

## Accumulation of the mitochondrial form of the sulphhydryl oxidase Erv1p/Alrp during the early stages of spermatogenesis

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Accepted 1 May 2002

### Summary

In this study, we investigated the expression of the mammalian FAD-dependent sulphhydryl oxidase Erv1p/Alrp in the rat and mouse and during mouse spermatogenesis. Up to three forms of Alrp were identified in protein extracts from different tissues and organs, but very little enzyme was present in blood samples. The three forms of Alrp represent the full-length protein of 23 kDa and fragments of 21 kDa and 15 kDa. All forms of Alrp were assembled into dimers *in vivo*. In contrast to samples from other organs, the protein analysis of mouse testis identified predominantly full-length 23 kDa Alrp. This finding prompted us to investigate in more detail the expression of Alrp during spermatogenesis. Testis samples of individual mice from postnatal days 13–29 were probed with an antibody specific for mammalian Alrp. In addition, cells from whole testis preparations were

fractionated on a bovine serum albumin column gradient. Protein expression of mouse Alrp was compared with those of testis-specific cyritestin, the cytoskeleton marker actin and mitochondrial subunit Vb of cytochrome oxidase and cytochrome *c*. These studies demonstrated a specific accumulation of full-length mouse Alrp during the early stages of spermatogenesis. The highest levels of Alrp were found in spermatogonia and primary spermatocytes. Levels of expression of Alrp did not correlate with the synthesis of components of the respiratory chain, indicating that full-length Alrp in the mitochondria of spermatogonia and spermatocytes has another function in addition to its role in oxidative phosphorylation.

Key words: rat, mouse, sulphhydryl oxidase, Erv1p/Alrp, spermatogenesis, mitochondria, iron/sulphur cluster.

### Introduction

Recently, we identified yeast Erv1p (Lee et al., 2000) and mammalian augments of liver regeneration (ALR) as FAD-linked sulphhydryl oxidases belonging to the new Erv1p/ALR protein family (Gerber et al., 2001; Lisowsky et al., 2001). These enzymes use FAD, O<sub>2</sub> and an internal redox-active cysteine pair to generate disulphide bridges in protein substrates (Hooper and Thorpe, 1999). The general importance of sulphhydryl oxidases in cellular development is indicated by the finding that, during evolution, the sulphhydryl oxidase fragment of the yeast ERV1 prototype was fused to thioredoxin/disulphide isomerase domains, thereby creating a new class of diverse growth factors forming the sulphhydryl oxidase/quiescin Q6 family (Coppock et al., 1998; Hooper et al., 1999a,b; Senkevich et al., 2000).

Previous studies have demonstrated that different forms of Alrp may have different cellular functions. After partial hepatectomy, a 15 kDa Alrp fragment accumulates in the cytosol and serum of regenerating liver (Francavilla et al., 1994; Giorda et al., 1996; Hagiya et al., 1994; Wang et al., 1999). Accumulation of this 15 kDa carboxyl-terminal fragment, which still retains the enzymatic activity of a sulphhydryl oxidase (Lisowsky et al., 2001), correlates with a

reduction in interferon-gamma levels, a reduction in the lytic activity of natural killer cells and an increase in levels of mitochondrial transcription factor A (TFAM) (Polimeno et al., 2000a,b). Overexpression of TFAM results in the production of greater amounts of proteins for the respiratory chain and, thereby, increases the oxidative phosphorylation capacity of mitochondria (Polimeno et al., 2000b), but the functional correlation between the expression of the 15 kDa Alrp fragment and the process of liver regeneration remains unclear.

So far, studies on mammalian Alrp have been dominated by its specific role as an extracellular factor in liver regeneration (Francavilla et al., 1994; Gandhi et al., 1999; Wang et al., 1999). We know that, under normal conditions, the vast majority of Alrp is located inside cells and that Alrp expression is not restricted to liver tissue (Hofhaus et al., 1999; Lange et al., 2001). Our latest studies identified Alrp as a ubiquitous eukaryotic protein (Hofhaus et al., 1999; Lange et al., 2001; Lisowsky et al., 2001; Polimeno et al., 1999). Under normal conditions, the full-length 23 kDa mammalian Alrp is predominant and localized in the mitochondrial intermembrane space. In this compartment, Alrp performs an essential function in the biogenesis of cytosolic iron/sulphur cluster (Fe/S) proteins

and is important for cellular iron homeostasis (Lange et al., 2001). Therefore, participation in the assembly of cytosolic Fe/S proteins appears to be the primary essential task of mitochondrial full-length Alrp (Lange et al., 2001). This function is crucial for all eukaryotic cell types, whereas the proposed role of 15 kDa Alrp as a hepatotrophic growth factor is restricted to liver cells, and it may be effective only after cell damage. This indicates that Alrp, like other redox-active proteins and sulphhydryl oxidases (Chivers et al., 1997; Kobayashi and Ito, 1999; Nakamura et al., 1997; Tanaka et al., 1997), may have diverse functions in the regulation of cell growth and differentiation (Francavilla et al., 1994; Gandhi et al., 1999; Li et al., 2000; Polimeno et al., 1999; Wang et al., 1999).

A consequence of these new data is the necessity to extend studies on mammalian Alrp to different tissues, organs and developmental processes. The finding that Alrp messenger RNA is synthesized in large amounts in the testis (Giorda et al., 1996; Hagiya et al., 1994) turned our attention to spermatogenesis. In addition, biochemical and immunohistochemical studies had indicated the important role of other sulphhydryl oxidases for testicular differentiation processes (Benayoun et al., 2001; Meinhardt et al., 1999; Oehmen et al., 1992). Possible functions of sulphhydryl oxidases and other redox-active proteins in growth regulation, differentiation, changes in mitochondrial and cellular membrane morphology and in the formation of the extracellular matrix have already been proposed (Benayoun et al., 2001; Coppock et al., 1998; Hooper et al., 1999b; Hooper and Thorpe, 1999; Lee et al., 2000; Lisowsky, 2001; Lisowsky et al., 2001; Meinhardt et al., 1999; Nakamura et al., 1997).

For our molecular analyses, a major breakthrough was the recent isolation and sequencing of rat seminal vesicle FAD-dependent sulphhydryl oxidase (Benayoun et al., 2001). The derived protein sequence identified the rat enzyme as highly homologous to human and chicken Q6 sulphhydryl oxidases. In fact, it appears plausible that rat seminal vesicle FAD-dependent sulphhydryl oxidase is synthesized from an alternative splicing product of the Q6 messenger RNA (Benayoun et al., 2001). Human Q6 and rat seminal vesicle FAD-linked enzymes are clearly distinct from the Erv1p/Alrp sulphhydryl oxidases. The Q6-related enzymes are monomeric proteins larger than 60 kDa that are excreted from cells (Benayoun et al., 2001; Coppock et al., 1998). In contrast, Erv1p/Alrp are small proteins of approximately 20 kDa that form dimers normally localized in the mitochondrial intermembrane space (Lee et al., 2000; Lange et al., 2001). Weak sequence homologies between Q6 and Erv1p/Alrp are restricted to the common ERV1 prototype domain harbouring the redox-active centre of these enzymes. This domain of approximately 100 amino acid residues contains the CXXC motif essential for disulphide bridge formation activity (Hooper and Thorpe, 1999; Lee et al., 2000; Lisowsky et al., 2001).

Mitochondrial localization of full-length Alrp is of special interest for spermatogenesis because it is known that morphological and functional changes in mitochondria are

associated with this highly complex cytodifferentiation process (Meinhardt et al., 1999; Russell et al., 1990). This was another important reason for investigating the expression of Alrp during mouse spermatogenesis. Full-length Alrp is identified as a new intratesticular sulphhydryl oxidase that clearly exhibits a regulated expression pattern and subcellular localization distinct from those of the Q6-related enzymes. Furthermore, our studies indicate a new function for mitochondria in the biogenesis of mature sperm cells.

## Materials and methods

### *Tissue and cell preparations*

Animals were provided by the animal research facility of the University of Düsseldorf. Organ and blood samples were prepared from male Wistar rats. For gradient probes, adult male C57BL/6J mice were killed by cervical dislocation. Testicles and epididymides were separately dissected and rinsed in Sorensen phosphate buffer. Mature sperm cells were isolated from the epididymis, and spermatids were released from the seminiferous tubules after cutting them into smaller pieces.

For the analysis of the testis at defined time points after birth, the mouse strain CD1 was used.

Isolation of the testis, separation of cells and fractionation on a 1% to 3% bovine serum albumin (BSA) gradient were essentially performed as described previously (Wolgemuth et al., 1985) using a CelSep gradient chamber (DuPont). Samples of the cell fractions were stained with 2% Toluidine Blue and inspected by oil immersion microscopy to characterize the cell types. Fractions containing predominantly the same cell type were pooled: 80–90% of the cell population in the pooled fractions was made up of just one cell type.

### *Antibodies*

To raise antibodies in rabbits against human Alrp, a purified hexahistidinyl-tagged carboxyl-terminal fragment of Alrp (residues 81–205) was used (Lange et al., 2001; Lisowsky et al., 2001). Antibodies against mouse cyritestin (Chemicon), actin (Oncogene) and mitochondrial subunit Vb of cytochrome oxidase (CoxVb) (Molecular Probes) were purchased from the indicated suppliers or used as described previously (Linder et al., 1995). Cy3 and Cy5 fluorescent secondary antibodies were kindly supplied by Dr H. A.-J. Müller, Düsseldorf, Germany.

### *Western blotting*

For immunological studies, samples containing approximately 20 µg of total protein were applied to 4% to 12% SDS/polyacrylamide gels (Novex/Invitrogen). One exception was the separation and identification of cyritestin. For western blot analysis of this protein, it was essential to use conventional 10% polyacrylamide gels according to Laemmli (1970). Most of the primary antibodies were detected using alkaline-phosphatase-conjugated secondary antibodies and chemiluminescence. Cyritestin was identified using peroxidase-conjugated secondary antibodies and staining.

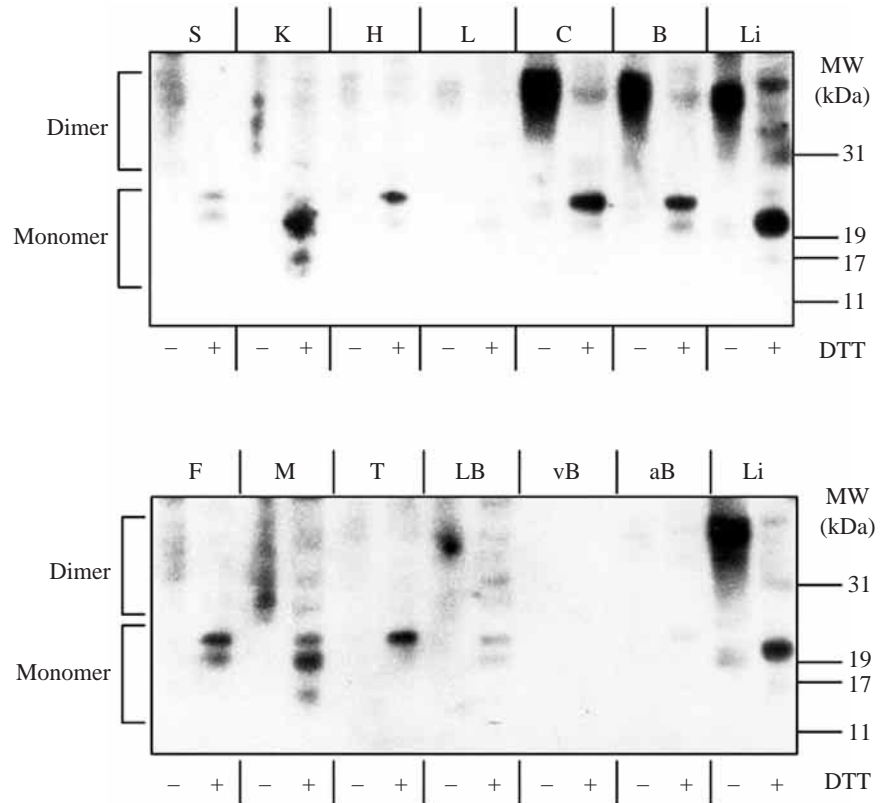


Fig. 1. Expression of Alrp in rat organs and blood samples. Samples (20 µg) of total protein extract were separated in non-reducing 4% to 12% gradient gels. The respective western blot was probed with an antibody specific for mammalian Alrp. Proteins were analysed with (+) and without (-) reducing agent (20 mmol l<sup>-1</sup> dithiothreitol) in the sample buffer. The molecular masses (MW) of marker proteins are listed in kDa. Three forms of Alrp with molecular masses of 23, 21 and 15 kDa were identified. Under non-reducing conditions, all three forms of Alrp exist as dimers. S, spleen; K, kidney; H, heart, L, lung; C, cerebellum; B, brain; Li, liver; F, fat tissue; M, muscle, T, testis; LB, liver blood; vB, venous blood; aB, arterial blood. DTT, dithiothreitol.

#### Immunocytochemistry

For alkaline-phosphatase-aided detection of *Erv1p*, tissues were fixed in paraformaldehyde or ethanol/acetic acid and embedded in Paraplast as described by Heinlein et al. (1994). Sections (6 µm) were mounted on SuperFrost slides and dewaxed through xylene and descending alcohol concentrations, washed in 10 mmol l<sup>-1</sup> phosphate buffer, 0.27 mmol l<sup>-1</sup> KCl, 140 mmol l<sup>-1</sup> NaCl, 0.05% Tween-20 (PTw) and incubated for 10 min in PTw containing 2% normal goat serum (PTwG). Rabbit anti-*Erv1p* antiserum was applied at a dilution of 1:50 in PTwG and incubated for 45 min at ambient temperature. The slides were then washed twice for 10 min in PTw, before the second antibody, goat anti-rabbit IgG, was added (dilution 1:500) in PTwG; the mixture was incubated for a further 45 min. Bound alkaline phosphatase activity was monitored by adding the substrate (0.75 mg ml<sup>-1</sup> Nitroblue Tetrazolium and 0.38 mg ml<sup>-1</sup> 5-bromo-4-chloro-3-indolyl phosphate in alkP reaction buffer: 100 mmol l<sup>-1</sup> Tris-HCl, pH 9.5, 100 mmol l<sup>-1</sup> NaCl, 5 mmol l<sup>-1</sup> MgCl<sub>2</sub>). After development of the colour reaction (5–10 min), the slides were washed in phosphate-buffered saline and dehydrated. Coverslips were mounted with DePex. For immunofluorescence, Cy3 and Cy5 secondary antibodies were used, and the coverslips were mounted with Mowiol. Images were collected with a confocal laser scanning microscope and digitally processed.

## Results

### Expression analysis of Alrp in rat

A first systematic investigation of the distribution of Alrp in

the rat was performed with total protein extracts from a number of tissues and blood samples. Separation of these samples on protein gels was carried out under reducing and non-reducing conditions to include the investigation of dimer formation in this analysis. After transfer to nitrocellulose membranes, the separated proteins were probed with a polyclonal antibody specific for the conserved carboxy-terminal domain of mammalian Alrp (Lange et al., 2001). The results of these immunological studies are shown in Fig. 1. The expression of at least three different forms of Alrp was observed: the full-length 23 kDa form and fragments of 21 kDa and 15 kDa. Under non-reducing conditions, all forms of Alrp were shifted to higher molecular masses that corresponded to dimers. The samples from heart and testis contained predominantly the 23 kDa full-length Alrp, whereas all three forms were present in, for example, kidney and muscle extracts. In blood samples from liver, only traces of Alrp were detected, whereas the other blood samples did not contain Alrp.

### Comparison of testicular Alrp expression with those of actin, mitochondrial *CoxVb* and cytochrome *c*

Western blots with selected samples from the probes shown in Fig. 1 were successively probed with four different antibodies, as listed in Fig. 2. Again, the antibody against Alrp identified up to three different forms of this protein. Actin served as an internal control for protein concentrations. The smaller amount of actin in some of the samples corresponds with the reduced presence of cytoskeleton in tissues such as the spleen, lung or blood. The mitochondrial proteins *CoxVb* and

Fig. 2. Comparison of rat Alrp expression with that of actin, mitochondrial subunit Vb of cytochrome oxidase (CoxVb) and mitochondrial cytochrome *c* (Cyt *c*). Samples (20 µg) of protein were separated in a non-reducing 4% to 12% gradient gel. The respective western blot was probed with antibodies specific for mammalian Alrp, actin, CoxVb and Cyt *c* as indicated. Proteins were analysed with (+) and without (-) reducing agent (20 mmol l<sup>-1</sup> dithiothreitol) in the sample buffer. The sample from muscle demonstrates dimer formation. The molecular masses (MW) of marker proteins are listed in kDa. Li, liver; K, kidney; H, heart; M, muscle, S, spleen; L, lung; B, brain; F, fatty tissue; LB, liver blood; vB, venous blood; aB, arterial blood; T, testis. DTT, dithiothreitol.

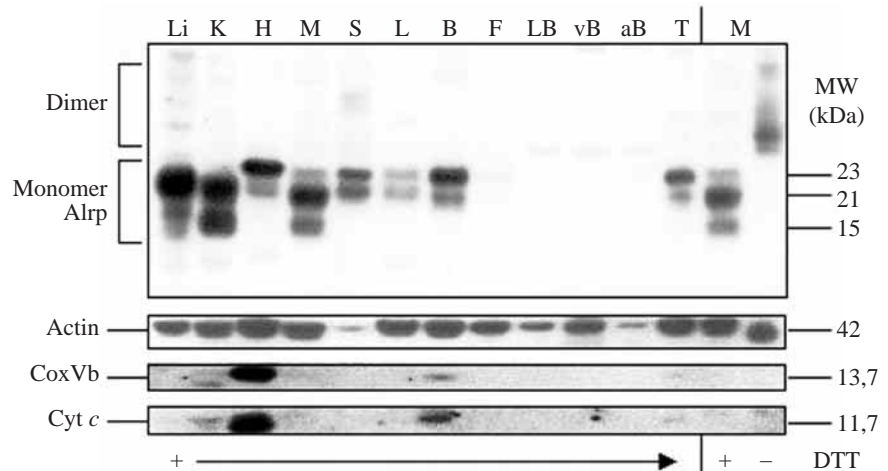
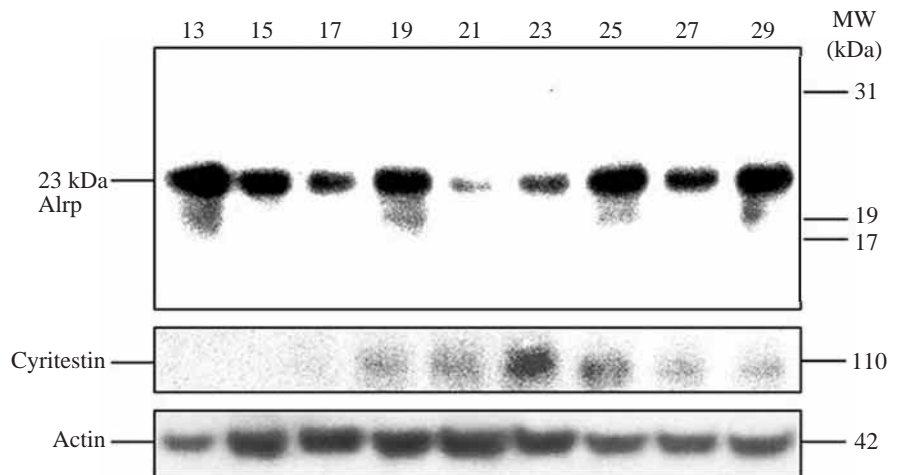


Fig. 3. Expression of Alrp in mice from postnatal days 13–29. Testis samples from two animals were prepared for each listed day. Samples (20 µg) of protein were separated in a 4% to 12% gradient gel. The respective western blots were probed with antibodies specific for mammalian Alrp, mouse cyritestin and actin. Alrp is detectable at the earliest stages of spermatogenesis examined. The largest amounts of Alrp are observed at days 13, 19, 25 and 29. In contrast, cyritestin is expressed during the later stages of spermatogenesis. The molecular masses (MW) of marker proteins and of cyritestin (110 kDa) and actin (42 kDa) are listed.



and cytochrome *c* are markers for the expression status of proteins from the respiratory chain. Comparison of the expression of CoxVb and cytochrome *c* with that of Alrp demonstrates that there is no correlation between the expression levels of proteins for the respiratory chain and that for Alrp.

#### Testicular expression of Alrp in postnatal mice from days 13–29

Male mice from the same day of birth were grown for 13 days. Pairs of animals were then removed from the population and used for the preparation of whole testis samples. Identical samples from total protein extracts were used for the immunological studies. The testis-specific marker protein cyritestin (Heinlein et al., 1994; Lemaire et al., 1994; Linder et al., 1995) was included in the analysis together with the actin control. Cyritestin accumulates during the late stages of sperm differentiation (Forsbach and Heinlein, 1998) and is therefore present at the highest levels around post-natal day 23. In contrast, Alrp is present at high levels from the very early days of spermatogenesis. High levels of Alrp were also detected on post-natal days 19, 25 and 29 (Fig. 3). This demonstrates that

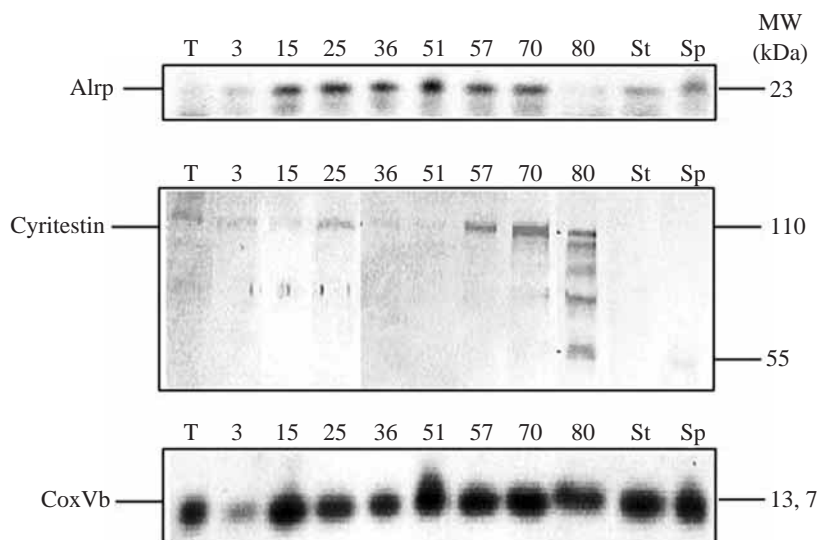
peaks of Alrp synthesis in the testis are observed approximately every 6–7 days after birth. This cycle of expression coincides with the generation of spermatogonia and spermatocytes. These experiments were repeated three times with different sets of animals. The results were essentially the same.

#### Analysis of Alrp expression in gradient-separated testis cells

Dissociated testes cells from eight mice were applied to a BSA gradient. Fractions were collected, and samples were stained and inspected microscopically. Fractions containing predominantly the same type of cell were pooled. Samples of these cells were used to prepare total protein extracts for the immunological studies shown in Fig. 4. The distribution, differentiation-dependent expression and processing of the germ-cell-specific marker cyritestin served as an internal control (Forsbach and Heinlein, 1998). Cyritestin is synthesized as a 110 kDa precursor (Forsbach and Heinlein, 1998) and integrated into the membrane of the acrosome (Linder et al., 1995). The intra-acrosomal half of the transmembrane protein is then released, and a 55 kDa fragment is retained in the membrane as the functional protein. The 55 kDa fragment therefore accumulates during the last stages



Fig. 4. Gradient separation of testis cells and expression analysis for Alrp, cyritestin and mitochondrial subunit Vb of cytochrome oxidase (CoxVb). Samples (20 µg) of protein were separated in 4% to 12% gradient gels, and western blots were probed with the listed antibodies. Expression levels of Alrp are clearly distinct from those of testis-specific cyritestin. Alrp is found predominantly in spermatogonia and early spermatocytes. The 110kDa form of cyritestin accumulates in the later stages of spermatogenesis, with the typical processing into the 55kDa fragment during the final maturation steps of sperm cells. In contrast, mitochondrial CoxVb is present in comparable amounts in nearly all samples. T, total testis extract; numbers 3–80 represent the first number of the pooled fractions collected from the gradient. Pooled fractions contained predominantly one type of differentiating sperm cell as listed: 3, 3–14/pachytene spermatocytes; 15, 15–24/type A spermatogonia; 25, 25–35/leptotene–zygotene spermatocytes; 36, 36–50/Sertoli cells; 51, 51–56/type B spermatogonia; 57, 57–69/secondary spermatocytes; 70, 71–79/early spermatids; 80, 81–91/late spermatids. St, isolated spermatids from seminiferous tubules; Sp, mature sperm cells from epididymis.



of sperm cell maturation. The expression profile for Alrp was different from that of cyritestin: Alrp was detected in fractions associated with cells characteristic of the early stages of sperm cell development. The largest amount of Alrp was found in fractions 51–56, which contain type B spermatogonia. In fractions containing cells from the last stages of sperm maturation or in isolated spermatids and spermatozoa, only small amounts of Alrp were detected. Fractionation experiments were repeated three times with testis preparations from different animals. The expression analysis for Alrp gave comparable results in all these experiments.

*Immunological detection of Alrp in tissue sections*

Immunocytochemistry was used to corroborate the results obtained in cell separation experiments. Paraffin-embedded tissue sections were probed with anti-Alrp antiserum and chromogenic or fluorescent secondary antibodies. The signals generated provided additional evidence that Alrp is expressed in spermatogonia (Figs 5, 6).

**Discussion**

Here, we report the first characterization of full-length Alrp sulphhydryl oxidase as a new enzyme the expression of which is regulated during spermatogenesis. The greatest amounts of this protein are observed in spermatogonia and early spermatocytes. Expression levels of Alrp do not correlate with the synthesis of marker proteins for a functional respiratory chain. This indicates the importance of the mitochondrial form of the sulphhydryl oxidase Alrp for other functions besides oxidative phosphorylation during sperm cell differentiation. In this respect, it is of special interest that the most recent studies identify full-length Alrp as a component of the mitochondrial intermembrane space with an essential function in the

assembly of cytosolic Fe/S cluster proteins (Lange et al., 2001). This opens interesting new lines of investigation for the analysis of sperm development.

Protein analysis of numerous rat and mouse tissues

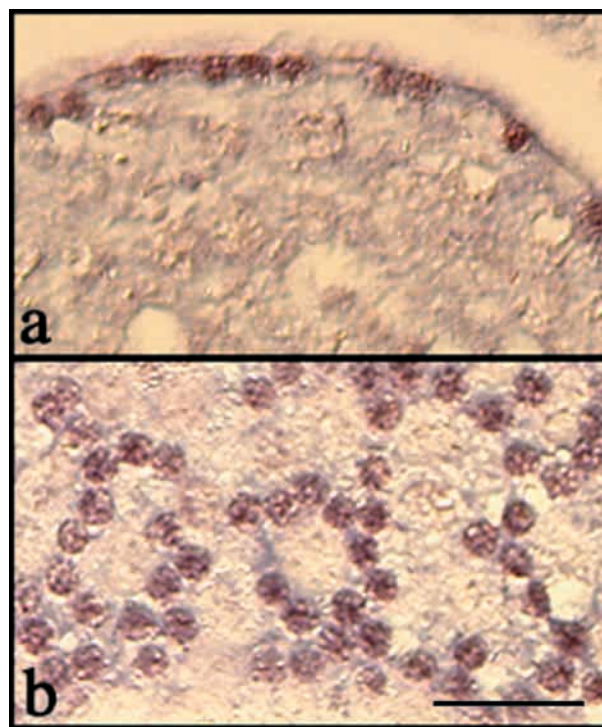


Fig. 5. Alrp immunodetection with alkaline-phosphatase-conjugated secondary antibody. (a) Sagittal section through seminiferous tubule with immunopositive (brown colour) spermatogonia along the basal lamina. (b) Lateral section through the spermatogonial layer of the seminiferous epithelium. Scale bar, 50 µm.

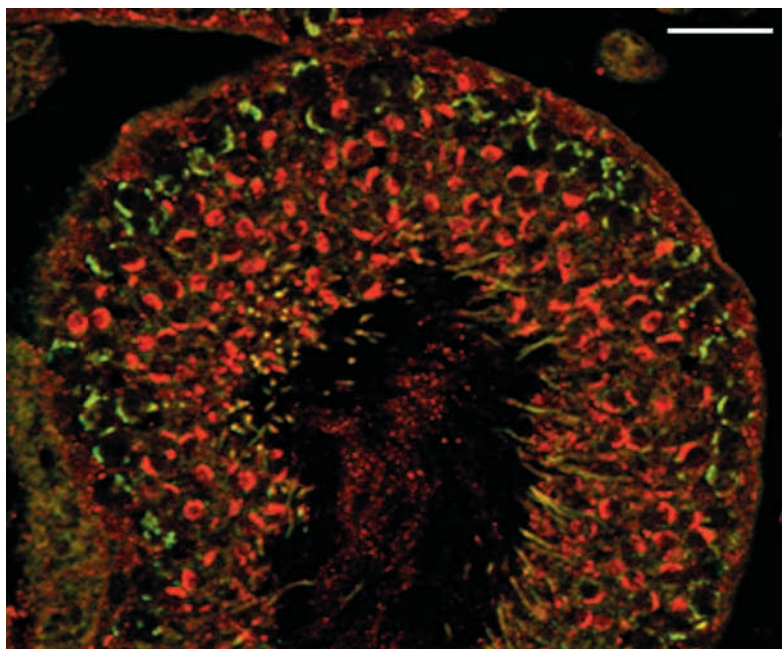


Fig. 6. Confocal laser scanning microscope image of double-immunofluorescence assay using anti-Alrp (green) and anti-CoxVb (mitochondrial subunit Vb of cytochrome oxidase) (red). The highest levels of Alrp are observed in spermatogonia and elongated sperm cells, whereas the highest levels of CoxVb are found in other cells. The yellow colour therefore indicates that co-localisation of Alrp and CoxVp inside mitochondria occurs only when approximately equal amounts of the two proteins are present. Scale bar, 50  $\mu$ m.

demonstrated that Alrp is a ubiquitous protein. In accordance with the finding that, under normal conditions, Alrp is not secreted from cells, blood samples do not contain Alrp. The very small amount of Alrp that is present in blood samples taken directly from the liver had probably been released from damaged hepatocytes. The concentrations of Alrp vary among tissues. Our important new findings are the high level of Alrp in the brain and cerebellum and the identification of three different forms of Alrp. Previous studies indicated that different forms of Alrp may be associated with different localizations and functions (Gandhi et al., 1999; Giorda et al., 1996; Hofhaus et al., 1999). During liver regeneration, there is specific accumulation of a cytosolic 15 kDa Alrp fragment (Giorda et al., 1996; Hagiya et al., 1994) that may be associated with its function as a secondary growth factor (Francavilla et al., 1994; Gandhi et al., 1999; Wang et al., 1999).

Our data characterize full-length Alrp as a testicular sulphhydryl oxidase distinct from the Q6-related FAD-dependent rat seminal vesicle sulphhydryl oxidase. The rat Q6 enzyme is a secretory protein found preferentially in epididymis and seminal vesicles (Benayoun et al., 2001). In contrast, Alrp is associated with spermatogonia and early spermatocytes, as verified by the analysis of testis from postnatal mice of different ages. The distribution of Alrp in gradient fractions of separated testis cells supports this finding.

Current research suggests that sulphhydryl oxidases and other redox proteins have a broader than expected role in the regulation of cell growth and differentiation (Eickhoff et al., 2001; Lisowsky, 2001). In addition to a defined enzymatic function in modifying amino acid residues in target proteins, these enzymes also act like cytokines or growth factors and occur at a variety of subcellular locations (Benayoun et al., 2001; Coppock et al., 1998; Eickhoff et al., 2001; Hooper and Thorpe, 1999; Lee et al., 2000). One recent new example is the

macrophage migration inhibitory factor MIF. This redox protein was first described as a classical T-cell cytokine (Bloom and Bennett, 1966; David, 1966). MIF has since been identified as a new secretory protein of rat epididymis that is transferred to spermatozoa and is localized in the outer dense fibres (Eickhoff et al., 2001). Another important example of the synthesis of two isoforms from a nuclear gene during mouse spermatogenesis has also been described: the alternative splicing products from the same mouse gene encode mitochondrial transcription factor A and a testis-specific nuclear HMG box protein whose precise cellular function is still unknown (Larsson et al., 1996).

According to current data, there are two possible functions for Alrp in the mitochondrial intermembrane space of spermatogonia and spermatocytes. First, as a sulphhydryl oxidase, Alrp could be responsible for the introduction of specific disulphide bridges into integral mitochondrial membrane proteins, and changes in the level and activity of Alrp would result in morphological changes in mitochondria. This would make sense because mitochondria, in particular, exhibit a highly complicated change in their structure during spermatogenesis (Meinhardt et al., 1999). In addition, for the homologous Erv1p from yeast, we have shown previously that reduced levels of mitochondrial Erv1p cause dramatic changes in the morphology of mitochondria (Becher et al., 1999).

Second, Alrp could play a role in the biosynthesis of a product important for spermatogenesis. Our latest data demonstrate that full-length Alrp is essential for the biogenesis of cytosolic Fe/S proteins. This could indicate a specific demand for particular Fe/S-cluster-containing proteins during sperm development. Recent studies demonstrate that the mitochondria of all eukaryotes perform a central task in the biogenesis of cellular Fe/S proteins (for reviews, see Craig et al., 1999; Lill et al., 1999; Lill and Kispal, 2000). They harbour



a complex apparatus, termed the iron/sulphur cluster (ISC) assembly machinery, consisting of some ten proteins. The ISC assembly machinery is required for the biogenesis of Fe/S proteins within mitochondria as well as for the maturation of cytosolic Fe/S proteins (Kaut et al., 2000; Kispal et al., 1999; Lange et al., 2000; Li et al., 2000).

*Erv1p/Alrp*, like the central constituents of the yeast ISC assembly machinery, is indispensable for life (Lisowsky, 1992, 1996). Cytosolic and nuclear Fe/S proteins perform important regulatory functions as enzymes or transcription factors. The synthesis of Fe/S clusters inside mitochondria is therefore an essential task independent from the activity of the respiratory chain. Even under anaerobic conditions, Fe/S clusters have to be synthesized. Therefore, one does not observe a correlation in the expression levels of mitochondrial proteins for the respiratory chain and for the Fe/S assembly machinery. This would also be in accordance with the finding that elevated levels of mitochondrially localised *Alrp* in spermatogonia and elongated sperm cells do not correlate with the levels of expression of mitochondrial *CoxVb* or cytochrome *c*, which are both constituents of the respiratory chain.

*Erv1p/Alrp* appears to be the only sulphhydryl oxidase detected in the mitochondrial intermembrane space. This opens new lines of investigation for the analysis of sperm development. Future elucidation of the molecular function of *Alrp* during spermatogenesis will require the identification of sperm proteins harbouring Fe/S clusters whose assembly is dependent on the mitochondrial Fe/S cluster machinery.

Our work was partially supported by the Deutsche Forschungsgemeinschaft.

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