Centrosomal proteins and lactate dehydrogenase possess a common epitope in human cell lines
(nonimmune sera/affinity purification of immunoglobulins/isolated centrosomes)

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ABSTRACT A spontaneously arising rabbit anti-centrosome serum with strong human specificity, used to identify specific antigens in isolated centrosomes, was shown to react with several noncentrosomal proteins including a 36-kDa protein that appeared to be the major cellular antigen. To explore the immunological relationship between noncentrosomal and centrosomal antigens, immunoglobulins were affinity purified using the individual noncentrosomal antigens (from lymphoblastoma KE37 cells) and were tested for their capacity to bind to human centrosomes in situ and to proteins from isolated centrosomes. In this way, the 36-kDa antigen, an abundant cytosolic protein, was shown to be at least one antigenic determinant with high molecular weight centrosomal proteins. This antigen was further identified by mild proteolysis as the glycolytic enzyme lactate dehydrogenase. In all the analyzed human cell lines, the centrosomal staining in situ was correlated with a strong labeling of purified lactate dehydrogenase in immunoblots. Conversely, the absence of centrosomal staining in rodent cells was always correlated with the absence of lactate dehydrogenase labeling. These data suggest an evolutionary relationship between centrosomal proteins and this "housekeeping" enzyme.

Several cytoskeletal components have been studied at the cellular and molecular level (1–3), or even discovered (4), with nonimmune sera. In some instances, these sera are still the exclusive tools for tracing cellular structures. This is the case for microtubule-organizing centers such as kinetochores (4–6) and centrosomes (7–10). Among the anti-centrosome sera, some have immunoglobulins with a specificity toward antigenic determinants that are highly conserved from plants to mammals. This is true for the human autoimmune serum S051 (7, 11). Others, such as the rabbit serum 0013, specifically reacted with determinants that were restricted to primates (12). Using the latter serum and a highly enriched preparation of centrosomes, we have identified a family of high molecular weight proteins (140–250 kDa in molecular size) as the major centrosomal antigens (13). However, this serum was complex as shown by noncentrosomal immunofluorescent staining on fixed cells and by antibodies that bound to noncentrosomal proteins from a whole-cell extract. These proteins formed a small set of noncentrosomal immunoreactive proteins that included a 36-kDa protein as its major component (13).

Although polyspecificity might be expected from a nonimmune serum, we decided to explore the antigenic relationship between the 36-kDa cellular antigen and the characterized centrosomal antigens. We demonstrate here that the immunoglobulins affinity purified with the 36-kDa protein decorate centrosomes in situ and react with the high molecular weight antigens of the isolated centrosomes. The 36-kDa protein is shown to be a cytosolic protein and identified as a subunit of lactate dehydrogenase (LDH).

MATERIAL AND METHODS

Material. Human erythrocyte glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and LDH and rabbit muscle LDH were from Sigma. Rabbit muscle GAPDH was a generous gift from F. Bernier-Valentin and B. Rousset. Staphylococcus aureus V8 protease was from Sigma.

Cell Culture. Human monolayer cell cultures used were Hela, D-17, SA4, SA39, SA44, SA45, SA101, SA52, SA72, and SA87 (14, 15). The unattached human cells used were the KE37 cell line of T-lymphoblastic origin (16). Mouse and hamster monolayer cell cultures used were 3T3, C1 1D, NIE 115, CHO, and BHK-21.

Serum. In the course of a study on the inflammatory process, eight rabbits were injected with Streptococcus type 24 (17). When tested on cultured cells from various origins by the indirect immunoperoxidase technique, the preimmune serum of one rabbit (serum 0013) was shown to strongly label centrosomes and weakly label nucleoli in primate cells (12). The experimental immunization induced a strong and transient increase of this preimmune specificity. The fact that cellular labeling did not disappear after extensive preabsorption of the serum with Streptococcus type 24 showed that these specificities were antigenically unrelated to the Streptococcus (17). Moreover, these specificities were not detected in the sera of the seven rabbits immunized in the same way.

Immunofluorescence Microscopy. Cells were fixed in PBS (150 mM NaCl/10 mM sodium phosphate, pH 7.4) containing 3% (vol/vol) formaldehyde for 30 min at room temperature and then in methanol for 6 min at −20°C. The antibodies were diluted in PBS containing 3% (vol/vol) bovine serum albumin. All the washing steps were performed in PBS containing 0.1% Tween 20.

Cell Fractionation. Cells were Vortex mixed briefly (1 min) in 1% Nonidet P-40, 0.5% sodium deoxycholate in TNE buffer (10 mM Tris-HCl, pH 7.4/10 mM NaCl/5 mM MgCl2) containing 1 mM phenylmethylsulfonyl fluoride. The detergent-insoluble cell fraction (P1), which contains nuclei and perinuclear cytoskeleton (18), and the detergent-soluble fraction (S1) were recovered after centrifugation at 300 × g for 7 min. Alternatively, cells were mechanically disrupted in

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase.

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TNM buffer using a tightly fitting Dounce homogenizer. After centrifugation at 100,000 × g for 30 min at 4°C, a pellet fraction (P2) and a cytosolic fraction (S2) were recovered.

**Centrosome Isolation.** The method of Mitchison and Kirschner (19) was modified and will be detailed elsewhere (unpublished results). The main modifications were the addition of a reaction mixture of antiproteases during the lysis, a DNase II treatment of the lysate supernatant, and a direct sedimentation of the lysate on a discontinuous sucrose gradient. Fractions were assayed as described by Mitchison and Kirschner (19) after sedimentation on a glass coverslip and fixation in methanol at −20°C. The final yield was between 50 and 70%, assuming one pair of centrioles per cell.

**Immunoblots.** Gel electrophoresis analysis of each proteic fraction was performed according to Laemmli (20). After electrotransfer to nitrocellulose filters (21, 22), proteins were autoradiographically visualized in situ with a 1:500 dilution of serum 0013 followed by radiiodinated protein A (Amersham). Antibodies were affinity purified on nitrocellulose strips according to Olmsted (23), as modified by Krohne et al. (24).

**Limited Proteolysis.** Mild proteolytic digestion with *S. aureus* V8 protease was performed according to Cleveland et al. (25) using three concentrations of enzyme (0.2, 0.5, and 2 μg/mg) and a 90-min incubation at room temperature.

**Immunoprecipitation Experiments.** KE37 cells (2 × 10⁶ cells per ml) were incubated for 4 hr in RPMI 1640, without methionine (Seromed, Strasbourg, France) containing 0.2 mCi/ml of [35S]methionine (specific activity, 1000 Ci/mmol; 1 Ci = 37 GBq; Amersham). Cells were washed twice in PBS containing 5 mM iodoacetamide and then lysed in 200 μl of a buffer containing 0.1 M Tris, pH 8.3, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, aprotinin at 0.1 mg/ml, leupeptin at 2 μg/ml, pepstatin at 1 μg/ml, and 0.5% NaDodSO₄. After sonication the NaDodSO₄ concentration was lowered to 0.1% by dilution in the same buffer. Nonidet P-40 and sodium deoxycholate were added to give a final concentration of 0.5% each. Before mixing, both cellular lysate and antibodies were separately centrifuged at 12,000 × g (20 min, 4°C). Another centrifugation was performed after a 16-hr incubation at 4°C. Antigen–antibody complexes were recovered after a 2-hr incubation at 4°C of the sample with 25 μl (packed bead volume) of protein A-conjugated Sepharose 4B (Pharmacia). The immunoadsorbent beads were washed five times with the immunoprecipitation buffer, and the immunoprecipitated proteins were eluted from the immunoadsorbent by incubating the Sepharose beads for 5 min, at 100°C in NaDodSO₄ sample buffer (20).

**RESULTS**

**Antigens in KE37 Cells Recognized by Serum 0013.** When blots of proteins from purified centrosomes were probed with serum 0013, high molecular weight proteins were detected together with doublet bands at 60–65 kDa (13). When cellular proteins were probed with the same serum, in addition to the 60- to 65-kDa centrosomal component, three noncentrosomal proteins with molecular weight of 80, 75, and 36 kDa were detected. The 36-kDa component appeared to be the major cellular antigen labeled with 0013 immunoglobulins (Fig. 1). None of these three proteins appeared to be an oligomer since no new bands were seen in the absence of reducing agent (data not shown). Cell disruption in the presence or absence of nonionic detergent showed that the 36-kDa antigen was the only true cytosolic protein. The other antigens could be localized to cellular structures in addition to the centrosome—i.e., the 80-kDa protein was localized to the nucleoli (26) and the 75-kDa protein to the reticular cytoplasm (see below). Moreover, two-dimensional gel analysis revealed that the 36-kDa antigen was an abundant protein compared to the other antigens (data not shown).

The 36-kDa Antigen and Centrosomal Proteins Are Antigenically Related. Using the approach of Olmsted (23) as modified by Krohne et al. (24), affinity-purified immunoglobulins binding to the 36-kDa antigen were tested both on fixed cells and immunoblots. These immunoglobulins were shown to stain centrosomes and nucleoli in situ without staining the cytoplasmic reticulum observed with the whole serum (compare Fig. 2A and B). When total cellular proteins or centrosomal proteins were electrophoresed and blotted onto nitrocellulose filters, the affinity-purified anti-36-kDa immunoglobulins reacted with the same antigens identified by whole serum except for the 75-kDa antigen (Figs. 2E and 3D). A similar result was obtained with the immunoglobulins purified using the 80-kDa and the 65- to 60-kDa antigens (data not shown). By contrast, affinity-purified immunoglobulins isolated with the 75-kDa antigen stained only a reticular cytoplasmic network in cells (Fig. 2C) and reacted only with itself on immunoblots (Fig. 2E). From this, we concluded that the 36-kDa cytosolic antigen shares one (or several) epitope(s) with several centrosomal [and one nuclear (26)] proteins.

The 36-kDa Antigen Is the Subunit of the Human LDH. In an attempt to identify the 36-kDa antigen, we examined whether the 36-kDa protein shared other properties with centrosomal proteins, such as the capacity to interact with microtubules or tubulin. Several laboratories have reported the copurification of tubulin and the 35-kDa subunit of...
FIG. 2. Affinity-purified immunoglobulins using immunoreactive proteins from KE37 cells was tested by immunofluorescence on Hela cells fixed with the formaldehyde/methanol method (A-D) and by immunoblots containing KE37 cellular proteins (E). (A) Cytochemical staining with the whole serum. Three types of staining can be observed. A centrosomal staining (long solid arrow), a nuclear, speckled staining (small solid arrow), and a weak, reticular cytoplasmic staining (long, dotted arrow). (B) The 36-kDa affinity-purified immunoglobulins stained centrosomes (long arrows) and nuclei (short arrows). Note the total absence of the reticular cytoplasmic labeling. (C) The 75-kDa affinity-purified immunoglobulins stained only the cytoplasmic reticular network (dotted arrows). Note the total absence of centrosomal and nuclear staining. (D) Control experiment. “Immunoglobulins” were eluted from nonreactive zone on the nitrocellulose filter. (Bar = 10 μm.) (E) Immunodetection of detergent-soluble proteins with the whole serum (lane 1), with the 75-kDa affinity-purified immunoglobulins (lane 2), and with the 36-kDa affinity-purified immunoglobulins (lane 3). Note that the latter bind to all the reactive bands revealed by the whole serum except the 75-kDa protein, whereas the 75-kDa affinity-purified immunoglobulins bind only to the 75-kDa protein. The arrowhead on the top points to a high molecular weight signal revealed by the 36-kDa affinity-purified immunoglobulins in lane 3 that is erratically observed with the whole serum (lane 1). It is likely to correspond to high molecular weight centrosomal proteins (see Fig. 3).

FIG. 3. Immunodetection of centrosomal proteins with 36-kDa affinity-purified immunoglobulins. (A and B) Centrosome preparations, used as a source of proteins, were observed by double immunofluorescence using serum 0013 (A) or a monoclonal anti-tubulin antibody (B) (Amersham). Mixed rhodamine-conjugated goat anti-mouse (Cappel Laboratories, Cochraneville, PA) and fluoresceine-labeled sheep anti-rabbit (Institut Pasteur Production, Marne-la-Coquette, France) secondary antibodies were used. Note that the isolated centrosomes maintain a paired configuration. Note also that serum 0013 stains the pericentriolar material. (×3000.) (C) Ultrastructural aspect of an isolated centrosome. (×40,000.) (D) Lane 1: centrosomal proteins (5 μg) stained with Coomassie blue. Lanes 2 and 3: Immunoblots of centrosomal proteins using 36-kDa affinity-purified immunoglobulins (lane 2) or whole serum (lane 3).

GAPDH and suggested that GAPDH mediates the lateral interactions between microtubules (27).

Two independent experiments designed to compare the 36-kDa protein with GAPDH gave conflicting results. (I) GAPDH from human erythrocytes reacted strongly with serum 0013 (Fig. 4A), whereas GAPDH from rabbit muscle
protein did not (compare blots a and b in Fig. 4A). (ii) However, when \textsuperscript{35}S-radiolabeled proteins from KE37 cells were immunoprecipitated with serum 0013 or with an anti-human GAPDH serum and hydrolyzed with \textit{S. aureus} V8 protease, different peptides were obtained with human GAPDH and the 36-kDa antigen that showed the two proteins were not related (Fig. 3B). Moreover, the two proteins appeared different even in terms of molecular weight (Fig. 4B Inset). This discrepancy was resolved when use of a more discriminating gel method resolved a contaminant migrating slightly ahead in the human GAPDH preparation (Fig. 4A, blot c). Serum 0013 clearly reacted with the contaminant but did not cross-react with the major band of human GAPDH preparation (Fig. 4A, blot d), whereas an anti-human GAPDH serum stained only the major band (Fig. 4A, blot e), demonstrating the absence of cross-reaction between GAPDH and the contaminant. The 0013 immunoglobulins affinity-purified using the contaminant stained centrosomes (and nucleoli) on fixed cells (data not shown). Finally, the protein contaminant of the GAPDH preparation was shown by mild proteolysis to be identical to the 36-kDa protein (data not shown).

According to the affinity-purification protocol used by the supplier, we knew that human GAPDH could be contaminated by other dehydrogenases. A likely candidate was LDH, the subunit molecular size of which is 33.5 kDa (28). That the 36-kDa antigen was indeed the LDH subunit was shown by mild proteolysis (Fig. 4B and C). Accordingly, serum 0013 strongly reacted with purified human LDH on immunoblots but not with rabbit LDH, and the preincubation of serum 0013 with human LDH abolished centrosomal staining (data not shown).

We have, therefore, demonstrated that in human species, the major antigenic determinant of serum 0013 is shared by centrosomal proteins, a nucleolar protein (26), and the glycolytic enzyme LDH. As serum 0013 has a strong specificity for human or monkey cell lines, the question arises as to the species-specific distribution of this determinant on centrosomes and in the cytosol. In other words, does serum 0013 recognize LDH in species where no centrosomal (and nucleolar) labeling is observed?

The Major Antigenic Determinant of Serum 0013 Is Present in Rodent Cells. Indirect immunofluorescence staining of rodent cells from different origins with serum 0013 showed an important and homogeneous staining of the whole cell. Treatment of cells with detergent before fixation removed only part of this staining (data not shown). In immunoblots containing either detergent-soluble or -insoluble proteins a

![Fig. 4](image-url)  Identification of the 36-kDa antigen recognized by serum 0013 as the subunit of the human LDH. (A) Immunodetection of human (H) and rabbit (R) GAPDH by immunoblot after 8.75% (blots a and b) or 15% (blots c, d, and e) NaDodSO_4/PAGE. Gels a and c, Coomassie-blue stained gel. Blots b and d, immunoblot using serum 0013. Blot e, immunoblot using an anti-human GAPDH. Note that in 8.75% NaDodSO_4/PAGE, human GAPDH specifically reacts with serum 0013 and migrates as a single band (large arrowhead), whereas in 15% NaDodSO_4/PAGE a contaminating protein (small arrowhead in blot c), migrating slightly ahead of the major GAPDH band (middle arrowhead in blot c), is the only one to react with serum 0013 (blot d). GAPDH and the contaminant are distinct proteins as shown by the specific binding of the anti-human GAPDH to GAPDH alone. (B) Mild proteolysis analysis (0.5 μg of \textit{S. aureus} V8 protease for 90 min at room temperature) of GAPDH and 36-kDa antigen immunoprecipitated, respectively, with anti-human GAPDH and with serum 0013 (Ag 36) from the same biosynthetically labeled KE37 cells. The molecular size difference between these two proteins is clearly seen in the autoradiogram of immunoprecipitates before proteolysis (Inset). (C) Silver-stained blot of human LDH proteolytic products (0.5 μg of \textit{S. aureus} V8 protease for 90 min at room temperature) obtained in a separate experiment.

![Fig. 5](image-url)  Identification of antigens binding to 0013 immunoglobulins in rodent cell lines. Cells were disrupted and detergent-insoluble (lanes 1 and 3) and detergent-soluble (lanes 2 and 4) proteins from 3T3 (lanes 1 and 2) and BHK-21 (lanes 3 and 4) cells were analyzed on immunoblots with serum 0013. For each cell line a single band at 70 kDa appears to be stained in both fractions.
single or double heavily labeled band was observed at an apparent molecular size of 70 kDa (Fig. 5). Affinity-purified immunoglobulins purified using these bands labeled both centrosome and nucleolus on human cells (data not shown). The absence of the immunoreactive 36-kDa protein in rodent cell lines agrees with the absence of reactivity of serum 0013 with rabbit LDH (see above).

**DISCUSSION**

Nonimmune sera have proved to be useful cytological tools to probe cellular structures (1–6). The molecular analysis of the antigens is, however, sometimes complicated by the polyclonality of these sera. To be conclusive, the identification of an antigen must rest not only on its immunoreactivity with the serum, but also on an independent demonstration of its presence in the cellular structures decorated by the serum. In this way, using a nonimmune rabbit serum known for its reactivity against centrosomes in human cells (12), we have identified high molecular weight proteins that were highly enriched in centrosome preparations, whereas other immunoreactive proteins were shown to be noncentrosomal (13). Among the latter, the major antigen had a molecular size of 36 kDa.

We have shown in this work that immunoglobulins affinity-purified using the 36-kDa antigen decorate centrosomes in fixed cells and react with centrosomal high molecular weight proteins on immunoblots. This demonstrates an antigenic relationship between the 36-kDa antigen and the centrosomal proteins. Immunoglobulins purified using another noncentrosomal antigen (75 kDa) did not stain centrosomes on cells and did not react with centrosomal proteins, providing a negative control for this technique. The 36-kDa protein was shown to be an abundant cytosolic protein and was identified as a subunit of human LDH.

Therefore, centrosomal proteins and LDH appear to share one (or several) antigenic determinants. In nine human cell lines analyzed in addition to KE37, the centrosomal labeling was always accompanied by a strong labeling of LDH. Conversely, the absence of centrosomal labeling in rodent cells was always shown (in six experiments) to be correlated with the absence of LDH labeling, suggesting a possible evolutionary relationship between some centrosomal proteins and this important enzyme of the glycolytic pathway. Molecular characterization of centrosomal antigens must be further investigated to identify the extent of homology between these proteins and LDH, and thus the physiological significance of the intriguing result reported in this work can be fully appreciated.

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