated with lysosomes (samples not treated with proteinase K) and transported into the lysosomal lumen (samples treated with proteinase K) was detected and quantified by exposure of dried gels to a PhosphorImager screen in a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). Any additions were dissolved in the incubation buffer before adding to lysosomes.

Proteolysis Measurements—Degradation of annexins by lysosomes was assayed as described previously (23, 26, 27). Briefly, intact rat liver lysosomes or lysosomes broken by 10 cycles of freezing and thawing in a hypotonic buffer (25 μg of protein) were incubated with the *in vitro* synthesized annexins (approximately 75,000 dpm) in 10 mM MOPS, pH 7.5, 0.3 M sucrose, 1 mM dithiothreitol, and 5.4 μM cysteine (MOPS/dithiothreitol buffer) for 1.5 h at 25 °C. Reactions were stopped by the addition of trichloroacetic acid to a final concentration of 10%. Acid-soluble material (amino acids and small peptides) was collected by filtration through a Millipore Multiscreen assay system (Millipore, Bedford, MA) using a 0.45-μm pore filter, and the acid-precipitable material (protein) was collected in the filter. Radioactivity in the samples was converted to disintegrations per minute in a P2100TR Packard liquid scintillation analyzer by correcting for quenching using an external standard (Packard Instruments, Meriden, CT). Proteolysis was expressed as the percentage of the initial acid-insoluble radioactivity converted to acid-soluble radioactivity at the end of the incubation. In some experiments aliquots taken at different times of the incubation were subjected to SDS-PAGE and proteolysis was followed after exposure of the dried gel to a PhosphorImager screen as the decrease in the amount of initial protein.

General Methods—SDS-PAGE (31) and immunoblotting (32) were performed by standard procedures. Protein was determined by the Lowry method (33) using bovine serum albumin as a standard. Standard procedures were used for the determination of enzymatic activities as reported previously (26, 27). Densitometric analysis of the immunoblots, was performed with a Digital Imaging System IS-1000 (Innotech S-100, Sunnyvale, CA).

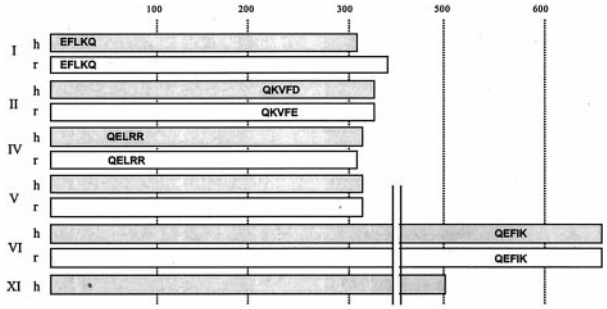
RESULTS

Effect of Nutrient Deprivation on the Half-lives of Annexins—Removal of serum from confluent cultures of fibroblasts activates chaperone-mediated autophagy (16). We measured the half-lives of two KFERQ-containing annexins (II and VI; Table I) and two annexins lacking the motif (V and XI) in confluent human fibroblasts cultured in the presence or absence of serum (Fig. 1). In the presence of serum, the half-lives of the four annexins in those cells were similar (40–52 h). After serum removal we found reduced half-lives of annexin II (30%) and annexin VI (65%), but no changes in the half-lives of annexins V and XI. These results suggest that the annexins containing the KFERQ-like motifs may be degraded by chaperone-mediated autophagy.

TABLE I

KFERQ-like motifs in human and rat annexins

The location and sequence of KFERQ-like motifs in the human (h) and rat (r) annexins analyzed in this work is shown.



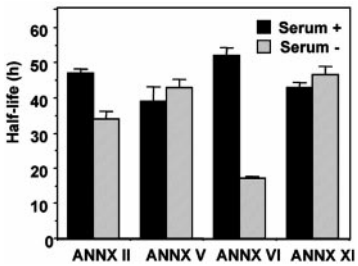


FIG. 1. **Effect of nutrient deprivation on annexin degradation rates in cultured cells.** Confluent human fibroblasts radiolabeled for 48 h as described under "Experimental Procedures" were maintained in the presence (*Serum +*) or absence of serum (*Serum −*), and at different times cells were lysed and annexins (*ANNX*) were immunoprecipitated. Radioactivity in the annexin bands were measured after SDS-PAGE and exposure to a PhosphorImager screen. Half-lives were calculated by the formula $t_{1/2} = ln2$/degradation rate. Values (half-lives in hours) are the average + S.D. of three to five different experiments.

Location of Annexins in Rat Liver Lysosomes—We then analyzed isolated rat liver lysosomes for their content of different annexins. Annexins have been detected in the cytosol of most cell types but also in association with various intracellular membranes (9–12), but to our knowledge only annexin VI has been previously reported to be associated with lysosomes (11). We found that the four annexins analyzed in this work (annexins II, V, VI, and XI) (Fig. 2*A*), but also annexins I and IV (data not shown) can be detected to some extent in the isolated lysosomes (0.2%, 0.6%, 0.1%, and 0.04% of the total annexins II, V, VI, and XI in the homogenate, respectively). The lysosomal fraction used in this study has been extensively characterized (26, 27), and it consists mainly of mature lysosomes as indicated by their acidic pH (pH 5.2–5.5), the complete processing and maturation of cathepsins in their lumen, and the absence of markers for other vesicular organelles such as for late endosomes (*i.e.* the mannose 6-phosphate receptor). The annexins detected in the lysosomal fraction had the same molecular weight as those in the cytosolic fraction. In addition, in some preparations we detected two immunoreactive lower molecular mass bands of 35 and 30 kDa for annexins II and VI, but never for annexins V or XI (data not shown). Those lower molecular weight forms might correspond to proteolytic fragments of annexins as described below.

Although we found all annexins analyzed associated with lysosomes, when we separated lysosomal membranes and matrices we found a preferential location of annexins II and VI in the lysosomal matrix (83% in the matrix *versus* 17% in the membrane) and of annexin XI in the lysosomal membrane (24% in the matrix *versus* 76% in the membrane) (Fig. 2*A*). Annexin V lacks the KFERQ motif but was still more abundant in the lysosomal matrix, although not as strikingly so as the KFERQ-containing annexins (Fig. 2*A*).

We then incubated the isolated rat liver lysosomes in an isotonic medium in the presence or absence of the protease inhibitor, leupeptin, and followed changes in levels of different annexins by immunoblot (Fig. 2*B*). The lysosomal levels of annexin II and VI, but not of annexin V and XI, decreased with the incubation time, and that decrease was almost completely blocked by the protease inhibitor. These results suggest that the two annexins containing the KFERQ motif, but not the two lacking the motif, were degraded in lysosomes.

Direct Transport of Annexins into Isolated Lysosomes—To analyze the mechanism that the annexins containing the KFERQ motif follow for their transport and further degradation into the lysosomal matrix, we used a previously developed *in vitro* system (23, 26, 27). That system allowed us to analyze the ability of isolated rat liver lysosomes to take up and degrade different annexins. A portion of the annexin II, and to a lesser extent annexin VI, synthesized in an *in vitro* transcription/translation system was degraded after incubation with intact lysosomes (Fig. 3*A*). We found much less degradation of annexin V incubated under the same conditions. The different degradation rates for those annexins were not due to differences in their susceptibility to lysosomal proteases since all of them were degraded at similar rate by broken lysosomes (Fig. 3*B*, *bottom*).

When we analyzed the annexin pattern of lysosomal degradation in autoradiograms, we found an intermediate proteolytic fragment for annexins II and VI of 35 and 38 kDa, respectively (Fig. 3*A*, *top*). In agreement with our previous observations, annexin V incubated with intact lysosomes remained intact (Fig. 3). Interestingly, we found similar intermediate fragments for annexins II and VI after their incubation with broken lysosomes (Fig. 3*B*, *top*), suggesting that the fragments were probably the result of preferential cleavage by lysosomal protease(s). After incubation with broken lysosomes, we did not detect any intermediate fragment for annexin V during its complete degradation (Fig. 3*B*).

We separately analyzed binding and uptake of proteins by isolated lysosomes *in vitro* after inhibiting their proteolytic activity with specific protease inhibitors (27). When we added the *in vitro* synthesized annexins II, V, and VI to that system, we found that a portion of all of them bound to lysosomes (Fig. 4*A*, *lanes 4–6*). Interestingly, despite the pretreatment of lysosomes with the protease inhibitor, chymostatin, we still detected the two lower proteolytic fragments for annexin II and VI described above (Fig. 4*A*, *lane 6*). To determine the amount of each annexin transported into the lysosomal lumen, we then removed the proteins bound to the external surface of the lysosomes by treatment with proteinase K. We found that a portion of annexin II and VI, but also of annexin V, was presumably located in the lysosomal lumen since it was not degraded by proteinase K (Fig. 4*A*, *lanes 7–9*). The amount of proteinase K added was sufficient to remove all the extralysosomal annexins since we did not detect any remaining annexin in the supernatant after lysosomal sedimentation (Fig. 4*A*, *lanes 10–12*).

To better determine the location of the different annexins associated with lysosomes, we then performed similar uptake assays but separated lysosomal membranes from matrices at the end of the incubation (Fig. 4*B*). We found that a portion of the total annexin II and VI associated with lysosomes (*lanes 4* and *6*) was bound to the lysosomal membrane (*lanes 7* and *9*), but both annexins and their corresponding proteolytic fragments were also present in the lysosomal matrix (*lanes 10* and *12*). In contrast, we only detected annexin V in the lysosomal

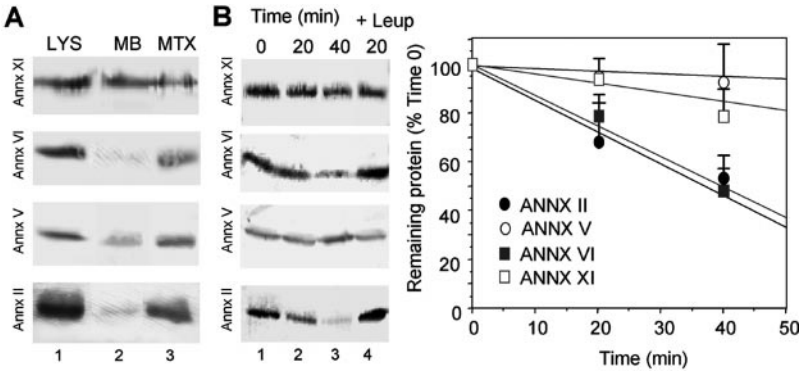


FIG. 2. **Immunolocalization and degradation of annexins in lysosomes.** *A*, rat liver lysosomes (100 μg of protein) (*LYS*) and their corresponding membranes (*MB*) and matrices (*MTX*) were subjected to SDS-PAGE and immunoblotted for annexins II, V, VI, and XI as labeled. *B*, intact rat liver lysosomes (100 μg of protein) were incubated at 37 °C in MOPS buffer without additions (*lanes 1–3*) or in the presence of leupeptin (100 μM; + *Leup*). At the indicated times aliquots were taken and subjected to SDS-PAGE and immunoblotting for annexins II, V, VI, and XI as labeled. The graph corresponds to the average values + S.D. of the densitometric quantification of the full-sized protein in three experiments similar to the one shown here. *Annx* and *ANNX*, annexin.

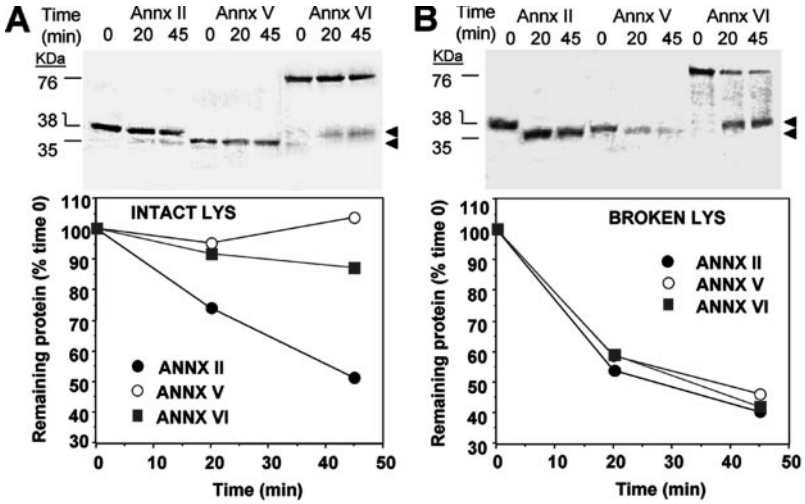


FIG. 3. **Degradation of *in vitro* synthesized annexins by rat liver lysosomes.** Annexins II, V and VI synthesized *in vitro* using a transcription/translation system as described under "Experimental Procedures," were incubated with intact (*A*) or broken (*B*) lysosomes (25 μg of protein) in MOPS/dithiothreitol buffer. At the indicated times samples were taken, subjected to SDS-PAGE, and exposed to a PhosphorImager screen. *Arrowheads* indicate proteolytic fragments for annexins II (35 kDa) and VI (38 kDa). Graphs correspond to the densitometric quantification of the full-sized protein from the gels shown. *Annx* and *ANNX*, annexin.

membrane fraction (*lane 8*) but never in the lysosomal matrix (*lane 11*). These results suggest a portion of annexin V associates with the lysosomal membrane in a fashion that makes it resistant to the attack of proteinase K but that annexin V is not transported into the lysosomal lumen.

Association of Annexins to the Lysosomal Membrane—Binding of annexins to phospholipids is often modulated by calcium. We found that the binding of *in vitro* synthesized annexin II and VI to the membrane of intact lysosomes was not significantly modified in the presence of calcium (Fig. 5*A*). However, we detected a slight increase in the amount of annexin V associated to the lysosomal membrane after calcium supplementation (Fig. 5*A*).

One possibility is that the normal stimulatory effect of calcium on annexin binding properties might be masked by the presence of calcium already in the transcription/translation reactions or in the isolated lysosomes. Some of the annexins, such as annexin II, have low calcium requirements for phospholipid binding. Thus, we then analyzed the effect that calcium chelating agents had on the fraction of annexins II, V, VI, and IX detected endogenously bound to the lysosomal membrane (Fig. 5*B*). We found that a portion of all annexins can be released from the lysosomal membrane after a single wash with unsupplemented buffer (Fig. 5*B*). However, in the presence of EDTA or EGTA, the amount of annexin V and XI released from the lysosomal membranes was clearly higher. Addition of calcium during the single wash significantly reduced the release of those two annexins from the lysosomal membrane, but did not modify the release of annexin II or VI (Fig. 5*B*). These results suggest that the association of annexins lacking the KFERQ motif to the lysosomal membrane is regulated by different mechanisms than for the KFERQ-containing annexins, and that association does not lead to their transport into the lysosomal matrix.

Effect of Changes in the Activity of Chaperone-mediated Autophagy on the Lysosomal Content of Different Annexins—We then analyzed under different conditions *in vivo* the lysosomal content of annexins. In rat liver it is possible to separate two different groups of lysosomes with different activities for chaperone-mediated autophagy (22). The more active group of lysosomes is enriched in lysosomal hsc73, the lysosomal chaperone required for substrate uptake (Fig. 6*A*). We found that the total lysosomal levels of annexins II and VI were significantly higher in the group of lysosomes with higher chaperone-mediated autophagy activity (Fig. 6*A*). Those differences were even more evident when we separated the lysosomal membranes and matrices. As shown in Fig. 6*A*, the amount of annexin II and VI associated with the lysosomal membrane was similar for both groups (*lanes 5* and *6*), but their levels in the matrix of the more active group of lysosomes were clearly higher (*lanes 3* and *4*). In contrast, we did not find differences in levels of annexins V and XI between both groups of lysosomes. The enrichment in the lysosomes more active for chaperone-mediated autophagy is a common characteristic of protein substrates for this pathway (22, 24, 28).

Finally, the main characteristic of chaperone-mediated au-

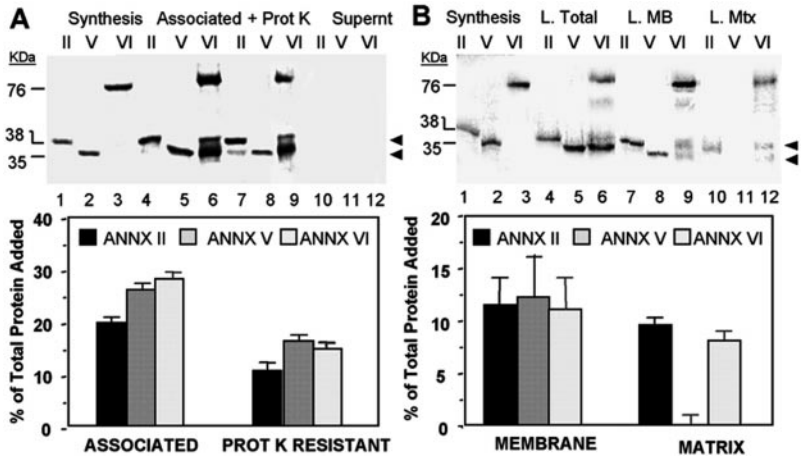


FIG. 4. **Direct transport of annexins into rat liver lysosomes.** *A*, annexins II, V, and VI, synthesized *in vitro* using a transcription/translation system as described under "Experimental Procedures," were incubated with chymostatin-treated intact rat liver lysosomes (100 μg of protein) in MOPS buffer under standard conditions. At the end of the incubation, part of the samples were treated with proteinase K (+ *Prot K*). Lysosomes were recovered by centrifugation, and the supernatants (*Supernt*) of the proteinase K-treated samples were subjected to SDS-PAGE and exposed to a PhosphorImager screen. *Arrowheads* indicate proteolytic fragments for annexins II and VI as described in the legend to Fig. 3. The *lower panel* corresponds to the densitometric quantification (average + S.D.) of five different experiments similar to the one shown. *B*, *in vitro* synthesized annexins II, V, and VI were incubated with intact lysosomes as in *A*. At the end of the incubation, lysosomes were recovered by centrifugation (*L. Total*) and in part of the samples lysosomal membranes (*L. MB*) and matrices (*L. Mtx*) were isolated as described under "Experimental Procedures." Samples were processed as in *A*. *Lanes 1–3* contain 1/10 of annexins added per incubation. *Arrowheads* indicate annexin II and VI proteolytic fragments as described in the legend to Fig. 3. *Lower panels* correspond to the densitometric quantification (average + S.D.) of five different experiments similar to the one shown. *ANNX*, annexin.

tophagy, when compared with other lysosomal proteolytic pathways, is its activation during prolonged nutrient deprivation (18, 23). When we compared levels of different annexins in lysosomes isolated from rats starved for increasing periods of time, we found that levels of annexins II and VI, but not of annexins V and XI, increased with the starvation time (Fig. 6*B*). These results suggest that also *in vivo* the annexins containing the KFERQ-like motifs reach the lysosomal matrix by chaperone-mediated autophagy.

Degradation of Other KFERQ-containing Annexins by Chaperone-mediated Autophagy—To determine if KFERQ-containing annexins other than annexins II and VI were also likely to be degraded by chaperone-mediated autophagy, we analyzed the association of annexins I and IV (Table I) to lysosomes. We were able to detect both annexins in isolated rat liver lysosomes (Fig. 7*A*). High levels of annexins I and IV have been previously reported to be associated with endosomes (34, 35). We did not detect in our lysosomal preparation endosomal proteins such as the mannose 6-phosphate receptor, but the mature form of cathepsin D, a lysosomal marker, was abundant (Fig. 7*A*). We conclude that annexins I and IV are located in lysosomes as well as endosomes.

Annexins I and IV were also more abundant in the group of lysosomes with higher activity of chaperone-mediated autophagy (Fig. 7*B*). In addition, lysosomal levels of both annexins increased with the starvation time (Fig. 7*C*). We conclude that chaperone-mediated autophagy is probably a common mechanism for degradation of KFERQ-containing annexins.

DISCUSSION

In this work we present experimental evidence for the degradation of some of the members of the annexin family in lysosomes by chaperone-mediated autophagy. The four annexins initially analyzed in this study (annexins II, V, VI, and XI) can be detected in lysosomes (Figs. 2*A* and 7*A*), but of those tested only the ones containing the KFERQ motif undergo degradation in that compartment (Fig. 2*B*). Facts supporting the lysosomal degradation of annexins II and VI by chaperone-mediated autophagy are: 1) their increased degradation rates during serum deprivation when chaperone-mediated autophagy is activated (Fig. 1), 2) their direct transport into and degradation by isolated lysosomes (Figs. 3 and 4), and 3) their higher levels in lysosomes with higher activity of chaperone-mediated autophagy (Fig. 6*A*) including lysosomes from starved animals (Fig. 6*B*). In addition to annexins II and VI, two other annexins containing a KFERQ motif, annexins I and VI, are also enriched in lysosomes with high chaperone-mediated autophagy activity (Fig. 7), suggesting that this is a common pathway for the degradation of KFERQ-containing annexins.

In contrast to those annexins containing the KFERQ motif, annexins V and XI associate with lysosomes but are not degraded (Fig. 2*B*). The association of those two annexins to lysosomes has different requirements and characteristics from those for the KFERQ-containing annexins. Thus, their binding to the lysosomal membrane is increased in the presence of calcium (Fig. 5) and, in the case of annexin V, results in a conformation resistant to proteinase K (Fig. 4*A*). These results for annexin V are in agreement with previous studies describing a conformational change of this annexin upon binding to lipid monolayer membranes (34, 35). In addition to the lysosomal membrane, annexin V can also be detected in the lysosomal matrix (Figs. 2*A* and 6*A*). The mechanism(s) by which it reaches the matrix seems to be other than chaperone-mediated autophagy since we did not find direct transport of annexin V into isolated lysosomes as shown for other annexins (Fig. 4). Part of the matrix annexin V might result from macroautophagy or microautophagy (16).

Some of the KFERQ-containing annexins might associate with lysosomes for reasons other than their degradation. A calcium-independent binding of certain annexins to lipids has been described (36, 37). In many of those examples, pH changes seem to be important in determining the independence on calcium of the annexin-phospholipid interaction (36, 37). The acidic lysosomal environment might promote binding of some of those annexins to the lumenal side of the membrane. Those annexins might have roles as intralysosomal calcium sensors or for protection of the membrane phospholipids against lysosomal phospholipases (38).

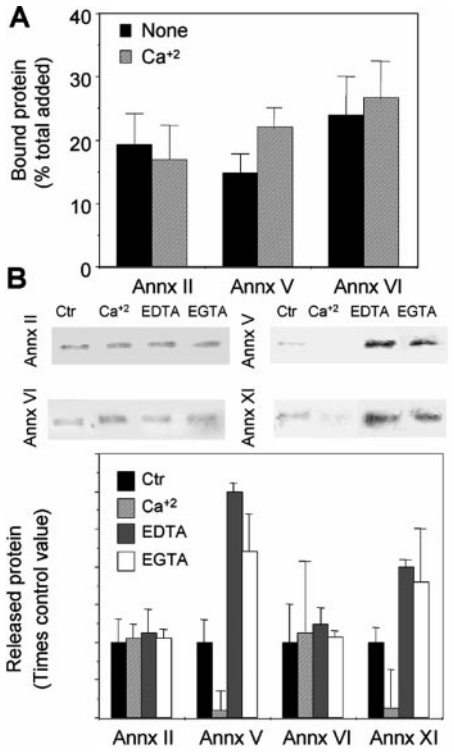


FIG. 5. **Calcium requirements for the binding of annexins to the lysosomal membrane.** *A*, annexins II, V, and VI were incubated with intact rat liver lysosomes (100 μg of protein) without additions (*None*) or in the presence of 1 mM $CaCl_2$ (Ca^{+2}). At the end of the incubation, the lysosomes collected by centrifugation were subjected to SDS-PAGE and exposed to a PhosphorImager screen. Values are the average + S.D. of the quantification of four different experiments, and binding is expressed as a percentage of the total amount of annexins added in the incubation. *B*, lysosomal membranes isolated as described under "Experimental Procedures" were incubated in MOPS buffer without additions (*Ctr*) or in the presence of 1 mM $CaCl_2$ (Ca^{+2}), 3 mM EDTA, or 3 mM EGTA. After centrifugation pellets and supernatants were subjected to SDS-PAGE and immunoblotted for annexins II, V, VI, and XI. The gel shows the annexins recovered in the supernatants. Values represent the amount of protein released during the single wash as a percentage of the total protein bound and are the average + S.D. of the quantification of three different experiments similar to the one shown. *Annx*, annexin.

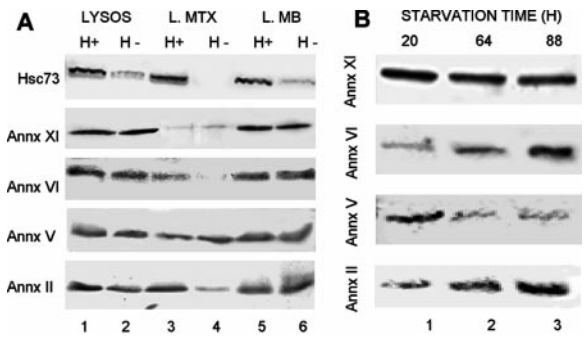


FIG. 6. **Differences in the lysosomal levels of annexins with the activity of chaperone-mediated autophagy.** *A*, lysosomes (*LYSOS*) with high (*H*+) and low (*H*−) activity for chaperone-mediated autophagy and their corresponding matrices (*L. MTX*) and membranes (*L. MB*) were subjected to SDS-PAGE and immunoblotted for annexins II, V, VI, and XI and for hsc73 as labeled. *B*, liver lysosomes isolated from rats starved for 20, 64, and 88 h, as labeled, were subjected to SDS-PAGE and immunoblotted for annexins II, V, VI, and XI as labeled. *Annx*, annexin.

It is interesting that we detected truncated forms of annexin II (35 kDa) and annexin VI (38 kDa) within lysosomes (Figs. 3 and 4). These truncated forms of annexins II and VI were especially resistant to further proteolysis for unknown reasons.

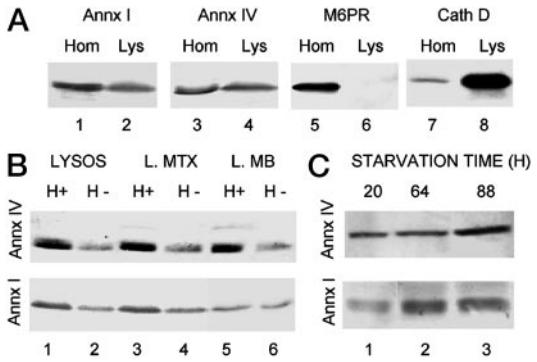


FIG. 7. **Lysosomal association of other KFERQ-containing annexins.** *A*, rat liver homogenates (*Hom*) and lysosomes (*Lys*) (100 μg of protein) were subjected to SDS-PAGE and immunoblotted for annexins I and IV, mannose 6-phosphate receptor (*M6PR*), and cathepsin D (*Cath D*) as labeled. Only the mature form of cathepsin D is shown. *B*, lysosomes with high (*H*+) and low (*H*−) activity for chaperone-mediated autophagy and their corresponding matrices (*L. MTX*) and membranes (*L. MB*) were subjected to SDS-PAGE and immunoblotted for annexins I and IV as labeled. *C*, liver lysosomes isolated from rats starved for 20, 64, and 88 h, as labeled, were subjected to SDS-PAGE and immunoblotted for annexins I and IV as labeled. *Annx*, annexin.

Truncated forms of some annexins originated by splicing (39) or by cleavage by calpain (40), elastase (41), or a metalloprotease in the plasma membrane (42) have been described. In all the cases, the truncated forms display different calcium binding regulation than the intact protein and sometimes reduced (41) and sometimes increased (40) activity. The resistance of the truncated annexins to degradation might result from their association to the lumenal side of the lysosomal membrane (Fig. 4*B*) or their aggregation after cleavage. Whether or not the lysosomal partial cleavage of annexins may have a physiological role will require further investigation.

Other than the limited proteolysis of some annexins described above, very little is known about their intracellular degradation. For the EF-hand family of calcium-binding proteins, the presence of signals for rapid proteolysis (regions rich in the amino acids P, E, S, and T) (43) in more than 50% of their members has been reported (14, 44). In addition, several of those calcium-binding proteins can be ubiquitinated under certain conditions (45). Both calpains and the 26 S proteasome have been proposed as the proteolytic systems for the cleavage and/or degradation of those proteins (46, 47). Annexins I, II, ,V and VI also possess weak regions rich in the amino acids P, E, S, and T regions (14, 44), but only limited cleavage by calpains have been demonstrated (40).

hsc73 has been shown to bind to the KFERQ sequence of RNase A (48). The KFERQ-like motifs in the four annexins analyzed in this study (Table I) are located in α-helical regions buried in the core of the protein (49, 50). Thus, the interaction of those annexins with the cytosolic hsc73 might require some degree of unfolding of the protein. Like most of the described hsc73-binding peptides, the annexin KFERQ-like motifs contain one or two central aliphatic hydrophobic amino acids that are believed to interact with the hydrophobic-binding pocket of the hsc73, flanked by one or two basic residues, which are thought to maintain the affinity of the interaction (51, 52). According to previous studies KFERQ-like motifs have a low ability to stimulate hsc73 ATPase activity (53), which will guarantee a stable interaction between the chaperone and the annexins during their transport to lysosomes.

Annexins have been detected along the different compartments of the endocytic pathway and in phagosomes (10–12), but mainly associated to the membranes of those vesicles. We were able to detect to some extent all annexins analyzed in this study endogenously in the lysosomal lumen. That finding sug-

gests that, as for other cytosolic proteins, a continuous basal degradation of those proteins might take place inside lysosomes probably by macroautophagy or microautophagy (16, 40). In addition, we present evidence of regulated lysosomal degradation of specific annexins by chaperone-mediated autophagy. Of course, other proteolytic pathways may also be responsible for the degradation of a smaller faction of annexin molecules.

The degradation of some annexins, but not others, by chaperone-mediated autophagy might be of physiological relevance since it could allow specific changes in the levels of some annexins without affecting levels of others. As mentioned in the Introduction, common and specific functions have been described for each annexin (5–7). In addition, opposite functions have been described for some annexins. For example, although most of the annexins are considered secretion mediators, annexin VI has an inhibitory effect on the secretory process (40). Under different cellular conditions, the selective degradation of some annexins in lysosomes might regulate specific annexin-mediated cellular functions without affecting other more general functions.

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