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Unique Subcellular Distribution of Five Annexins in Resting and Insulin-Stimulated Rat Adipose Cells

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Several lines of evidence suggest that annexins, a family of phospholipid-binding proteins, play a role in cellular trafficking. Five annexins (I, II, V, VI, VII) were detected in rat adipose cells. They were primarily associated with the plasma membrane in a calcium-dependent manner. None of them redistributed with insulin treatment of the cells, in contrast to the glucose transporter GLUT4, which moved from intracellular membranes to the plasma membrane. Although the actual function of annexins in adipose cells remains to be determined, our data indicate that insulin-stimulated GLUT4 trafficking does not rely on a change in subcellular location of any of the five annexins detected so far in these cells.

In adipose cells, insulin increases glucose transport by promoting the translocation of GLUT4 glucose transporters from an intracellular vesicular compartment to the plasma membrane (1-5). Recent evidence further indicates that GLUT4 recycles inside the cell (6, 7) at least in part via the coated pit-endosome pathway (4). Although these movements have been well characterized, the components mediating GLUT4 trafficking at the molecular level are still poorly documented.

Annexins (Anxs) constitute a family of structurally related Ca^{2+}-dependent phospholipid-binding proteins which have been implicated in trafficking and other membrane related events (8). Anxs I, II, and VII promote Ca^{2+}-dependent aggregation and fusion of secretory granules by a process thought to represent exocytotic fusion reactions (9, 10). Anxs I and VII also directly drive fusion of acidic phospholipid liposomes (see ref 11 for review), while Anx II retards the ''run down'' of Ca^{2+}-activated hormone release from digitonin-permeabilized chromaffin cells (12, 13). In addition, studies have shown a close association of Anxs I, II and VI with endosomal particles (14-16). In a fusion assay, Anx II transfers from one endosomal vesicle to another (15) and it may also be able to regulate intracellular localization of specific endosomal vesicles (17). Finally, although recently challenged (18), Anx VI has been described as an essential factor in endocytotic budding from clathrin-coated pits (19). Thus, annexins have received strong consideration as potential mediator of different trafficking processes.

This prompted us to examine whether members of the Anx gene family are expressed in adipose cells and whether they could be involved in GLUT4 trafficking. At a first attempt to answer this question, we looked for Anxs subcellular localization in basal (no insulin) and insulin-stimulated cells. The distribution of Anxs in different cellular compartements has been poorly documented whatever the cells (8, 20) and adipose cells have neither been studied from

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Abbreviations used: Anx, annexin; PM, plasma membrane; HDM, high-density microsomes; LDM, low-density microsomes.

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this perspective. Our data indicate that five Anxs are expressed in rat adipose cells and that their subcellular distribution is stable in response to insulin.

MATERIALS AND METHODS

Isolated rat adipose cell preparation. Isolated adipose cells were prepared by collagenase digestion of epididymal fat pads from 170-200 g Sprague Dawley rats (CD strain, Charles River Breeding Laboratories, Boston, MA, USA) as previously described (21).

Incubation with insulin and subcellular fractionation. Prior to fractionation, isolated adipose cells were incubated in the absence or presence of insulin (7 nM) at 37°C for 15 min. The cells were then homogenized in 10 mM Tris pH 7.4, 255 mM sucrose, 1 mM EDTA containing protease inhibitors and fractionated by differential ultracentrifugation as described (22). Three fractions were recovered: plasma membrane (PM), high density microsomes (HDM) enriched in endoplasmic reticulum, and low density microsomes (LDM) enriched in Golgi vesicles. These subcellular membrane fractions have been characterized elsewhere (21). Part of the homogenate was subjected to a single ultracentrifugation at 200,000 $\times$ g for 20 min. to obtain a cytosolic fraction (supernatant) and total cellular membranes (pellet). In some experiments, the homogenizing buffer was modified by addition of 1 mM CaCl2 without EDTA. Due to the very low protein recovery under these conditions, only one intracellular fraction (IM) was recovered comprising HDM plus LDM. Membrane pellets were resuspended in homogenization buffer containing protease inhibitors and the protein content was determined by the BCA assay (Pierce, Rockford, IL, USA).

Immunoblotting. Cytosolic and membrane proteins were subjected to denaturing electrophoresis using 10% polyacrylamide gels (Novex, San Diego, CA, USA). The content of the gels was transferred by electroblotting onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). Immunological detection of GLUT4 was carried out with a rabbit polyclonal antiserum prepared against a C-terminal peptide (peptide and antiserum were kindly provided by Hoffmann-La Roche, Nutley, NJ, USA) in conjunction with ^{125}I-labeled protein A. A panel of antibodies which specifically detect various Anxs was tested on the same fractions. Anx II was detected using a monoclonal antibody from Zymed Laboratories (San Francisco, CA, USA). Rabbit polyclonal antisera against purified bovine or human proteins were used to detect Anxs I, V, and VI (23). Finally, Anx VII was detected with a rabbit anti-peptide rat lung Anxs (23) served as a control of signal specificity. Following incubation with the first antibody, the filters were exposed to either goat ^{125}I-anti-rabbit IgG or goat ^{125}I-anti-mouse IgG (ICN, Costa Mesa, CA, USA). The immunolabeled bands were visualized and quantitated by phosphorimaging (Molecular Dynamics). The apparent Mr of the immunodetected proteins were determined using Mr standards (Novex, San Diego, CA, USA).

Membrane extraction. Following cell fractionation without Ca^{2+}, 100 μg of protein from PM or LDM was aliquoted. Different agents were then added: homogenization buffer alone, 1M NaCl or 1% Triton $\times$ 100 (final concentration in homogenization buffer). The samples were processed as follows: sonication 3 sec., 15 min. on ice, sonication 3 sec. and sedimentation at 200,000 $\times$ g for 15 min. AnII and GLUT4 were detected by immunoblotting in the supernatants and pellets obtained from PM and LDM, respectively.

RESULTS

As shown in Figure 1, each of the five antibodies tested reveals immunolabeled bands in rat adipose cell cytosol. The signals detected on the same blots in various cellular membrane fractions are markedly fainter, with the striking exception of Anx II which is readily detected in PM. The intensity of the Anx II signal is similar in PM and in cytosol for the same amounts of proteins loaded. Since 7 % of the proteins from the starting homogenate are routinely recovered in PM, versus 85 % in cytosol (24, 25), this indicates that approximately 8 % of total Anx II cellular content is associated with PM, in a Ca^{2+}-free environment. Anxs I, V, and VI are also present in PM but at a markedly lower level than Anx II and Anx VII is not detected in this fraction. All five Anxs are faintly detected in LDM and only Anx I, V, and VI, in HDM.

We next looked for the effect of insulin on the subcellular localizations of Anxs in rat adipose cells. Figure 1 (bottom) illustrates that insulin treatment induces a marked increase in immunodetected GLUT4 in PM with a corresponding decrease in LDM, as expected. In contrast, no difference is observed in the signals specific for the five Anxs in subcellular fractions from basal and insulin-stimulated cells.

It is known that the presence of Ca^{2+} is a critical factor for the association of Anxs with biological membranes. Thus, to further biochemically characterize Anxs in rat adipose cells, we fractionated the cells in a modified buffer containing 1 mM CaCl2 without EDTA. As

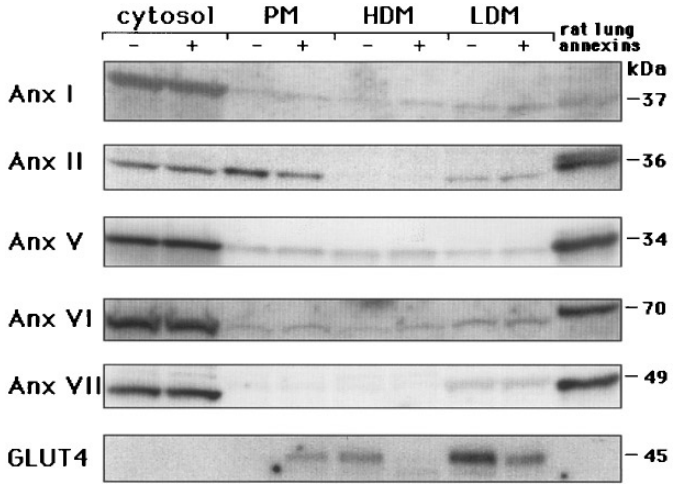

FIG. 1. Immunoblot analysis of various Anxs and GLUT4 in subcellular membranes from rat adipose cells. Cells were incubated for 15 min at 37°C with (+) or without (−) 7 nM insulin. Subcellular fractionation was performed by differential ultracentrifugations in the presence of EDTA. 80 μg of protein was applied for immunoblotting with anti-Anx antibodies and 20 μg for GLUT4. Due to mishandling of this particular sample, GLUT4 signal (known to be unchanged by insulin) was lost in HDM (+). 10 μg of an extract from rat lung was used as positive control. These blots are representative of three separate experiments.

demonstrated in Figure 2, Anxs I, V, and VI are then detected exclusively in cellular membranes and mainly in PM. This is also true for Anx II, although instead of the unique signal observed in the absence of calcium (Figure 1) we detect several immunoreactive bands of 36 kDa and lower molecular weight in total membranes and PM. This observation suggests partial proteolysis of Anx II despite the presence of protease inhibitors in the fractionation buffer. Under these conditions, we do not detect any signal for Anx VII (not shown). Given the strong Anx VII signal observed in the absence of Ca^{2+} (Figure 1), this lack of detectable Anx VII is likely due to a high sensitivity of this protein to Ca^{2+}-dependent proteases.

Similar to the data obtained when the cells were fractionated in the absence of Ca^{2+}, the

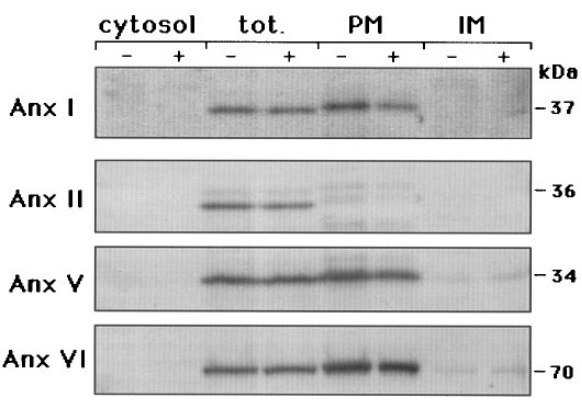

FIG. 2. Immunoblot analysis of Anxs I, II, V, and VI in rat adipose cell subcellular membranes prepared with Ca^{2+}. Cells were incubated for 15 min at 37°C with (+) or without (−) 7 nM insulin, then homogenized and fractionated in the presence of 1mM Ca^{2+}. 40 μg of protein was applied in each lane. Tot, total membranes; PM, plasma membranes; IM, intracellular membranes comprising HDM + LDM.

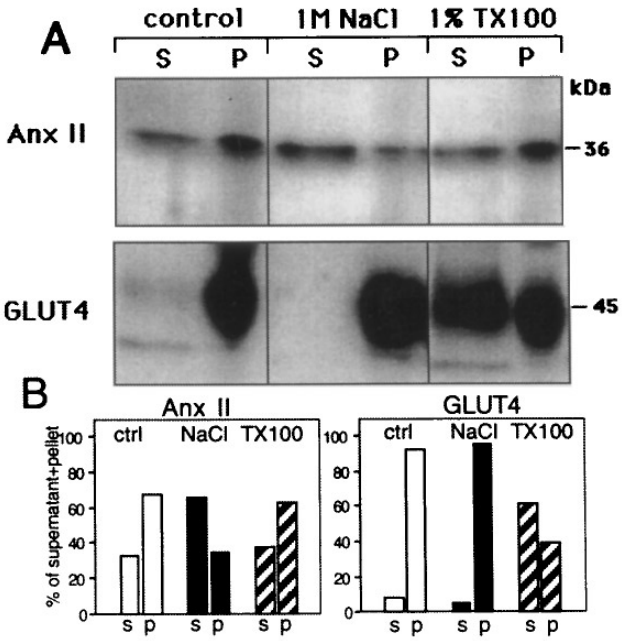

FIG. 3. Immunoblot analysis of Anx II and GLUT4 in rat adipose cell membranes extracted by high salt (1M NaCL) and detergent (1% Triton (T) X 100). Anx II was detected in PM and GLUT4 in LDM. The membranes were processed as described under Materials and Methods. (A) Phosphorimager data. (B) Relative quantification of the signals. S, supernatant; P, pellet.

subcellular distribution of Anxs in basal and insulin-treated cells does not show any significant differences.

Figure 1 shows that a fairly high amount of the cell content of Anx II is recovered with PM in a Ca^{2+}-free environment. In a first attempt to characterize this association, PM were treated with high salt concentration or detergent. These treatments were also applied to LDM to compare the behavior of Anx II with that of GLUT4, known as an integral membrane protein (26). The membrane fractions were sonicated in the presence of 1M NaCl or 1% Triton X-100. After centrifugation, Anx II and GLUT4 were immunologically detected in the pellets and supernatants. Figure 3 shows that sonication alone and following high salt treatment releases 30% and 70 % of the PM associated-Anx II, respectively. In contrast GLUT4 remains entirely membrane-associated. Treatment with Triton X-100 however, partially solubilizes both proteins, with approximately 40 % of Anx II and 60 % of GLUT4 recovered in the supernatant.

DISCUSSION

Based on immunoblotting and Ca^{2+}-dependent binding to cell membranes, we have detected Anxs I, II, V, VI and VII in rat adipose cells. To our knowledge, this is the first report of the presence of Anxs in these cells. Subcellular fractionation indicates that Anxs are primarily associated with PM in the presence of calcium, with very faint signals detected in microsomal membranes. Due to the method of cell fractionation used, it cannot be excluded that low intracellular signals result actually from contamination by plasma membrane proteins. In contrast with Anxs, GLUT4 is mainly present in LDM in resting adipose cells. Our data further show that the subcellular distribution of Anxs is stable after insulin treatment, when GLUT4 is translocated from LDM to PM.

Efforts to understand the molecular mechanisms of GLUT4 trafficking have led to the

detection in rat adipose cells of several proteins involved in vesicular movements. These include secretory carrier membrane proteins (SCAMP) (27), vesicle-associated membrane proteins (VAMP) (28), and several low-molecular weight GTP-binding proteins of the Rab family (29-32). Among these, SCAMP, VAMP and Rab 4 have been localized on GLUT4-vesicles isolated by immunoabsorption with GLUT4 antibodies and move out of this compartment in response to insulin (27, 28, 31). In contrast, two other Rab proteins, Rab 8 and Rab 3D, are mainly associated with PM and do not redistribute with insulin (31, 32) reminicent of Anxs. The translocation and the fusion of GLUT4-vesicles with PM are thought to occur through several steps. Thus, it is likely that numerous proteins are involved, each of them playing a definite but yet unknown function in concert with various partners. If the Anxs expressed in rat adipose cell play a role in this process, our data suggest that this does not rely on change in their subcellular localization. On the other hand, it has been previously shown that Anx I and Anx II are in vitro substrates of the insulin receptor tyrosine kinase (33). Although it is not known if phosphorylation also occurs in adipose cells in response to insulin, these observations raise questions with regard to the potential implication of certain phosphorylated Anx isoforms in GLUT4 trafficking.

This report also describes that a tangible amount of Anx II is Ca^{2+}-independently associated with PM. The rationale for this observation is unknown since the sequence of Anx II is highly homologous to the sequence of other Anxs, and it has no hydrophobic segment (8). Extraction experiments show that Anx II from adipose cell PM does not behave as an integral membrane protein such as GLUT4, but rather like a protein that is superficially attached to the membrane. This suggests that a nonhydrophobic component of PM interacts with Anx II, possibly a protein from the Triton X-100-precipitable cytoskeleton. Anx II has been reported to bind to actin and spectrin, but the binding is Ca^{2+}-dependent (34, 35). Further investigations are necessary to identify the factor(s) promoting Ca^{2+}-independent association of Anx II to PM in adipose cells.

In conclusion, our data reveal the presence of several Anxs in PM of insulin-sensitive rat adipose cells. These cells could provide an interesting model to search for the authentic biological function of these proteins. The data reported here indicate that insulin-stimulated GLUT4 trafficking does not involved the redistribution within different cellular compartments of the Anxs detected so far in adipose cells.

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