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An abundant and ubiquitous homo-oligomeric ring-shaped ATPase particle related to the putative vesicle fusion proteins Sec18p and NSF

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We have discovered a ring-shaped particle of 12.5 nm diameter, 14.5S and apparent molecular weight of ~570 000 that displays 6-fold radial symmetry and is composed of a single kind of an acidic (pI ~5.5) polypeptide of M_r 97 000 (p97). Using antibodies to this protein we have detected its occurrence in a wide range of cells and tissues of diverse species from frog to man, including highly specialized cells such as mammalian erythrocytes and spermatozoa. In *Xenopus laevis* ooctyes, the particle is found in both isolated nuclei and in manually enucleated ooplasms, which corresponds to immunofluorescence staining dispersed over both nucleoplasm and cytoplasm. The particle has a *N*-ethylmaleimide (NEM)-inhibitable Mg^{2+}-ATPase activity, and its amino acid sequence, as deduced from cDNA clones, displays considerable homology to the mammalian NEM-sensitive fusion protein (NSF) and yeast Sec18p believed to be essential for vesicle fusion in secretory processes, indicating that these three proteins belong to the same multigene family.

Key words: ring-shaped particles/protein homo-oligomers/ATPases/Sec18p/NSF

Introduction

Post-ribosomal ('S-100') fractions of prokaryotic and eukaryotic cells are known to contain a number of oligomeric protein particles which are sufficiently large to be visualized by electron microscopy. Such particles have been categorized according to the symmetry of subunit organization as cyclic, dihedral or cubic (Klotz *et al.*, 1975), and those of the cyclic type usually appear as polygons (e.g. pentamers, hexamers, heptamers, etc.) or rings. Some of the ring-like protein oligomers occurring in 'S-100' fractions of different cells have gained special attention because of their characteristic structure and their widespread occurrence. Well known examples of bacterial origin are the transcription termination factor 'rho' (Roberts, 1969; Oda and Takanami, 1972; Bear *et al.*, 1988) and the 'bacteriophage head assembly factor' groE (Hendrix, 1979; Hohn *et al.*, 1979), which has recently been shown to be a member of a large protein family of chaperonins with relatives in chloroplasts and mitochondria of eukaryotic cells (Hemmingsen *et al.*, 1988; McMullin and Hallberg, 1988; Hutchinson *et al.*, 1989).

In eukaryotic cells ~20S cylinder particles which are composed of a characteristic set of polypeptides of M_r 19 000 – 36 000 have attracted particular interest because of their occurrence in both the nucleus and the cytoplasm of different cell types of such diverse organisms as plants (Kremp *et al.*, 1986), yeast (Arrigo *et al.*, 1987; Kleinschmidt *et al.*, 1988), insects (*Drosophila*; Schuldt and Kloetzel, 1985; Arrigo *et al.*, 1985), amphibians (Kleinschmidt *et al.*, 1983) and man (Domae *et al.*, 1982; Kleinschmidt *et al.*, 1983; Schmid *et al.*, 1984), suggesting that they perform a fundamental function in eukaryotic cells. These ~20S particles have also been called 'prosomes' as RNA was found in them by some authors (Schmid *et al.*, 1984; Schuldt and Kloetzel, 1985) but not by others (Harmon *et al.*, 1983; Kleinschmidt *et al.*, 1983; for a review, see Kloetzel, 1987). More recently, protease activity was detected in these particles (Hough *et al.*, 1987; Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Kleinschmidt *et al.*, 1988; Tanaka *et al.*, 1988) and they were therefore referred to as 'proteasomes' by some authors. However, their precise cellular function is still obscure.

In analyses of nuclear 'S-100' extracts of *Xenopus laevis* oocytes we have noticed an abundant M_r 97 000 polypeptide (p97) sedimenting slower than ~20S particles, i.e. in the 14–15S range. Examinations of the purified protein by electron microscopy showed ring-like particles of a distinct appearance. We have characterized these 14.5S particles and p97 by electron microscopical and biochemical methods, respectively, studied their species and subcellular distribution by immunological techniques, and identified it, on the basis of its amino acid sequence, as a member of a protein family of the 'vesicle fusion proteins' which includes the *N*-ethylmaleimide(NEM)-sensitive fusion protein (NSF) (Wilson *et al.*, 1989) and Sec18p (Eakle *et al.*, 1988).

Results

Purification of p97

When proteins obtained in supernatants after high speed centrifugation (100 000 *g*, 1 h) of isolated nuclei from *X. laevis* oocytes were fractionated by sucrose gradient centrifugation, followed by SDS–PAGE, we consistently observed a prominent M_r 97 000 polypeptide (p97) which caught our attention because of its high S value (peak 14.5S; Figure 1a and b). A polypeptide of identical M_r and S value was recognized in high speed supernatants ('S-100' extracts) from *Xenopus* liver (data not shown) and from manually enucleated cytoplasms of *Xenopus* oocytes (Figure 1c), indicating that the polypeptide occurs in both the nucleoplasm and the cytoplasm and in oocytes as well as in somatic cells.

When we pre-fractionated 100 000 *g* supernatants of isolated nuclei of *Xenopus* oocytes by ammonium sulphate (AS) precipitation and analysed the precipitate formed at 40% AS by sucrose gradient centrifugation we obtained a 14.5S peak which was enriched in p97 (data not shown). Chromatography of this peak material on a Mono-Q column resulted in a fraction containing almost pure p97, as judged by SDS–PAGE (data not shown) and electron microscopy.

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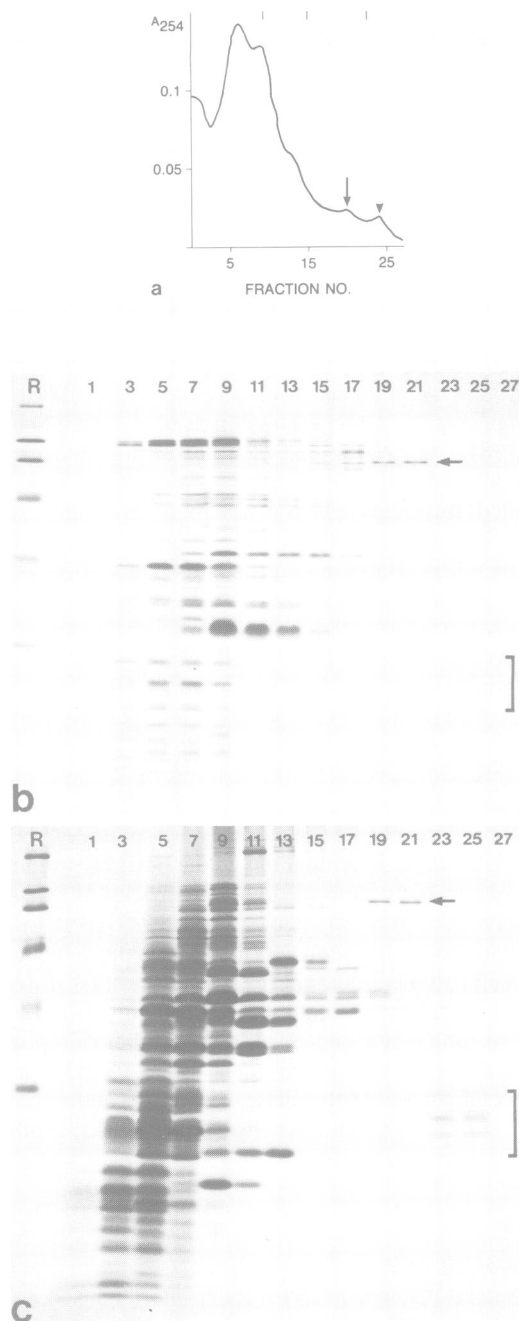

Fig. 1. Identification of the 14.5S particle forming protein p97 in 100 000 *g* supernatant fractions from isolated nuclei (**a**,**b**) and cytoplasms (**c**) of *Xenopus laevis* oocytes as obtained by sucrose gradient centrifugation. (a) Absorbance profile (A_{254}, absorbancy at 254 nm) of fractionated nuclear proteins, showing a peak in the 14−15S range (arrow) jut above the peak of the ~20S particles (arrowhead). Vertical bars indicate, from left to right, the positions of BSA (4.5S), catalase (11.3S) and thyroglobulin (16.5S). (b) Coomassie blue stained nuclear proteins fractionated as shown in (a) and separated by SDS−PAGE, revealing p97 (arrow) in the fractions corresponding to the 14.5S peak of (a). Polypeptide bands seen in lane R contain, from top to bottom, myosin heavy chain (M_r 223 000), β-galactosidase (M_r 116 000), phosphorylase b (M_r 97 400), BSA (M_r 68 000), ovalbumin (M_r 43 000), and carbonic anhydrase (M_r 29 000), used as reference proteins. (c) Coomassie blue stained cytoplasmic proteins fractionated in the same manner as the nuclear proteins in (a) and (b), showing p97 (arrow) in the 14−15S range. Lane R, reference proteins as in (b). Brackets in (b) and (c) indicate the position of the polypeptides of the ~20S particles.

Assuming that p97 occurs in the nucleus and the cytoplasm of *Xenopus* oocytes, a purification procedure for large-scale preparation starting from whole ovaries was designed. The 100 000 *g* supernatant fractions of homogenized ovaries were prepared and frozen until use. Freezing and thawing resulted in the precipitation of ~40% of total protein, as judged by absorbance at 280 nm, and >90% of p97 was recovered in the clear supernatant obtained after centrifugation (estimated from immunoblot results using antibodies to p97, followed by densitometric scanning of the autoradiograms). The clarified supernatant was passed through a DEAE−Fractogel column and the fractions obtained by elution with a salt gradient were analysed by SDS−PAGE (Figure 2b). Analysis of pooled fractions by sucrose gradient centrifugation showed that the fractions eluting between ~280 and ~295 mM KCl were greatly enriched in p97 which sedimented at 14.5S (Figure 2c). The relative amount of nucleic acids present in this fraction was <0.5% of dry mass, as estimated from the absorbance at 280 and 260 nm. From a typical preparation starting with five to eight ovaries (~100 ml) we were able to purify 3−4 mg of p97 (Figure 2c, lane 19). In some experiments, additional chromatography on a Mono-Q column, followed by another sucrose gradient centrifugation, was applied to achieve purification to homogeneity (Figure 2d).

Biochemical characterization of p97

Two-dimensional gel electrophoresis of the purified protein showed that p97 had an isoelectric pH (in 9.5 M urea) of ~5.5 (Figure 2e). Gel filtration on a Superose 12 column revealed an apparent molecular weight of ~570 000 ± 10 000 which indicates an oligomeric state. Two-dimensional separation of tryptic peptides of [^{125}I]p97 resulted in a peptide map (Figure 2f) which was different from a number of other, similarly fixed proteins from the same cell type. SDS−PAGE of p97 immunoprecipitated from a 100 000 *g* supernatant prepared from oocytes labelled *in vivo* with [^{32}P]phosphate, using affinity-purified p97 antibodies, showed that this polypeptide was phosphorylated (data not shown).

When recoveries of p97 after differential centrifugation were determined, ~70% was found in the pellet formed after one high speed centrifugation of total ovary material, compared to 30% in cultured kidney epithelial A6 cells and <10% in the case of isolated oocyte nuclei. As we could recover additional amounts of p97 in supernatants after repeated resuspension and centrifugation, we think that most of this protein is contained in 'free', i.e. not constitutively structure bound, 14.5S particles.

When testing our purified 14.5S particle fractions for several enzyme activities, especially kinase and nucleotidase activity, we detected an ATPase activity which was dependent on Mg^{2+}, the amount of p97 added, pH and temperature. The specific activity was ~5 μmol/mg/h when measured in the presence of 5 mM ATP, 10 mM Mg^{2+} at pH 9.0 and 27°C. Under the same conditions, a lower GTPase activity could be measured (~1.5 μmol/mg/h). The activity could be inhibited or significantly reduced by addition of Ca^{2+}, EDTA, KNO_3 or by treatment with NEM (Table I). Lowering the pH from 9.0 to 7.0 reduced the activity to 22.7±1.3% of the control. The influences of several ATPase inhibitors are shown in Table I.

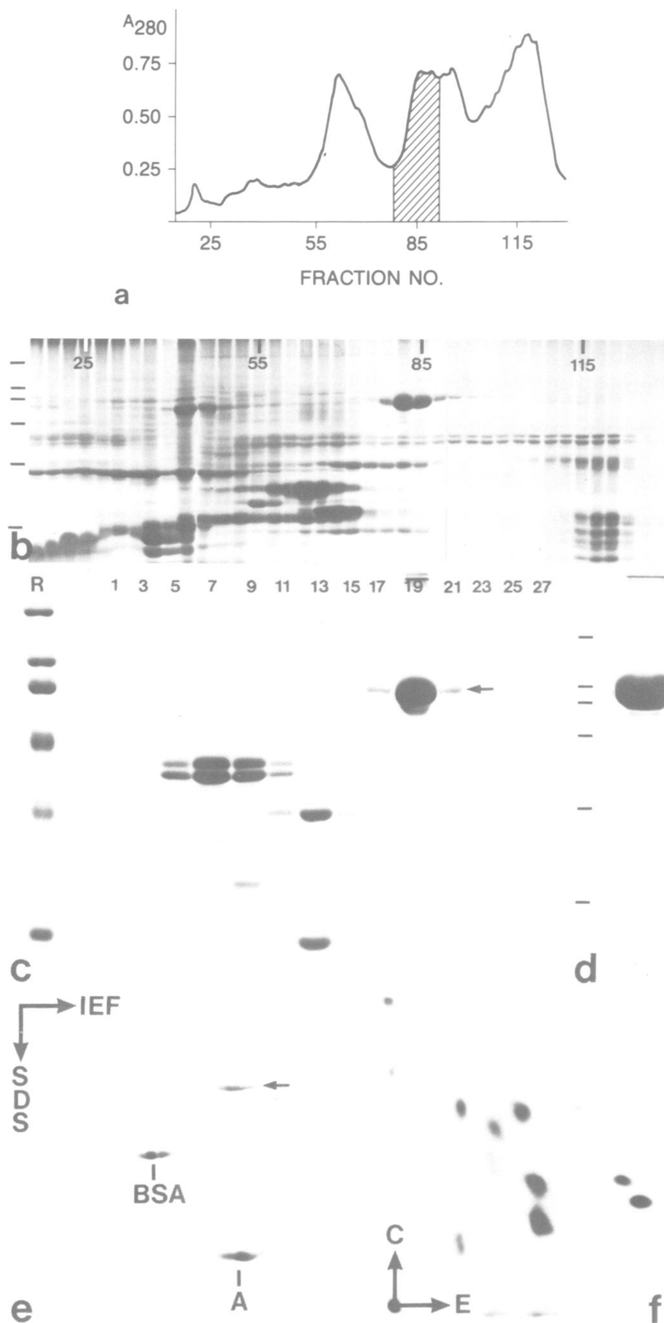

Fig. 2. Purification and biochemical characterization of p97. (**a**) Absorption profile (A_{280}, absorbancy at 280 nm), showing the result of the fractionation of 100 000 *g* supernatant proteins from *X. laevis* ovaries by DEAE–Fractogel chromatography. The hatched area indicates fractions containing p97. (**b**) Coomassie blue stained proteins after SDS–PAGE analysis of the fractionation shown in (a). Lane R, reference proteins as in Figure 1(b). (**c**) Coomassie blue stained proteins after SDS–PAGE analysis of fractions obtained by sucrose gradient centrifugation of DEAE–Fractogel fractions containing p97. p97 is denoted by an arrow. Bars indicate the positions of reference proteins as in Figure 1(b). (**d**) Coomassie blue stained proteins after SDS–PAGE analysis showing the purified p97 obtained after Mono-Q chromatography and another sucrose gradient centrifugation of the p97 containing fractions shown in (a), (b) and (c). (**e**) Coomassie blue staining of a purified fraction containing p97 analysed by two-dimensional gel electrophoresis (first dimension, isoelectric focussing, IEF; second dimension, SDS–PAGE) using BSA and α-actin (A) as reference proteins. (**f**) Autoradiograph showing a two-dimensional tryptic peptide map analysis of radio-iodinated p97 (first dimension, electrophoresis, E; second dimension, chromatography, C).

Table I. Effect of divalent ions and ATPase inhibitors on ATPase activity in the purified 14.5S particle fraction

Addition	Concentration	[γ-^{32}P]ATP hydrolysed (percentage of control)
Control		100
Divalent ions		
EDTA (no $MgCl_2$)	5 mM	7.7 ± 0.7
$CaCl_2$ (no $MgCl_2$)	10 mM	2.5 ± 0.2
ATPase inhibitors		
molybdate	250 μM	93.6 ± 2.2
vanadate	50 μM	91.2 ± 1.7
ouabain	1 mM	102.5 ± 6.7
KNO_3	50 mM	24.1 ± 2.9
NEM	1 mM (15 min, 0°C)	38.1 ± 1.0
NEM	1 mM (15 min, 23°C)	31.8 ± 1.5
oligomycin a	10 μg ml^{-1}	92.4 ± 0.5
NaN_3	2.5 mM	74.8 ± 1.7

ATPase activity is expressed as a percent of the control reaction which contained 10 mM $MgCl_2$ and had a pH of 9.0 (mean ± SD).

Electron microscopy

Negatively stained samples of purified fractions of the p97 were strongly enriched in 14.5S ring-like particles (Figure 3a and b show survey micrographs). The typical appearance of the negatively stained particles (Figure 3c and d) was a ring- or pinwheel-like structure with a maximal outer diameter of ~12.5 nm and a hollow core of ~1.5 nm [measured with tobacco mosaic virus (TMV) as internal size standard]. In several samples (Figure 3a), but not in thickly-stained specimens (Figure 3b), we observed, in addition, some faintly stained, similar structures (Figure 3a and d) which are either artifacts of locally insufficient heavy metal staining or represent 'footprints' of particles washed off during the staining procedure.

Many particles revealed a 6-fold symmetry when studied at higher magnification (Figure 3c and d) and this became clearer upon rotational analysis (Figure 3f–i; Markham *et al.*, 1963). While these particles were by far the most predominant in all the different preparations, very rarely a different structure was also observed: ~12.5 nm cylinders of 'stacked rings' with a long axis of ~15 nm and a short axis of ~4 nm (Figure 3e). These forms probably represent stacked rings each composed of four 14.5S particles rather than contaminating ~20S cylinder particles (cf. Kleinschmidt *et al.*, 1983), because the 20S particles were efficiently separated on the DEAE column (~20S particles appear as a distinct peak in fraction ~40 of Figure 2b). Also we could not detect any of the characteristic lower M_r polypeptides of the ~20S particles in our fractions by immunoblotting with specific antibodies (data not shown; cf. Hügle *et al.*, 1983). The ring-shaped character of the 14.5S particles was also confirmed by electron microscopy of rotary-shadowed samples (Figure 3j).

In view of the purity of our preparations, we suspected that the 14.5S particles observed by electron microscopy in our fractions were homo-oligomers of p97. Considering the gel filtration data (see above) and the hexameric appearance of the particles it seems most likely that six monomers formed one such ring-like particle.

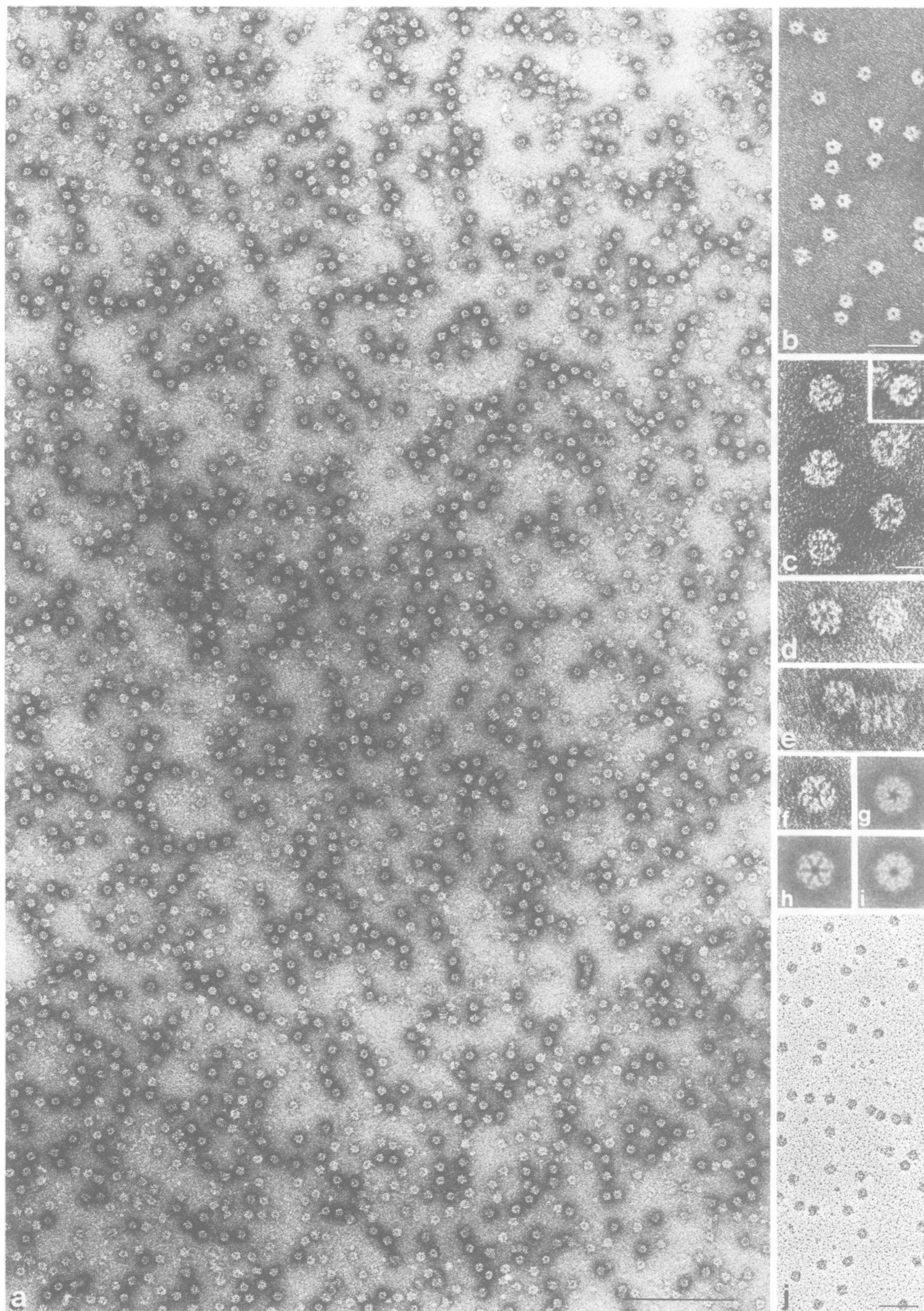

Fig. 3. Electron microscopy of negatively stained (**a–i**) and rotary shadowed (**j**) samples of purified fractions containing the 14.5S particles and p97, respectively. (a) Survey electron micrograph illustrating the purity and homogeneity of the 14.5S particle fraction prepared from 100 000 *g* supernatant of total *X.laevis* ovaries as obtained after sucrose gradient centrifugation. (b) 14.5S particles [same fraction as in (a)] embedded in a thicker staining film of uranyl acetate, clearly showing the central hollow core in each particle. (c–e) Panel of higher magnification electron micrographs, showing various negative staining aspects of the 14.5S particles: while some of the particles reveal a widely stained hollow core (insert in c) the interior of some adjacent particles is not, or only partially, penetrated by the uranyl acetate. Note also six symmetrically distributed, centripetal projections (c,d) and an occasional stack of rings (e; for discussion of significance see text). The faintly stained structure in the right half of (d) may represent a 'footprint' of a particle washed off during the staining procedure. The rotational symmetry is enhanced in a rotational analysis according to Markham *et al.* (1963; f, picture selected for analysis, $n = 0$; g, $n = 5$; h, $n = 6$; i, $n = 7$). (j) Rotary shadowing of a sample of the same fraction as in (a), illustrating the homogeneity of the preparation and the hollow core structure of the particles. Bars: 100 nm (a), 50 nm (b), 10 nm (c–i), 75 nm (j). Magnification: 81 000 × (a), 145 000 × (b), 431 000 × (c–i), 78 000 × (j).

Immunological detection of p97 in other cells and species

Guinea pig antibodies were raised against purified p97, affinity-purified and analysed for specificity by immunoblotting experiments. Immunoblotting of proteins of 100 000 *g* supernatant fractions from *Xenopus* oocyte nuclei and total ovaries showed that the affinity-purified antibodies reacted

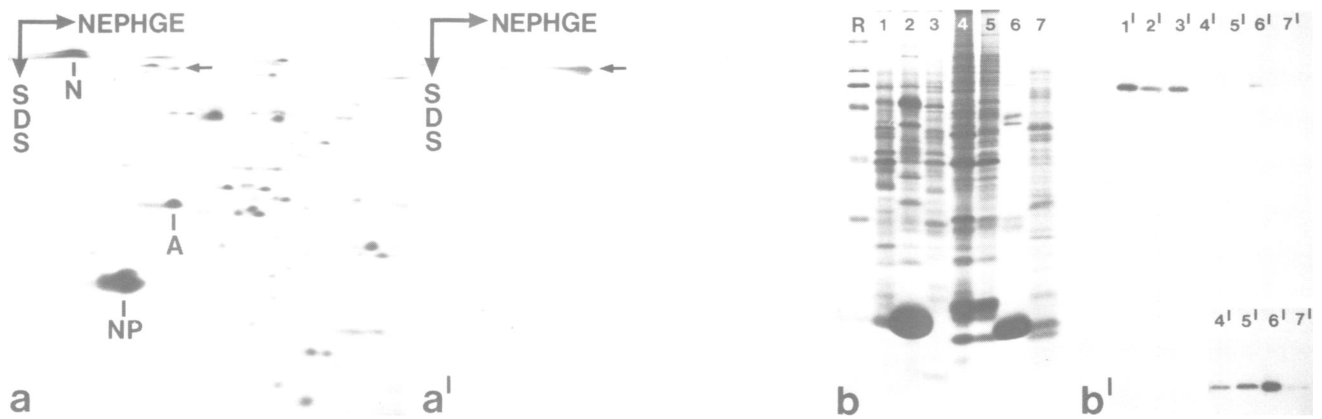

Fig. 4. Detection of p97 in different subcellular fractions and in different tissues from different species by electrophoresis and immunoblotting. (**a**) Coomassie blue staining of proteins of 100 000 *g* supernatant fractions from isolated *X.laevis* oocyte nuclei after separation by two-dimensional gel electrophoresis (first dimension, non-equilibrium pH gradient gel electrophoresis (NEPHGE); second dimension, SDS−PAGE). A, α-actin; N, N1/N2 polypeptides; Np, nucleoplasmin. (**a'**) Immunoblot of proteins separated as in (a) using affinity-purified antibodies to p97. Arrows in (a) and (a') indicate the position of p97. (**b**) Analysis of different cellular fractions by SDS−PAGE and Coomassie blue staining. Lane R, reference proteins as in Figure 1(b). Lanes 1, 2 and 3 contain proteins of 100 000 *g* supernatants of homogenized *X.laevis* ovary (1), liver (2) and cultured kidney epithelial A6 cells (3), respectively. Lanes 4 and 5 contain total extracts of cultured rat RVF cells (4) and human PLC cells (5), respectively. Lane 6 contains proteins of a 14−15S fraction from 100 000 *g* supernatant of human erythrocytes obtained by sucrose gradient centrifugation. Lane 7 contains total extract of bovine spermatozoa. (**b'**) Corresponding autoradiograph obtained after immunoblotting with affinity-purified p97 antibodies. Lanes 1'−7' correspond to lanes 1−7 in (b). The insert (lower right corner) shows the region of lanes 1'−7' that corresponds to the M_r ~ 80 000−120 000 proteins as visualized after longer exposure. Note positive reaction of p97 in all lanes.

specifically with p97 but not with any other polypeptide (e.g. Figure 4a').

From our biochemical studies we were aware that a M_r 97 000 polypeptide sedimenting at 14−15S was present not only in oocytes but also in liver cells of *Xenopus*. Using immunoblotting, a M_r 97 000 polypeptide band was also recognized in S-100 extracts from ovary and liver tissue and cultured kidney cells of *Xenopus* (Figure 4b', lanes 1'−3'), showing again that p97 occurs in oocytes as well as in somatic cells. An immunoreactive polypeptide of identical M_r value was also detected in total extracts from rabbit liver, kidney and skeletal muscle tissues (data not shown) and in extracts from cultured rat and human cells (Figures 4b' and 5'), indicating that p97 is also a common mammalian protein. We also detected the antigen in highly specialized cell types such as human erythrocytes and bovine sperm cells (Figure 4b', lanes 6' and 7'), indicating that p97 might be a molecule with 'housekeeping' functions independent of the cell's specific specialization.

p97 and the 14.5S particle were not detected in corresponding fractions from *Escherichia coli* cells. When proteins of yeast 20 000 *g* supernatants were fractionated by sucrose gradient centrifugation, and analysed by SDS−PAGE and immunoblotting three polypeptides reacted with the affinity-purified p97 antibodies, one of which had a M_r of ~120 000 and sedimented in the 14−15S range. To find out whether this polypeptide is related to p97 of *Xenopus* requires further investigation.

Using Coomassie blue staining of proteins separated by SDS−PAGE combined with densitometry and immunoblotting, we also determined the relative amounts of p97 in total cultured cells of line A6 and found that this protein was among the more abundant proteins, comprising ~14% of the amount of actin (data not shown). To estimate the proportion of p97 present in the nucleus we manually separated oocyte nuclei and ooplasms, separated the proteins by SDS−PAGE and reacted them with p97 antibodies by immunoblotting. The results showed that the concentration of this protein was similar in nucleus and cytoplasm.

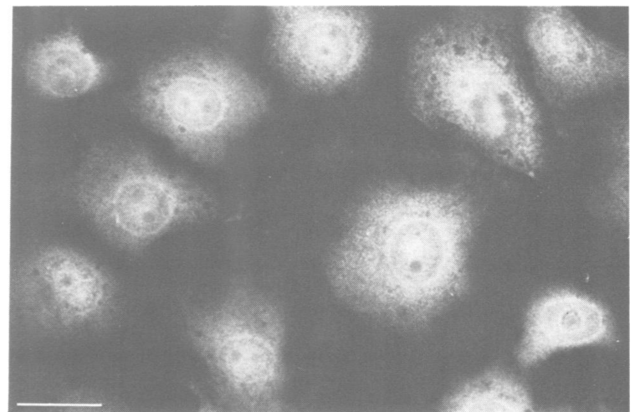

Fig. 5. Immunofluorescence microscopy, showing the localization of p97 on formaldehyde−ethanol fixed cultured kidney epithelial A6 cells of *X.laevis* using affinity-purified p97 antibodies. Bar: 25 μm.

Immunolocalization of p97 in cultured cells

Our biochemical results indicated that the majority of p97 occurred in the soluble ('S-100') protein fraction from both the nucleus and the cytoplasm of *Xenopus* oocytes (see above). To determine the intranuclear and intracytoplasmic distribution and location of the antigen we performed immunofluorescence microscopy on cultured cells, using the affinity-purified antibodies. As we were aware from studies of other soluble proteins that elution of the antigen may occur during preparation for immunofluorescence microscopy (cf. Krohne and Franke, 1980; Hügle *et al.*, 1983) we tested several fixation methods to optimize immunolocalization of p97.

After methanol−acetone fixation we observed a very weak and diffuse staining over the nucleoplasm and the cytoplasm

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X.l.    1  GAATTCTTTGGCTGTCACTATGGATGACTTCAGGTGGGGCTTAGTCAGAGTAACCCCTCGGCTCTACGAGAGACCGTTGTGGAGGTGCCG
S.s. 1516  GAAcTCccTGGCaGTtACcATGGATGACTTCcGaTGGGCCCTaAGcCAGAGcAACCCaTCaGCaCTtCGgGAaACtGTgGTaGAGGTGCCa
X.l.    1   N  S  L  A  V  T  M  D  D  F  R  W  A  L  S  Q  S  N  P  S  A  L  R  E  T  V  V  E  V  P
S.s.  443

X.l.   92  CAGGTCACATGGGAGGATATTGGGGGTTTGGAAGACGTCAAGAGGGAGCTCCAGGAGCTGGTTCAGTATCCTGTGGAGCATCCAGACAAG
S.s. 1606  CAGGTgACcTGGGAGGAcATTGGGGGcTTGGAgGAtGTCAAacGtGAGCTtCAGGAGCTGGTcCAGTATCCTGTGGAGCAcCCAGACAAa
X.l.   31   Q  V  T  W  E  D  I  G  G  L  E  D  V  K  R  E  L  Q  E  L  V  Q  Y  P  V  E  H  P  D  K
S.s.  473

X.l.  182  TTCCTGAAGTTCGGAATGACTCCATCAAAGGGTGTCCTATTCTATGGTCCACCTGGTTGTGGTAAGACTTTGCTGGCTAAGGCCATTGCC
S.s. 1696  TTCCTcAAGTTtGGcATGACACCCTCCAAGGGAGTGCTATTCTATGGACCTCCTGGCTGTGGAAAACCTTGCTGGCCAAAGCCATTGCT
X.l.   61   F  L  K  F  G  M  T  P  S  K  G  V  L  F  Y  G  P  P  G  C  G  K  T  L  L  A  K  A  I  A
S.s.  503

X.l.  272  AACGAATGCCAGGCCAACTTCATCTCCATCAAAGGGCCCGAACTTCTCACTATGTGGTTTGGAGAGTCTGAGGCCAACGTCCGAGAGATA
S.s. 1786  AATGAGTGCCAGGCCAACTTCATCTCCATCAAAGGtCCtGAgCTgCTCACcATGTGGTTTGGgGAaTCaGAGGCCAATGTtaGgGAaATc
X.l.   91   N  E  C  Q  A  N  F  I  S  I  K  G  P  E  L  L  T  M  W  F  G  E  S  E  A  N  V  R  E  I
S.s.  533

X.l.  362  TTTGATAAGGCTCGGCAGGCTGCTCCCTGTGTCCTCTTCTTTGATGAATTGGACTCCATCGCTAAGGCTCGAGGTGGGAACATTGGAGAC
S.s. 1876  TTTGAcAAGGCcCGcCAaGCTGCcCCCTGTGTaCTgTTCTTTGATGAgcTGGAtTCaATtGCcAAGGCcCGtGGTGGcAACATTGGAGAt
X.l.  121   F  D  K  A  R  Q  A  A  P  C  V  L  F  F  D  E  L  D  S  I  A  K  A  R  G  G  N  I  G  D
S.s.  563

X.l.  452  GGGGGTGGAGCTGCTGACAGAGTTATTAACCAGATCCTTACTGAGATGGACGGAATGTCTATAAAGAAGAATGTCTTCATCATCGGAGCC
S.s. 1966  GGtGGTGGgGCTGCcGACcGAGTcATcAACCAGATCtTgACaGAaATGGAtGGcATGTCcAcAAAaAAaAATGTgTTtATCATtGGcGCt
X.l.  151   G  G  G  A  A  D  R  V  I  N  Q  I  L  T  E  M  D  G  M  S  I  K  K  N  V  F  I  I  G  A
S.s.  593                                                               T

X.l.  542  ACAAACAGACCAGATATCATTGACCCCGCCATCCTGCGTCCTGGCCGTCTAGATCAGCTCATTTACATCCCGCTGCCCGATGAGAAGTCT
S.s. 2056  ACcAACcGACCtGAcATCATTGAtCCtGCCATCtTGCGaCCTGGCCGcCTtGATCAGCTCATcTACATCCCaCTtCCtGATGAGAAGTCc
X.l.  181   T  N  R  P  D  I  I  D  P  A  I  L  R  P  G  R  L  D  Q  L  I  Y  I  P  L  P  D  E  K  S
S.s.  623

X.l.  632  CGTATGGCCATCCTGAAGGCCAACCTGAGGAAGTCTCCAGTTGCCAAGGATGTGGACGTGGACTTCCTGGCCAAGATGACCAATGGTTTC
S.s. 2146  CGTgTCGCCATtCTcAAGGCCAACCTGcGcAAGTCcCCtGTTGCCAAGGATGTGGAttTGGAgTTCCTGGCtAAGATGACtAATGGcTTC
X.l.  211   R  M  A  I  L  K  A  N  L  R  K  S  P  V  A  K  D  V  D  V  D  F  L  A  K  M  T  N  G  F
S.s.  653      V                                                     L  E

X.l.  722  TCCGGAGCCGATCTGACTGAGATTTGCCAGCGAGCCTGTAAACTGGCCATCAGGGAATCTATTGAGAATGAGATCAGACGAGAGCGGGAC
S.s. 2236  TCtGGAGCtGActTGACaGAGATTTGCCAaCGtGCtTGcAAgCTGGCCATCcGaGAATCaATcGAGAgTGAGATtAGgCGAGAaCGGGAg
X.l.  241   S  G  A  D  L  T  E  I  C  Q  R  A  C  K  L  A  I  R  E  S  I  E  N  E  I  R  R  E  R  D
S.s.  683                                                                     S                    E

X.l.  812  AGGCAGACTAACCCCTCCGCTATGGAAGTGGAAGAAGACGACCCCGTACCGGAAATCCGCAGAGATCACTTTGAAGAGGCCATGCGATTG
S.s. 2326  AGGCAGACcAACCCaTCgGCcATGGAgGTaGAgGAAGAtGAtCCaGTgCCtGAgATCCGacGgGATCACTTTGAgGAaGCCATGCGtTTt
X.l.  271   R  Q  T  N  P  S  A  M  E  V  E  E  D  D  P  V  P  E  I  R  R  D  H  F  E  E  A  M  R  L
S.s.  713

X.l.  902  GCCCGCCGCTCAGTCAGCGATAACGATATCCGCAAATACGAGATGTTCGCACAAACTCTTCAGCAGAGCAGAGGATTCGGCAGCTTCAGA
S.s. 2416  GCCCGCCGtTCtGTtAGtGATAAtGAcATCCGgAAgTAtGAGATGTTtGCtCAgACcCTTCAGCAGAGtcGAGGcTTtGGCAGCTTCAGA
X.l.  301   A  R  R  S  V  S  D  N  D  I  R  K  Y  E  M  F  A  Q  T  L  Q  Q  S  R  G  F  G  S  F  R
S.s.  743

X.l.  992  TTTCCCGCTGGGGGACAAAGTGGAGCGGGTCCAAGCCCAGGAGCCGGGGGAGGCAGTGGCGGGGGCCATTTCACTGAAGAA---GACGAC
S.s. 2506  TTcCCttCaGGaaacCAAgGTGGAGCtGGaCCcAGtCagGGcagtGGcGGtGGCAcaGGtGGcaGtgtgTaCACgGAAGAtaatGAtGAt
X.l.  331   F  P  A  G  G  Q  S  G  A  G  P  S  P  G  A  G  G  G  S  G  G  G  H  F  T  E  E  -  D  D
S.s.  773         S     N     G                 Q     S           T        S  V  Y        D  N

X.l. 1079  GATCTCTATGGTTAAAGGGTGGGCCGACCCCTCCTCGTTGTACAGCCAATCGATTCCTTCGTTAATGGACACTTTGCTTTTCATTCCCCA
S.s. 2596  GAcCTgTATGGcTAAgtGGTGGtagccagCaTgCagtgaGctggcCtggctGgaTttgTtccctgaGGtgggggcgcccgCAgcgaggg
X.l.  360   D  L  Y  G. *
S.s.  803

X.l. 1169  GTCCAACTAGAAGCACCCCCACTTCTGTTCTATGATTTTGTTACACGGGTGTGAGAAGAGGGGAGCCAAGTCTCTCAGGATTCTTAAACT
S.s. 2686  acCacgggtgcgcccacggCctTggTtccaTtcctcagTcTgAacaGttcagctacAGtcaGactctggacaggggttAactgTtggaa

X.l. 1259  TTTACTTTTCTCTTCTTTCCCTCCCTGTTTCCGAAGCGGCCCCTGTGTTTGCAGAGAGTCGTCCAGGGGGGGAATGAAAGTTCTTCATCC
S.s. 2776  aaaAaTTcaaaacaaaagtgaTaaaataaaagcAAttttCattTGgGaggcggagagtgaattaccaacaGGgAattggGccTtggcTat

X.l. 1349  ACCGAGTGACTTGGGGCTCCATTACCCCCCCGTGTGGATCCTGCAGATATTTCTCTTCTTTTCACTCCTCCAGTTCAGCTGTTTGGCCTT
S.s. 2866  gCacttTctgTTGtaGtTggggcAgtgCaggGgactctgtggGtgcgaAgaaaggcatTaTTgcactCcCCAcagtAaagcaTctGCacT

X.l. 1439  CAACTGTTCATCCAAAGTGATTCTGTTGGGAAATTAATGCTGAGATTAACTGTTTTGCTGCCAGTGTGCCTGTGAATGTTTAGCTGGGCA
S.s. 2956  tcACTcaatgctgcccaTGccctcccgttcttccccTaCccAacTTgggcaggagGaTGaagGgcTcCagtTGctgGgTgtttatatag

X.l. 1529  CTATTGCAGTAAAAGGGTTAATACAGAACTAGCAGGGCCCCGGGTGTTGCAGGTTTCTCCAGCTGTGAATGGGTCGCTCCCTTCTCCTTC
S.s. 3046  agtagGttGatttttatTTtAcAtgctttTgagttaatgttGGaaaactaAttccaCaagcagTtTctAaacccaaaaatgacaTgCTgt

X.l. 1619  TGGGTATAAGAAAAGCTTAATAAAGACATAAAATACGGAATTC
S.s. 3136  aaaGgAcAAtAAAcGtTTgggtcAaAtggAAAAaAaaaAAaaaaaaaaa
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Fig. 6. Sequence comparison of the *X.laevis* p97 partial cDNA clone X.1.p97-1 (designated X.1.) and the correspondig part of the cDNA clone pcD-VQY-3-1 encoding the porcine VCP, which appears to be the mammalian orthologue of p97 (designated S.s.; taken from Koller and Brownstein, 1987). Identical nucleotides are shown as upper case letters; nucleotides in the pig sequence that differ from the *Xenopus* sequence are shown as lower case letters. The deduced amino acid sequences are shown in one letter code below the nucleotide sequences. Of the porcine amino acid sequence (bottom line), only the changes are shown. Dashes in the sequences denote omissions introduced for optimal alignment. Underlined amino acid residues have been confirmed by peptide sequence analysis.

of cultured cells from *X. laevis* provided that incubation times were kept short (data not shown). When cells were fixed with 2% formaldehyde, either prior to or after the incubation with the primary antibodies, a much stronger immunofluorescence in both the nucleoplasm and the cytoplasm was seen which also displayed the diffuse to fine granular appearance typical for many other soluble antigens (cf. Krohne and Franke, 1980; Hügle *et al.*, 1983). In most specimens the

```
NSF     228 IGGLDKEFSD IFRRAFASRV FPPEIVEQMG CKHVKGILLY GPPGCGKTLL ARQIGKMLNA REPKVVNGPE
            :::  : .   :  . ..   .   : ..  .:  : ..:::::  :::: ::::.  :: .     :    . ..::::
S.s.p97 206 IGGCRKQLAQ I-KEMVELPL RHPALFKAIG VKPPRGILLY GPPGTGKTLI ARAVANETGA -FFFLINGPE
            .::  : .   :  . ..   .   : ..  .:  .   ..:.:::  :::::::::: :: .     :    . ..::::
Sec18p  241 VGGLDKEFTK IFRRAFASRI FPPSVIEKLG ISHVKGLLLY GPPGTGKTLI ARKIGTMLNA KEPKIVNGPE

NSF     298 ILNKYVGESE ANIRKLFADA EEEQRRLGAN SGLHIIIFDE IDAICKQRGS MAGSTGVHDT VVNQLLSKID
            :. :..:::: :.::.: .: :        :      ::..::  .:::   :      :   :   .: :::. .:
S.s.p97 274 IMSKLAGESE SNLRKAFEEA E-----KNA- --PAIIFIDE LDAIAPKREK THG--EVERR IVSQLLTLMD
            :.::..: :: :.: .: .: :     :      ::..::  ::  ..  :         :    .: :::  ::
Sec18p  311 ILSKYVGSSE ENIRNLFKDA EAEYRAKGEE SSLHIIIFDE LDSVFKQRGS RGDGTGVGDN VVNQLLAKMD

NSF     368 GVEQLNNILV IGMTNRPDLI DEALLRPGRL EVKMEIGLPD EKGRLQILHI HTARMRGHQL LSADVDIKEL
            :. :   ..:  . .::::   : : :: :.::. .   ..::.::    ::: :: :  ::  :.     :  :::.  .
S.s.p97 334 GLKQRAHVIV MAATNRPNSI DPALRRFGRF DREVDIGIPD ATGRLEILQI HTKNMK---- LADDVDLEQV
             . :   ..:  . .:::   : : :: :.::: .  :.: .::    ::: :.:   :: :.     . ::: :  .
Sec18    381 -VDQLNNILV IGMTNRKDLI DSALLRPGRF EVQVEIHLPD EKGRLQIFDI QTKKMRENNM MSDDVNLAEL

NSF     438 AVETKNFSGA ELEGL--VRA AQSTAMNRHI KASTKVEVDM EKAESLQVTR GDFLASLEND IKPAEGTNQE
            : ::.   ::  .:  :   :  .:       .          .:.  : . :: ::   ::   :       :
S.s.p97 400 ANETHGHVGA DLAALCSEAA LQAIRKKMDL IDLEDETIDA EVMNSLAVTM DDFRWALSQS NPSALRETVV
            :  :.   ::  ..  :   :     :: : . .           ...  : ::  .::  ::       :.  .
Sec18   450 AALTKNFSGA EIEGLVKSAS SFAINKTVNI GKGATKLNTK DIA-KLKVTR EDFLNAL-ND VTPAFGISEE

NSF     506 DYASYIMNGI IKWGDPVTRV LDDGELLV-Q QTKNSDR--T PLVSVLLEGP PHSGKTALAA KIAEESNFPF
            ... .    :      :.  .   .   ..:      :      :  :   ::. ::  :  :::..:   :: :   . :
S.s.p97 470 EVPQVTWEDI GGLEDVKREL QELVQYPVEH PDKFLKFGMT PSKGVLFYGP PGCGKTLLAK AIANECQANF
            ..      .    .  .  .           : .    :       .       .:. ::  .: :::.::   ::     . :
Sec18p  518 DLKTCVEGGM MLYSERVNSI LKNGARYV-R QVRESDK--S RLVSLLIHGP AGSGKTALAA EIALKSGFPF

NSF     573 IKICSPDKMI G-FSET-AKC QAMKKIFDDA YKSQLSCVV- VDDIERLLDY --VPI---GP RFSNLVLQAL
            : :  :. .    : :. :     .. ::: :     . ::.   .:... ..       :   :   .  .. :.:
S.s.p97 540 ISIKGPELLT MWFGESEAN- --VREIFDKA RQAAP-CVLF FDELDSIAKA RGGNIGDGGG AADRVINQIL
            : .  :  :.    . :: :    .   :  :    ..  ..   .: :....        :   :   .   : :.:
Sec18p  585 IRLISPNELS G-MSES-AKI AYIDNTFRDA YKSPLNILV- IDSLETLVDW --VPI---GP RFSNNILQML
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Fig. 7. Comparison of the central portion of the amino acid sequence deduced from the VCP cDNA clone corresponding to *Xenopus* p97 (designated S.s. p97; taken from Koller and Brownstein, 1987) with that of the putative vesicle fusion protein NSF from hamster (Wilson *et al.*, 1989) and that of Sec18p from yeast (Eakle *et al.*, 1988). Identical residues are denoted by two points and conservative exchanges by one. The underlined residues correspond to putative nucleotide binding sites of the motif A-type (for references see text). Symbols are as in Figure 6.

immunostaining appeared stronger in the nucleus than in the cytoplasm but some variation was noticed between different experiments, indicating that the relative intensity of nuclear versus cytoplasmic staining might be significantly influenced by the specific fixation and washing procedures. Similar results were obtained with cultured cells of rodent and human origin (data not shown). Immunostaining of nucleoli (Figure 5) and chromosomes of mitotic cells was greatly reduced.

Amino acid sequence data and cDNA cloning

N-terminal amino acid sequence analysis was performed with electro-eluted p97 but no sequence was obtained, indicating a blocked N-terminus. Therefore, p97 was digested with V8 protease and the fragments obtained were separated by gradient SDS–PAGE, electroblotted to Immobilon membranes, excised and sequenced (results not shown). A computer search with a 15 amino acid sequence obtained from one excised peptide (p2) in the NBRF_PIR protein database showed identity at positions 579–593 with the cDNA-deduced amino acid sequence of the so-called 'valosin-containing protein' (VCP), a molecule of 806 amino acids with a predicted molecular weight of 88 600 (Koller and Brownstein, 1987). A second excised band (p3) gave two clear amino acid sequences in approximately equimolar amounts which could be completely aligned with VCP amino acid residues 492–510 and 608–626, respectively, except for three residues corresponding to amino acids 499, 613 and 625 of VCP where unequivocal amino acid assignments were not possible. A 22 residue fragment (p4) obtained from a V8 digest of electro-eluted p97 corresponded to residues 757–778 of VCP; however, there were two differences noted: at positions 19 and 21 alanine and glycine were found instead of serine and asparagine, respectively, in the VCP sequence. Three additional sequences were obtained from digests of p97 immobilized on nitrocellulose: two V8 fragments (p5 and p6) and one tryptic fragment (p7) were identical in sequence with residues 133–144, 295–305 and 488–501 of VCP, respectively.

Therefore, we used a fragment of the cDNA coding for the VCP to screen a λgt10 library prepared from *Xenopus* embryonic mRNAs. From 199 positive clones 14 were analysed, and the one containing the largest insert (~1600 bp; designated X.1.p97-1) was cloned into M13mp18 and sequenced. The clone was 1661 nucleotides long and contained an open reading frame (ORF) coding for 363 amino acids (Figure 6). The deduced amino acid sequence contained five of the seven peptide sequences obtained from the purified *Xenopus* p97 (those derived from p2, p3, p4 and p7), con-

firming the two amino acid exchanges observed. The deduced amino acid sequence of the partial clone X.1.p97-1 was ~96% identical with the C-terminal portion of the VCP amino acid sequence, showing that the *X.laevis* p97 corresponds to the porcine VCP, which for simplicity we have termed *Sus scrofa* p97 (Figure 6). Northern blot hybridization of total RNA from *Xenopus* ovaries and cultured cells of line A6, using a probe prepared from a X.1.p97-1 fragment, showed an intense 3.6 kb band (data not shown).

In our analysis of the amino acid sequence of the *Xenopus* and the porcine p97 we observed that the sequences extending from amino acid residues 201 to 424 and 474 to 700 in the porcine p97 were very similar to each other (~45% identical amino acid residues). Both of these large repeats contain a putative nucleotide binding site of the 'motif A' type (Walker *et al.*, 1982; Möller and Amons, 1985; Fry *et al.*, 1986), the first between residues 245 and 252 and the second extending from 518 to 525 (Figure 7). In addition, we found several putative phosphorylation and autophosphorylation sites of the calmodulin kinase II, the casein kinase II and the protein kinase C types (Pearson *et al.*, 1985; Kuenzel *et al.*, 1987; Woodgett *et al.*, 1986).

Screening recent literature for other proteins involved in cellular functions requiring ATP-hydrolysis, we found that proteins Sec18p from yeast and NSF from hamster, which are both believed to be involved in secretory and endocytotic pathways (Novick *et al.*, 1980; Riezman, 1985; Block *et al.*, 1988; Eakle *et al.*, 1988; Malhotra *et al.*, 1988; Wilson *et al.*, 1989), showed significant homology to p97 from *Xenopus* and pig (Figure 7). This homology was high in the central parts of the molecules; residues 206–605 of porcine p97 are ~36% (p97 and NSF) or ~32% (p97 and Sec18p) identical. This homology is especially high in the regions surrounding the first putative nucleotide binding site (residues 234–414 of p97 from pig are ~49% identical with the corresponding part of NSF and ~40% with that of Sec18p). Like p97 from *Xenopus* and pig, Sec18p and NSF also contain a second putative nucleotide binding site (Figure 7), indicating that the catalytic domain(s) of these related molecules might have been conserved. The adjacent regions showed much less homology.

Discussion

The fraction of soluble components of the cell, also commonly referred to by the preparative term 'cytosol', contains a few major and widespread particles of discrete sizes and shapes which appear as monodisperse entities in biochemical and electron microscopical analysis. The latest addition to this group of particles has been the ~20S cylinder particle which has attracted exceptionally wide attention and controversy as it was hypothesized to represent 'aminoacyl-transferase I' (Shelton *et al.*, 1970), ribonucleoproteins ('prosomes'; Schmid *et al.*, 1984; Martins de Sa *et al.*, 1986), a heat shock protein (Schuldt and Kloetzel, 1985; see, however, also Arrigo *et al.*, 1985), a pre-tRNA 5′ processing nuclease (Castaño *et al.*, 1986), a protease complex (Hough *et al.*, 1987; Arrigo *et al.*, 1988; Falkenburg *et al.*, 1988; Kleinschmidt *et al.*, 1988; Tanaka *et al.*, 1988) and an RNase protein (Tsukahara *et al.*, 1989).

In the present study, we have detected in supernatant fractions from oocytes, cultured cells and various tissues of numerous species another widespread and abundant particle which sediments at 14.5S and appears so far to have escaped detection as a general cell component. This particle occurs in both the nucleoplasm and the cytoplasm and is also present in the cytoplasm of terminally-differentiated cells such as mammalian spermatozoa and erythrocytes. Specifically for the latter cell type, i.e. the human erythrocyte, White and Ralston (1976, 1980) as well as Harris (1984) have described an ATPase particle which seems to be identical with the 14.5S particle identified here as an apparently ubiquitous nuclear and cytoplasmic entity (p97 of human erythrocyte particles provided by Dr J.R.Harris reacted with our *Xenopus* p97 antibodies; data not shown).

Although the 14.5S particle has a similar size and overall shape to the ~20S cylinder particles it is clearly distinguished from it by its structural details, notably a slightly larger diameter and a 6-fold pinwheel arrangement of subunits, and by its protein composition: whereas the ~20S 'proteasome' particle is an exceptionally complex hetero-oligomer of 12 or more different polypeptides of relatively low M_r values (from ~19 000 to ~36 000; Kleinschmidt *et al.*, 1983; for review, see Kloetzel, 1987), the 14.5S particle is formed by a single much larger polypeptide (M_r 97 000; p97). The 14.5S particle and its constituent protein (p97) also differ from other known ring-shaped particles and particle-forming cytosolic proteins of similar sizes. Specifically glycogen synthase, which has also been reported to occur in both the cytoplasm and the nucleoplasm (Kopun *et al.*, 1982), differs by SDS–PAGE mobility (M_r ~ 88 000) and its tetrameric or dimeric organization (for reviews, see Cohen, 1978, 1986; Roach, 1986). Moreover, immunoblotting of glycogen synthase enriched from rabbit skeletal muscle did not show any reactivity with p97 antibodies (data not shown). Interestingly, however, these antibodies did react with a minor M_r 97 000 polypeptide band present as a trace contaminant in glycogen synthase and glycogen phosphorylase kinase preparations (referred to as 'component x' by Cohen, 1973; see, however, Shur *et al.*, 1986). Structurally, our 14.5S particle closely resembles the particles shown in the electron micrographs of Rebhun *et al.* (1973) which were believed by these authors to represent glycogen synthase, apparently an erroneous assignment considering the by now well-known properties of purified glycogen synthase (reviewed by Cohen, 1978, 1986; Roach, 1986).

The 14.5S particle constituent p97 is highly homologous with the M_r 88 660 polypeptide predicted from an ORF of the cDNA clone containing the sequence of the peptide 'valosin' (Koller and Brownstein, 1987). This enigmatic molecule was assumed by several groups to be a secretory peptide with stimulatory effects on cells of the gastrointestinal tract (e.g. Schmidt *et al.*, 1985; Konturek *et al.*, 1987; Sumi *et al.*, 1987). However, Koller and Brownstein (1987) rightly concluded that it was an artefactual product of a hitherto unknown cytoplasmic protein generated from a larger protein (VCP), a conclusion confirmed by Gill *et al.* (1989). The somewhat higher M_r value (97 000) estimated from SDS–PAGE of the 14.5S particle protein, compared to a value of ~89 000 as predicted from the porcine cDNA, is probably explained by special sequence features; there are numerous examples of marked differences between the M_r value estimated from SDS–PAGE mobility and the true molecular weight of certain polypeptides (for discussions, see Kaufmann *et al.*, 1984; Kleinschmidt *et al.*, 1986). We conclude that p97 is the *Xenopus* orthologue of porcine VCP, and the extremely high amino acid sequence homology

indicates that this protein has been strongly conserved during vertebrate evolution.

The abundant 14.5S particle is obviously a homo-oligomer of p97, and electron microscopy suggests a ring-like hexameric organization. It clearly has a considerable nucleotidase activity, with a specific ATPase activity in the same range as several other cytoplasmic ATPases, including myosin, but it differs in other aspects from all known classes of ATPases (for review, see Al-Awqati, 1986; Pedersen and Carafoli, 1987; Pratt, 1989; Vale and Toyoshima, 1989). In particular, the presence of two large duplicated domains in the amino acid sequence of the p97 ATPase, both containing a putative nucleotide binding site, is a feature not found in most other ATPases. Interestingly, the p97 ATPase displays a similar sensitivity to inhibitors to the membrane-bound ATPases of the V-type (reviewed in Bowman and Bowman, 1986), although it is a 'free', i.e. diffusible, component of the nucleo-cytoplasmic space. These observations suggest that p97 constituting the 14.5S particles belongs to a novel class of ATPases.

Surprisingly, the analysis of the amino acid sequence of the p97 ATPase revealed a remarkable homology to the putative vesicle fusion proteins Sec18p in yeast (Eakle *et al.*, 1988) and NSF in hamster (Wilson *et al.*, 1989), particularly in the central portion harbouring the two likely nucleotide binding sites, which are also present in Sec18p and NSF. Therefore, it is conceivable that the latter two proteins also function as ATPases. Like the p97 ATPase protein NSF also seems to assemble into homo-oligomers, as a sedimentation of ~ 12S and a Stokes radius of 6.3 nm has been reported by Block *et al.* (1988), who proposed a tetrameric organization. In view of the assumed functions of Sec18p and NSF in catalysing the fusion of transport vesicles with Golgi stacks (for discussion, see Wilson *et al.*, 1989) and the unexpected amino acid sequence homology, suggesting the existence of a multigene family of particle forming ATPases of this type, it is certainly tempting to speculate that p97—and hence the 14.5S particles—might also have membranotropic effects, be it in secretory or other processes involving vesicle formation and fusion. The observed distribution of p97 over both the cytoplasm and the nucleus and the recovery of most of the protein in cytosol fractions does not necessarily exclude a functional action on membranes. In fact, the preparative behaviour of the putative membrane fusion protein NSF also does not indicate an association with—or enrichment at—membrane structures (Block *et al.*, 1988). Clearly, the relationship between the proteins p97 and NSF dictates a new series of functional hypotheses and experiments that hopefully will help to explain the abundant and apparently ubiquitous occurrence of the 14.5S particles.

Materials and methods

Animals and cells

Clawed toads (*X.laevis*) were purchased from the South African Snake Farm (Fish Hoek, South Africa). Conditions used for culturing *X.laevis* kidney epithelial (XLKE) cells of line A6, rat vascular smooth muscle-derived fibroblastoidal RVF cells and human liver PLC carcinoma cells have been described (cf. Franke *et al.*, 1979; Knapp and Franke, 1989). Human erythrocytes were enriched from outdated blood samples obtained from the blood bank of the University of Heidelberg (FRG). Bovine spermatozoa were prepared as described (Longo *et al.*, 1987).

Cell fractionation and protein analyses

Large-scale isolation of nuclei from *X.laevis* oocytes and the preparation of 100 000 *g* supernatants have been described (Hügle *et al.*, 1985). Homogenates of *X.laevis* ovary, liver, human erythrocytes and cultured cells were fractionated in the same way. Manual isolation of nuclei and cytoplasms from *X.laevis* oocytes was done according to Krohne *et al.* (1989).

Density gradient analysis of soluble proteins was carried out using 5–30% (w/v) sucrose gradients (Hügle *et al.*, 1985) using '5:1 medium' (83 mM KCl, 17 mM NaCl, 10 mM Tris–HCl, pH 7.4), containing 2 mM dithiothreitol (DTT) and—in some experiments—1 mM phenylmethyl sulphonylfluoride (PMSF; buffer A). Bovine serum albumin (BSA), catalase and thyroglobulin (Pharmacia, Uppsala, Sweden) were used as S value reference proteins in the sample and in parallel gradients.

For gel filtration experiments, a FPLC system (Pharmacia) with a Superose 12 column, a flow rate of 0.5 ml/min, a fraction size of 0.4 ml, with buffer A containing 0.2 mM $CaCl_2$, and thyroglobulin, ferritin and catalase (Pharmacia) as reference proteins was used. Peak fractions were analysed by SDS–PAGE.

Proteins were phosphorylated *in vivo* by incubation of *X.laevis* oocytes in modified Barth's medium (Krohne and Franke, 1983) containing [^{32}P]orthophosphate (250 μCi/ml; Amersham International, Amersham, UK) for 18 h at 18–20°C, and p97 from a 100 000 *g* supernatant prepared from the labelled oocytes was isolated by immunoprecipitation.

In recovery experiments homogenates of different tissues were fractionated into 3500 *g* and 100 000 *g* pellets, and 100 000 *g* supernatants as described in Hügle *et al.* (1985), and proteins of these fractions were analysed by SDS–PAGE, immunoblotting and densitometric scanning of the autoradiographs.

ATPase and GTPase activity of 14.5S particle fractions freshly prepared by sucrose gradient centrifugation of AS-pelleted material, which had been stored at −20°C, were measured by quantitation of $^{32}P_i$ released from [γ-^{32}P]ATP or [γ-^{32}P]GTP (Amersham). p97 (0.5–5.0 μg) was added to a final volume of 50 μl of assay mixture that contained 17 mM NaCl, 83 mM KCl, 10 mM $MgCl_2$, 5 mM ATP and 20 mM Tris–HCl, pH 9. After up to 60 min of incubation at 27°C, the reaction was quenched by the addition of 250 μl ice-cold activated charcoal (0.8% w/v) suspended in 0.2 N HCl, 1 mM NaH_2PO_4. $^{32}P_i$ was separated from [γ-^{32}P]ATP or [γ-^{32}P]GTP as described by Stitt and Webb (1986) and the amount of $^{32}P_i$ released was quantitated by liquid scintillation counting of the resulting supernatant samples.

Protein purification

AS was added to the 100 000 *g* supernatant of isolated nuclei of *X.laevis* oocytes to a final concentration of 40% (w/v). The precipitate formed after 1 h at 0–4°C was resuspended in buffer A, dialysed for 30 min and fractionated by sucrose gradient centrifugation. Peak fractions were directly applied to a Mono-Q column using a FPLC system (Pharmacia) and eluted with a linear gradient (0–1 M KCl in buffer A with 1 mM $MgCl_2$) at room temperature and a flow rate of 2 ml/min. Fractions of 1 ml were collected and analysed by SDS–PAGE and electron microscopy.

For large-scale preparations, frozen 100 000 *g* supernatants of whole *X.laevis* ovaries were thawed and centrifuged for 20 min at 30 000 *g* (4°C). The pellets were discarded, and the supernatants obtained (~ 150 ml) were applied to a DEAE–Fractogel column (5 × 25 cm) which had been equilibrated with buffer A containing in addition 0.2 mM $CaCl_2$ and 60 mM KCl. After washing with the same buffer, the bound protein was eluted using a linear gradient (60–500 mM KCl in 4 l of buffer A, 0.2 mM $CaCl_2$) and a flow rate of 2 ml/min at 4°C. Fractions of 20 ml were collected. The fractions containing p97, as determined by SDS–PAGE, were pooled and precipitated at a final concentration of 80% (w/v) AS. Precipitated protein was resuspended, dialysed as described above and fractionated by sucrose gradient centrifugation. Peak fractions were analysed by SDS–PAGE and electron microscopy. For storage and further use, sucrose gradient fractions were either frozen or passed through a PD-10 column (Pharmacia) to remove the sucrose, and protein was eluted with 10 mM NH_4HCO_3 and lyophilized.

For comparison, fractions enriched in glycogen synthase from rabbit skeletal muscle (kindly provided by Dr Philip Cohen, University of Dundee, UK), 20 000 *g* supernatants from the yeast *Saccharomyces cerevisiae* (kindly provided by Dr D.Gallwitz, Max Planck Institute for Biophysical Chemistry, Göttingen, FRG) and fractions enriched in Mg^{2+}-ATPase from human erythrocytes (kindly provided by Dr James R.Harris, North East Thames Regional Transfusion Centre, Brentwood, UK) were examined.

Electron microscopy

Samples from sucrose gradient fractions were vacuum dialysed against buffer A for 30–60 min at 4°C and negatively stained as described (Kleinschmidt *et al.*, 1983). Specimens were examined using a Siemens electron microscope 101. TMV was used as an internal size standard (for TMV dimensions see Günther, 1960; Holmes, 1979). Rotational analysis of selected particles was done according to Markham *et al.* (1963). For rotary shadowing, sucrose

gradient fractions were dialysed as above and analysed as described by Franke *et al.* (1982) using carbon–platinum (95%/5%) for shadowing.

Preparation of antibodies

Antibodies were raised in guinea pigs by injecting 3×300 μg of purified p97 at days 1, 22 and 36, respectively, followed by blood collection at day 43. The serum obtained was precipitated twice with a final concentration of 24.3% (w/v) AS and antibodies were affinity purified as described (cf. Kapprell *et al.*, 1988).

Gel electrophoresis, immunoblotting and immunoprecipitation

Conditions used for SDS–PAGE were either as described by Thomas and Kornberg (1975) or according to Laemmli (1970). Two-dimensional electrophoresis was performed essentially according to O'Farrell (1975) and O'Farrell *et al.* (1977).

For peptide map analysis, polypeptide bands separated by SDS–PAGE were excised, radio-labelled with ^{125}I (Amersham) and digested with trypsin. Fragments obtained were analysed according to the method of Elder *et al.* (1977).

For immunoblotting experiments, electrophoretically-separated polypeptides were transferred to nitrocellulose membranes according to Towbin *et al.* (1979) or, alternatively, according to Kyhse-Andersen (1984). Immunoreactions of primary antibodies on the membranes were visualized using ^{125}I-labelled protein A (Amersham) or by the alkaline phosphatase method (Promega, Madison, WI, USA).

For quantitative analysis of autoradiograms or Coomassie blue stained gels the computer program Elscript 400 (Hirschmann, Munich, FRG) was used. Immunoprecipitations were done according to Krohne and Franke (1980).

Immunolocalization

Procedures used for immunofluorescence microscopy on cultured cells were as described (Hügle *et al.*, 1983) using Texas-Red conjugated goat anti-guinea pig IgG (Jackson Immuno Research, West Grove, PA, USA) as secondary antibodies. In addition two modified protocols were used. (i) In addition to methanol–acetone fixation, the cells were fixed with 2% formaldehyde in phosphate buffered saline (PBS) for 5 min at room temperature after incubation with the primary antibodies. (ii) Instead of methanol–acetone, 2% formaldehyde in 90% ice-cold ethanol was used to fix the cells (for 5 min at ~0°C).

Protein sequence analysis

Lyophilized purified p97 was dissolved in a buffer containing 20 mM Tris–HCl, pH 7.4, 2 mM DTT, 0.2% SDS, and digested with V8 protease (ICN, Lisle, IL, USA). Fragments obtained were separated by gradient SDS–PAGE as described (Walsh *et al.*, 1988) and electro-transferred to Immobilon membranes (Millipore, Molsheim, France) at 4°C in 25 mM Tris–HCl (pH 8.4) containing 0.5 mM DTT for ~7 h at 60 V. The membranes were stained with amido black and destained in water containing 2 mM DTT. Well-separated peptide bands were excised, washed several times with water containing 2 mM DTT, dried under nitrogen and directly applied to the cartridge of a pulsed liquid sequencer (model 477A; Applied Biosystems, Foster City, CA, USA). p97 was also blotted to nitrocellulose and digested on the membrane with either staphylococcal V8 protease (ICN) or sequencing grade trypsin (Boehringer, Mannheim, FRG) as described (Aebersold *et al.*, 1987). The peptides obtained were separated by HPLC using a Brownlee C4 column (100 × 2.1 mm) and selected peaks were rechromatogaphed using a Brownlee C8 column (100 × 2.1 mm) and a 130A HPLC separation system (Applied Biosystems). The buffer used for rechromatography was 20 mM sodium acetate–water as solvent A and 15 mM sodium acetate in 80% acetonitrile–water as solvent B. For N-terminal amino acid sequence determination, samples containing predominantly p97 were separated by SDS–PAGE, p97 was electro-eluted, chloroform–methanol-precipitated according to Wessel and Fluegge (1984) and sequenced.

Isolation and characterization of cDNA clones

A cDNA library in λgt10 which had been prepared with poly(A)$^+$ RNA from stage 17 embryos of *X.laevis* (kindly provided by Dr D.A.Melton, Harvard University, Cambridge, MA, USA) was screened with a hybridization probe prepared with a random priming kit (Boehringer) from a gel-purified fragment of the cDNA clone pcD-VQY-3-1 (Koller and Brownstein, 1987; kindly provided by Dr Kerry Koller, Genentech Inc., South San Francisco, CA, USA). The insert size of plaque-purified positive clones was determined by a mini-preparation method (Lewis and Cowan, 1986), followed by Southern blot analysis of *Eco*RI-digested DNA with the same hybridization probe as above. A ~1600 bp cDNA insert (X.1.p97-1) was cloned into the *Eco*RI site of the M13mp18 vector and sequenced by the dideoxy chain reaction method (Sanger *et al.*, 1977). Standard computer programs were used for sequence comparisons. Total RNA from *X.laevis* ovary and cultured kidney epithelial A6 cells was used for RNA (Northern) blot analysis, using a commercially available RNA molecular weight marker set (Bethesda Research Laboratories, Gaithersburg, MD, USA) for calibration. The blot was probed with randomly primed fragments derived from a gel-purified fragment of the X.1.p97-1 insert after cloning into the 'Bluescript' expression vector (Stratagene, La Jolla, CA, USA).

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