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Ultrastructural antibody localization of α_2-macroglobulin in membrane-limited vesicles in cultured cells

(immunocytochemistry/saponins/myosin/peroxidase)

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ABSTRACT We have been developing a procedure for localizing intracellular antigens in cultured cells, by using peroxidase-labeled antibodies, that allows good morphologic preservation. Although useful, our previous technique did not preserve the morphology of membranes, and the location of the peroxidase reaction product was difficult to establish. In this paper, we report major improvements on the basic technique that markedly enhance the quality of localization and of morphology. Saponin is used to permeabilize membranes without destroying their morphology. The amount of reaction product is enhanced with a peroxidase-antiperoxidase label. The clarity of morphologic detail and contrast of reaction product density are increased by using postsectioning staining with the osmium/thiocarbohydrazide/osmium and uranyl acetate/lead citrate procedures. We have applied this techniuqe to the ultrastructural localization of α_2-macroglobulin and demonstrated that it is localized in membrane-limited vesicles. We have also used this method to improve the preservation of structures for localization by fluorescence microscopy.

One approach to identifying the role of specific proteins in cell behavior is to localize the position of proteins within cells by immunological methods using either fluorescence microscopy or electron microscopy. For a method to be useful, the cell must be fixed so that its intracellular structures are preserved. The conventional fixation for fluorescence microscopy is formaldehyde/acetone. When cells fixed in this manner are processed and examined by conventional transmission electron microscopy, only the nuclear outline and some filamentous remnants can be identified. The morphology of membranes, endoplasmic reticulum, Golgi apparatus, and lysosomes is destroyed. Thus, the precise localization of proteins cannot be established by fluorescence microscopy.

It would be useful if intracellular proteins could be localized by electron microscopy by using antibodies coupled to peroxidase or ferritin. Although in specific cases some proteins have been localized by this electron microscopic approach, no general method has been available. We have found that the factors limiting localization are the poor penetrability of well-fixed cells to antibodies and the nonspecific binding of the antibodies used for localization. We recently developed a fixation technique (to be described elsewhere) that preserves intracellular structures intact, allows antibodies to penetrate the cytoplasmic matrix and bind to specific proteins, and minimizes nonspecific antibody binding. However, the method had two serious limitations. It failed to preserve membrane structure, and the contrast of the peroxidase reaction product was so low that the section could not be counterstained to bring out morphological details. We have now solved these deficiencies. Saponin (1) has been used instead of Triton X-100 to permeabilize membranes to antibodies without destroying membrane morphology. A soluble peroxidase–antibody complex (PAP) (2) was used to enhance the amount of enzymatic reaction product, and the osmium/thiocarbohydrazide/osmium (OTO) counterstain procedure (3) was used to enhance the contrast of this reaction product, allowing subsequent staining of the section with uranyl acetate/lead citrate (UALC) (4).

The main application of this new procedure is for ultrastructural localization of intracellular proteins. As an example of this use, we have chosen α_2-macroglobulin. This protein is ingested from the medium in endocytic vesicles by a saturable receptor-mediated process (5). In this paper, we demonstrate its presence inside "secondary lysosomal" membrane-bound cytoplasmic vesicles. This location is consistent with that expected from previous biological and functional studies.

Another application of this technique is its use with light microscopy to preserve proteins that are either difficult to fix or drastically changed in their relationships to other structures with the usual fixatives. As an example of this use, we studied the fluorescence localization of myosin in cultured cells. The localization of this protein has been widely studied in formaldehyde/acetone-fixed cells (6, 7, 8) in which most detailed structure is destroyed. By this new method, the periodic distribution of myosin associated with microfilament bundles has been confirmed, indicating that this pattern is not due to a fixation artifact.

MATERIALS AND METHODS

Cell Culture. Swiss 3T3-4 mouse fibroblasts were propagated in Dulbecco–Vogt modified Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.) at 37° as described (9). For localization of α_2-macroglobulin, cultures were incubated with α_2-macroglobulin (100 μg/ml) for 2 hr, incubated in fresh medium overnight, and fixed after washing in phosphate-buffered saline. Localization was detected by using epifluorescence with rhodamine-conjugated rabbit anti-goat gamma globulin (Cappel) (at 1:20 dilution). Goat antibody to α_2-macroglobulin was obtained from Miles Laboratories. The preparation and characterization of fibroblast myosin antibody has been described (10). The myosin antibody used in this study was affinity-purified using a human platelet myosin affinity column.

Fixation and Processing. The entire procedure is summarized in Table 1.

Saponin. Plastic dishes (35 mm) of cells were primarily fixed by the ethyldimethylaminopropyl carbodiimide (EDC)/glutaraldehyde procedure (to be described elsewhere) except that 0.25% glutaraldehyde was used instead of 0.35%. For electron microscopic localization, the procedure for membrane solubi-

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Abbreviations: PAP, complex of peroxidase and antiperoxidase antibody; EDC, ethyldimethylaminopropyl carbodiimide; EGS, EDC/glutaraldehyde/saponin; OTO, osmium/thiocarbohydrazide/osmium; UALC, uranyl acetate/lead citrate.

Table 1. Procedure for localization in cultured cells with peroxidase-labeled antibodies*

1. Primary fixation:
   1% EDC/0.25% glutaraldehyde/1.4% Tris/50 mM PO_4/50% P_i/NaCl, pH 7.0 (premix *precisely* 4 min at 23°); fix for 7 min; wash with P_i/NaCl
2. Permeability and control of nonspecific binding:
   0.02% saponin and 5 mg of NRG per ml in P_i/NaCl, 23°, 30 min
3. Antibody incubation:
   1 hr in 0.02% saponin/5 mg of NRG per ml/P_iNaCl, 23°; 1 hr in PAP as peroxidase-labeled step; wash 30 min each in 0.02% saponin/NRG (5 mg/ml)/P_i/NaCl
4. Secondary fixation and neutralization:
   3% glutaraldehyde/P_i/NaCl, pH 7.4, 15 min; wash in P_i/NaCl; 300 mM glycine in P_i/NaCl, pH 10, 23°, 10 min; wash in P_i/NaCl
5. Enzymatic reaction:
   diaminobenzidine (0.3 mg/ml)/0.03% H_2O_2 50 mM Tris/P_i/NaCl, pH 7.6, 23°, 30 min (12)
6. Osmium postfixation:
   1.5% OsO_4/0.1 M cacodylate, pH 7.0, 23°, 30 min; wash in P_i/NaCl
7. Dehydration:
   70 → 100% ethanol, 5 min each (once in 70%, four times in 100%)
8. Epon embedding:
   from ethanol directly to 100% Epon, overnight equilibration, 23°; fresh Epon, 2 days, 58°
9. Postsectioning staining:
   Sections onto nickel grids; OTO on thin section (3); UALC on thin section

Cells in plastic culture dishes were washed in P_i/NaCl and fixed at 23° in the primary fixative (step 1). This fixative solution was prepared by first mixing a buffer solution of Tris, PO_4, and P_i/NaCl and adjusting the pH to 7.0. EDC (Sigma; powdered form) and glutaraldehyde (Tousimis; as a 50% solution in H_2O) were added simultaneously at 23° to this buffer solution. During the subsequent 4 min, the solution was allowed to incubate (premix time) and the pH was readjusted to 7.0. Precisely at 4 min, this fixative solution was added to washed cells in the dish and allowed to incubate at 23° for 7 min. The cells were then washed with P_i/NaCl. This initial step was the only part of this procedure requiring such precise timing and pH adjustment. Subsequent steps were carried out as indicated. It is essential that the antibody incubations be carried out in the continuous presence of both saponin and normal globulin. The normal rabbit globulin indicated here was used when the primary antiserum (in this case goat anti-α_2-macroglobulin) was from a species such that it did not crossreact with rabbit globulin. If the primary antiserum used is prepared in a rabbit, then one could use normal goat globulin as the competing globulin. In these experiments, sequential incubation in goat anti-α_2-macroglobulin, rabbit anti-goat IgG, and goat PAP were used, each incubation lasting 1 hr, separated by 30-min washes in saponin/NRG/P_i/NaCl.

* P_i/NaCl, phosphate-buffered saline; NRG, normal rabbit globulin.

lization was modified by the use of 0.02% saponin (Sigma) in place of 0.1% Triton X-100 and saponin was included in all the antibody incubations with normal rabbit globulin as described for the use of Triton X-100. For light microscopic fluorescence, both saponin and Triton X-100 produced similar localization (detailed membrane morphology is not visible at this level of resolution).

PAP. Antibody incubations (with saponin) were carried out (as described elsewhere) but, instead of with rabbit anti-goat gamma globulin conjugated to peroxidase, we substituted a 1:20 dilution of unconjugated rabbit anti-goat IgG, followed sequentially by a 1:20 dilution of goat PAP (Cappel) as described by Sternberger *et al.* (2). Enhancement could also be achieved in principle by using the peroxidase "bridge" procedure (2, 11).

Counterstains. After sectioning on an ultramicrote, the sections were mounted on 200-mesh nickel grids (instead of copper) to allow them to be counterstained. Sections were counterstained with the OTO procedure essentially as described by Seligman *et al.* (2). Thiocarbohydrazide (Sigma) was used as described (2) at 1% (50°) for 1 hr (sections floating face down), followed by hot water (50°) washes, and exposure to OsO_4 vapor for 1 hr at 60°. This treatment markedly enhanced the contrast of the diaminobenzidine reaction product (12) previously exposed to OsO_4 as well as that of lipid-containing structures.

Additional counterstaining was also performed by using the UALC counterstain [saturated uranyl acetate in methanol, followed by lead citrate (4)].

Microscopy. Fluorescence microscopy was performed with a Zeiss RA epifluorescence microscope with a ×40 (NA 1.0) oil-immersion lens. Bright-field light microscopy was performed with a X63 (NA 1.4) oil-immersion lens and a (0.63) bright-field condensor. Electron microscopy was performed with a Hitachi HU-12A electron microscope. To achieve high contrast, electron micrographs were produced by using overexposed electron image plates at 50 kV. For lesser contrast, lower exposure at 75 kV was used.

RESULTS

The improvement in morphologic detail produced by use of saponin treatment in place of Triton X-100 is shown in Fig. 1. Membranes were virtually totally absent from the cells processed with Triton X-100 whereas clear membrane detail was evident when saponin was used. The Golgi membrane system, mitochondria with their cristae, and membrane-limited lysosomes and lipid vesicles were clearly evident. The nuclear membrane was also preserved and, at higher magnification, nuclear pores could be clearly seen (data not shown). Experiments performed to evaluate the need for the continuous presence of saponin as opposed to a single treatment step showed that fixed membranes were permeable *only* in the continual presence of saponin (results not shown). Therefore, 0.02% saponin was included in all antibody incubations and washes following antibody incubations. For brevity, we will refer to the fixation-permeabilization procedure with EDC, glutaraldehyde, and saponin as the "EGS procedure."

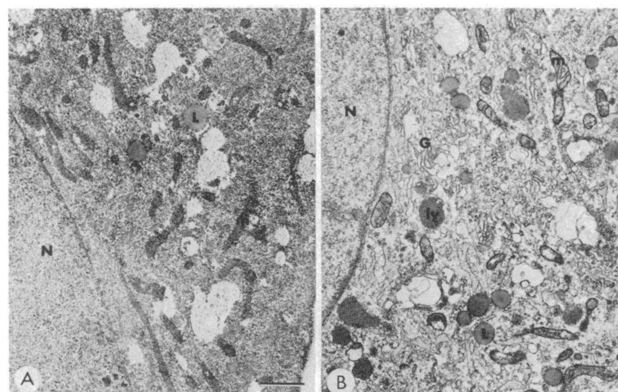

FIG. 1. Ultrastructural appearance of Swiss 3T3-4 cells fixed with the EDC/glutaraldehyde procedure and permeabilized with 0.1% Triton X-100 (*A*) or 0.02% saponin (*B*). The morphologic appearance after UALC staining is shown. N, nucleus; L, lipid; m, mitocondrion; G, Golgi; ly, lysosome. (Bar = 1 μm.)

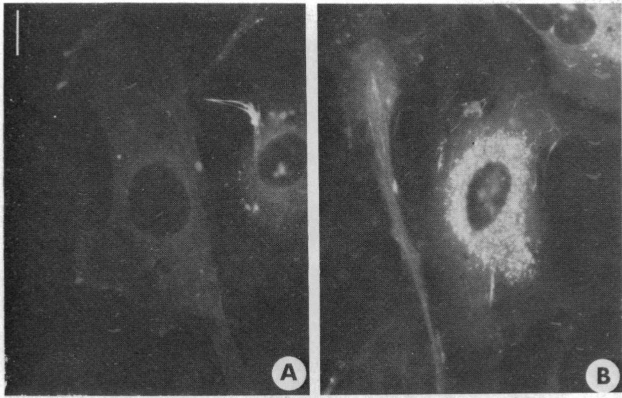

FIG. 2. Rhodamine fluorescent localization of antibody to α_2-macroglobulin in Swiss 3T3-4 cells with the EGS procedure. Cells were fixed and processed by the procedure described in Table 1 through the antibody incubations, except for the use of rhodamine-labeled rabbit anti-goat gamma globulin as the labeled antibody. The primary antibodies were normal goat globulin (control) (*A*) or goat anti-α_2-macroglobulin (*B*). (Bar = 20 μm.)

We used α_2-macroglobulin as a model antigen for localization because it has a discrete intracellular location in fibroblastic cells, and we have described its intracellular distribution in detail (5). The localization of α_2-macroglobulin in EGS-fixed cells by fluorescence is shown in Fig. 2*B*. As previously noted, the protein is found in a granular distribution within cells. Fluorescence is also a convenient way to monitor the immunospecificity of the reaction. As shown in Fig. 2, the reaction with antibodies to α_2-macroglobulin is immunospecific.

Because of its superior morphologic preservation, this procedure is useful for light microscopic localization using immunofluorescence, particularly for antigens whose intracellular distribution might change drastically with weaker fixatives. Its application to a protein found in endocytic vesicles and lysosomes was shown in Fig. 2. Myosin has been previously localized in formaldehyde/acetone-treated fibroblasts (6, 7, 8). In very flat cells, it showed an unusual periodic distribution along microfilament bundles. Because most cytoplasmic morphology

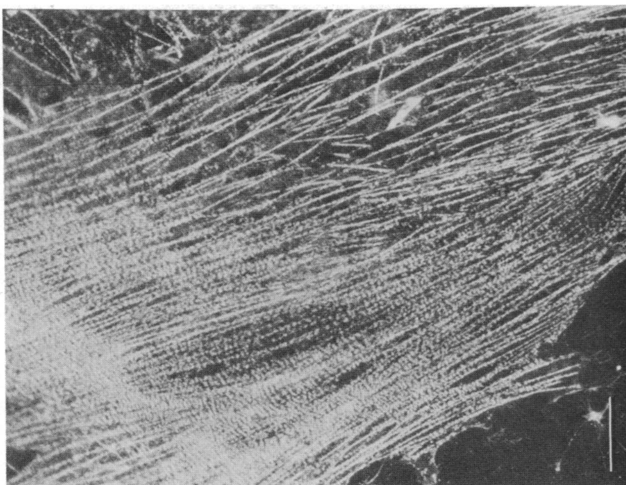

FIG. 3. Rhodamine fluorescent localization of antibody to fibroblast myosin in Swiss 3T3-4 cells with EDC/glutaraldehyde fixation. Cells were fixed with the procedure described in Table 1, except for the use of Triton X-100 and rhodamine-labeled rabbit anti-goat gamma globulin as the labeled antibody. Affinity-purified goat antibody to fibroblast myosin (20 μg/ml) was used as the first antibody step. (Bar = 20 μm.)

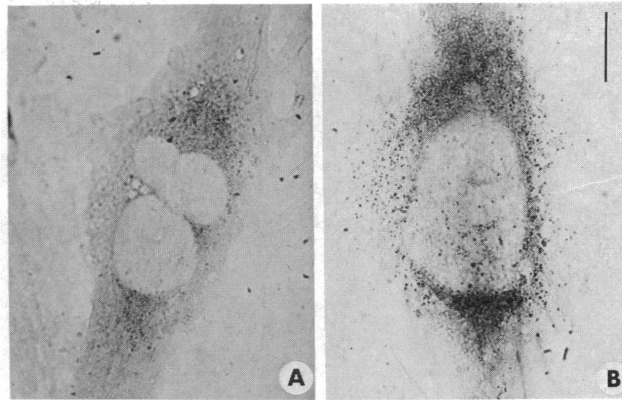

FIG. 4. Peroxidase-labeled or PAP-labeled antibody localization of α_2-macroglobulin with the EGS procedure. Cells were fixed and processed by the procedure described in Table 1 with either a peroxidase-labeled rabbit anti-goat γ globulin (*A*) or with the goat PAP labeling procedure (2) (*B*). (Bar = 20 μm.)

is destroyed by formaldehyde/acetone fixation, this periodicity could conceivably have been due to a fixation artifact. Therefore, we examined the localization of myosin in cells preserved with the EGS procedure. Fig. 3 demonstrates that the periodic distribution is preserved, indicating that this pattern is not due to some type of redistribution artifact and probably has a role in the function of myosin in fibroblastic cells.

For electron microscopy, peroxidase-conjugated antibodies have proven valuable for antigens concentrated in specific areas. However, the amount of reaction product produced is occasionally insufficient for antigens that are not concentrated. The PAP immunological complex (2), similar in principle to the earlier "bridge" peroxidase procedure (2, 11), is an improvement because it increases the amount of reaction product produced for each molecule of antibody. We have been able to use the PAP procedure with EGS-fixed cells. It is not necessary to process cells for electron microscopy to monitor the dramatic effect of the PAP procedure. Fig. 4 demonstrates the

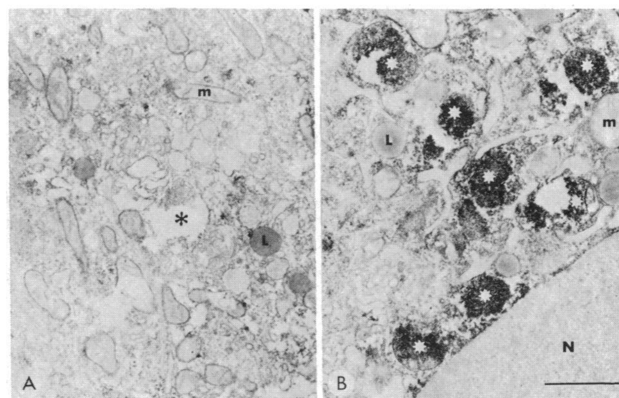

FIG. 5. Ultrastructural appearance of α_2-macroglobulin localization with the EGS procedure without counterstains. Cells were processed by the procedure in Table 1 without counterstaining (OTO or UALC) after sectioning. The ultrastructural appearance is shown with PAP labeling of cells incubated with normal goat serum (control) (*A*) or with goat anti-α_2-macroglobulin (*B*). The asterisk in *A* is in the lumen of a "secondary" lysosome, the expected site of localization of α_2-macroglobulin. The stars in *B* lie over large dense reaction product accumulations containing α_2-macroglobulin in "secondary" lysosomes. These micrographs were produced by overexposure of the negatives at 50 kV and printing on high-contrast paper to enhance the reaction product contrast. N, nucleus; L, lipid; m, mitochondria. (Bar = 1 μm.)

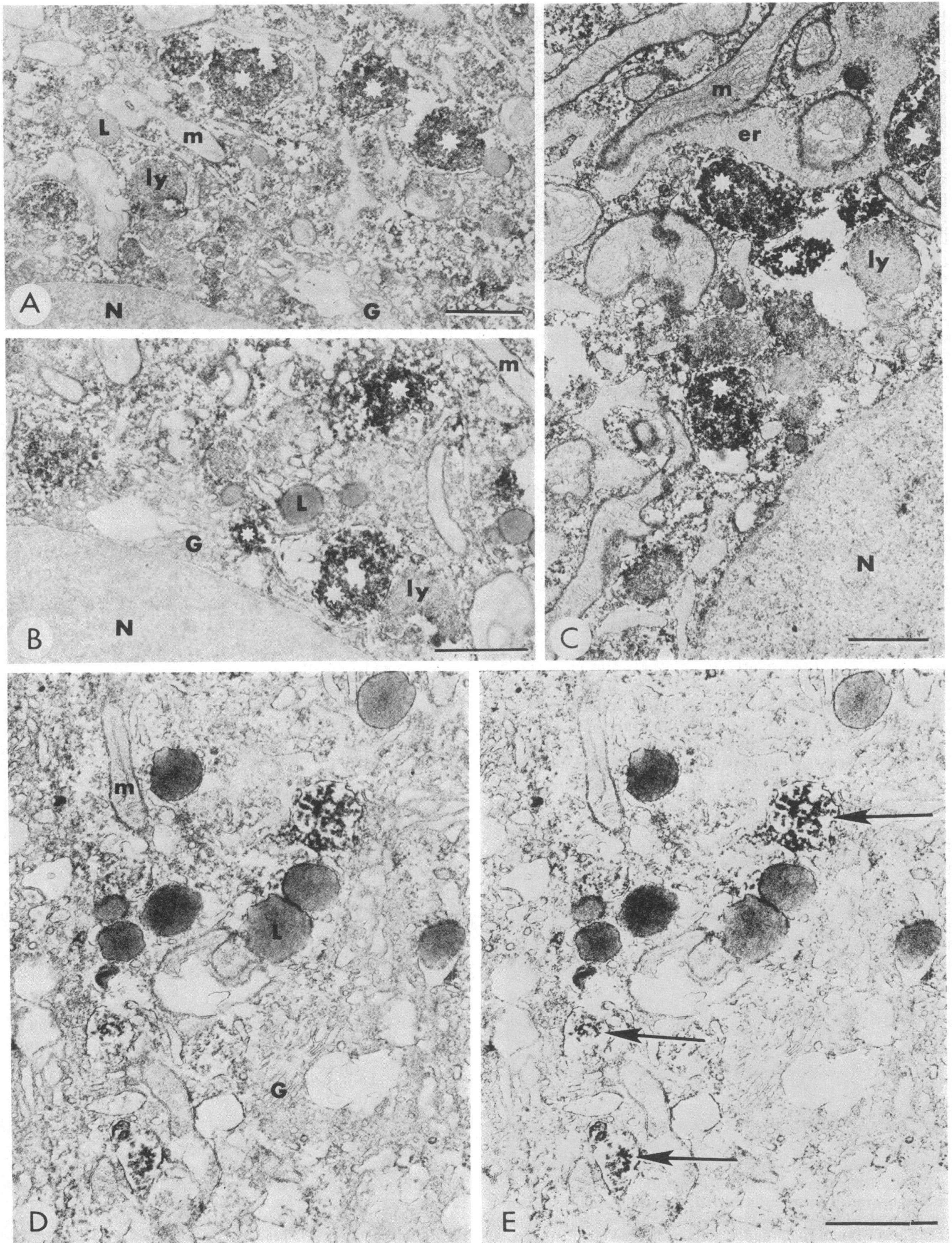

Fig. 6. (*Legend appears at the bottom of the next page.*)

bright-field light microscopic appearance of cells processed by the EGS procedure with antibody to α_2-macroglobulin detected by either a peroxidase-conjugate (Fig. 4*A*) or the PAP antibody-peroxidase complex (Fig. 4*B*). PAP clearly increased the amount of reaction product associated with the α_2-macroglobulin-containing vesicles and, as a result, has an advantage for both light and electron microscopic localization.

By electron microscopy, the reaction products in α_2-macroglobulin-containing vesicles can be identified by their electron-dense appearance, but this appearance is not striking. Nevertheless, by making high-contrast negatives and printing them on high contrast paper, it is possible to achieve interpretable pictures such as those shown in Fig. 5. The localization of α_2-macroglobulin in these cells is confined to "secondary" lysosomal vesicles which mainly accumulate in the perinuclear region.

To make the reaction product easier to see and to increase the detail of the other organelles in the cell, we used the OTO and UALC counterstain procedures. Fig. 6*A* shows the appearance of the reaction product (which had been optimized photographically in Fig. 5*B*) processed in a normal, medium-contrast manner. The reaction product contrast is significantly enhanced when the OTO procedure is applied to the thin section (Fig. 6*B*). To bring out the detail of the background organelles, we used additional postsectioning staining with UALC after the OTO procedure (Fig. 6*C*). With this preparation, one can use various types of photographic paper to show the cellular detail at medium contrast (Fig. 6*D*) or to enhance the reaction product detail (Fig. 6*E*). Thus, the interpretation of the precise localization of reaction product is greatly simplified. From these micrographs, one can see that α_2-macroglobulin is localized in "secondary" lysosomes and is absent from other organelles including mitochondria, Golgi saccules, endoplasmic reticulum, and the nucleus. In some cells, reactive vesicles also reside in the peri-Golgi region making it difficult to tell if those vesicles might also be condensing vacuoles of the Golgi apparatus (Fig. 6 *D* and *E*).

DISCUSSION

The purpose of this paper is to present a fixation procedure that produces excellent morphologic preservation of cells and enables one to localize proteins by immunological methods. The morphologic preservation is primarily a result of the EDC/glutaraldehyde primary fixation. The suppression of nonspecific binding by competing globulin improves the usefulness of this procedure. We have now been able to preserve membranous ultrastructure and achieve an appearance that rivals that seen with the best morphologic preservation available with any fixation procedure. With this procedure it should be possible to localize proteins present in small amounts, particularly if they are concentrated in specific places in the cell. This, of course, requires that the antigenic properties of the proteins involved are not damaged by this fixation.

The additon of increased contrast of the reaction product by using PAP and OTO and the ability to counterstain other organelles simultaneously with UALC alleviate some of the previous difficulty in direct observation created by low contrast. Extensive photographic enhancement is not required when ferritin conjugates are used; however, we have been unable to use ferritin-labeled antibodies because of nonspecific binding of ferritin conjugates. The best combination of labels and counterstains needs to be determined for the particular antigen and cell type under study.

There are still some aspects of the procedure that could be improved. Occasional mitochondrial swelling is observed (Fig. 6 *D* and *E*), although for most purposes this is not a severe problem. Longer incubations and washes (e.g., 24 hr) might improve labeling and minimize background for some antigens. The use of affinity-purified antibodies, as shown here for myosin, would also decrease background and ensure that specific labeling of proteins is observed.

With fluorescence light microscopy, the EGS procedure should be used when antigen localization with other fixatives shows unexpected or unusual patterns. We have chosen myosin to illustrate this point. In addition, proteins that are fixed poorly by the usual procedures may show superior preservation with the EDC/glutaraldehyde primary fixative.

We anticipate that, with electron microscopic localization, the pathways of synthesis, assembly, and secretion of specific proteins can be followed, particularly those produced in large amounts. Viral proteins would appear to be good candidates for this type of study. We have been studying the synthesis of two major membrane proteins induced by glucose starvation, GRP 78 and GRP 95 (13).

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FIG. 6 (*on preceding page*). Ultrastructural appearance of the localization of α_2-macroglobulin with the EGS procedure using OTO and UALC counterstaining. Cells were fixed and processed as in Fig. 5*B*, but the negatives were normally exposed at 75 kV and prints were made on medium-contrast paper. In *A*, the strikingly dark reaction product seen in Fig. 5*B* (stars) is considerably less prominent. Postsectioning staining with the OTO procedure increases the reaction product contrast (*B*, stars). Staining with UALC after the OTO procedure increases the contrast of other cellular organelles (*C*). The reaction product (arrows) can be compared in medium-contrast printing of OTO/UALC counterstained sections (*D*) or when the same negative is printed on high-contrast paper (*E*). This provides clear visualization of the reaction product (*E*), as well as the details of other organelles (*D*). N, nucleus; L, lipid; ly, lysosome; er, endoplasmic reticulum; m, mitochondrion; G, Golgi. (Bars = 1 μm.)