HSPA2 (formerly HSP70.2) is a testis-specific member of the HSP70 family known to play a critical role in the completion of meiosis during male germ cell differentiation. Although abundantly present in post-meiotic cells, its function during spermiogenesis remained obscure. Here, using a global proteomic approach to identify genome-organizing proteins in condensing spermatids, we discovered an unexpected role for HSPA2, which acquires new functions and becomes tightly associated with major spermatid DNA-packaging proteins, transition proteins 1 and 2. Hence, HSPA2 is identified here as the first transition protein chaperone, and these data shed a new light on the yet totally unknown process of genome-condensing structure assembly in spermatids.

Although several waves of genome-wide reorganization have long been known to occur during male germ cell post-meiotic maturation, the underlying mechanisms remain obscure. The most spectacular of these reorganizations are those associated with the replacement of histones by transition proteins (TPs)\(^2\) and of TPs by protamines (1–4). In somatic cells, specific sets of chaperones control the assembly of nucleosomes containing canonical and variant histones and hence are considered as major regulators of the establishment of differential genome organization (5). In differentiating spermatids, despite the fundamental role of TPs and protamines in genome organization, nothing is known on the chaperone system escorting these proteins and mediating their assembly into new DNA-packaging structures. In general, very little is known on spermatids specific for histone and non-histone genome organizers that are in action during spermiogenesis. The only characterized testis-specific histone chaperone is tNASP, which binds the male germ cell linker histone, H1t (6). Here, by analyzing the nature of proteins soluble in acids present in condensing spermatids, we have discovered an unexpected property of the testis-specific chaperone HSPA2 that sheds light on potential mechanisms controlling the post-meiotic genome reorganization. HSPA2 had already been shown to possess an essential and specific role in male germ cell meiosis (7–10). However, because of a meiotic blockade and massive apoptosis of spermatocytes in HSPA2 knock-out mice, the post-meiotic function of this protein has remained obscure. Here, HSPA2 is identified as the first chaperone of transition proteins TP1 and TP2. Our data therefore strongly suggest that, after meiosis, HSPA2 acquires new functions and contributes to the dramatic spermatid-specific genome-wide reorganization.

**EXPERIMENTAL PROCEDURES**

Detailed methods are available in supplemental information.

**Germinal Cell Fractionation**—The fraction composed of condensing spermatids (steps 12–16) used for mass spectrometry analysis was obtained as described in Ref. 11. Other spermatogenic cell fractions were obtained using the Bellvé sedimentation method (12, 13). Cell fractions were enriched at 80% in each major stage (Spc, spermatocytes; R, round spermatids; RE, mix of round and elongating spermatids; EC, mix of elongating and condensing spermatids).

**Acid Extraction**—Proteins were extracted by direct sonication of spermatogenic cell nuclei in sulfuric acid (0.2 N). The quality of extraction was controlled by SDS-PAGE gel stained with Coomassie.

**Immunoprecipitation**—25–40 \(\mu\)g of acid-soluble proteins were incubated with 1–2 \(\mu\)g of anti-HSPA2 or anti-hemagglutinin (Santa Cruz) antibodies in the appropriate buffer (see supplemental information). Protein G coupled to dynabeads (Invitrogen) were used following the manufacturer’s instructions. Bound proteins were washed, eluted in loading buffer, and analyzed by SDS-PAGE and silver staining (SilverQuest; Invitrogen) or Western blots using standard procedures.

**Chromatin Fiber Extraction**—Nuclei of pooled fractions obtained after fractionation were submitted to micrococcal nuclease digestion in the appropriate conditions (see supplemental methods). Nuclei were broken by osmotic shock by resuspension in \(H_2O\), trichostatin A 300 nM, and antiprotease mixture Complete (Roche Applied Science). Micrococcal
nuclease-released proteins were analyzed by SDS-PAGE, using core histone amounts as internal loading control.

**Immunofluorescence on Germ Cells**—Nuclei were prepared as described under “Chromatin Fibers” and fixed on Superfrost slides. Detection details are available in supplemental methods. Confocal images were taken using a CLSM microscope (Zeiss) with slices of 0.7–1 μm.

**RESULTS**

**Mass Spectrometry Analysis of Acid-soluble Proteins**—Many genome-organizing proteins including histones and testis-specific DNA-packaging proteins, such as TPs and protamines, are highly basic and can be purified thanks to their solubility in acid. We reasoned that a global identification of acid-soluble proteins extracted from condensing spermatids would allow us to gain an insight into the nature of genome-organizing proteins in these cells. Accordingly, nuclei from a population of sonication-resistant mouse spermatids, composed mostly of condensing spermatids at steps 12–16, were used to extract proteins soluble in 0.2 N sulfuric acid. A proteomic approach for the global identification of these proteins was undertaken. The identified proteins are listed in supplemental Table S1. As expected, many DNA-packaging proteins are on the list. Among the...
HSPA2, a Spermatid-specific Chromatin Chaperone

FIGURE 3. Stage-specific redistribution of HSPA2. A, stage-specific micrococcal nuclease-dependent release of HSPA2 from spermatogenic cell nuclei. Nuclei were prepared from pooled fractions of spermatocytes (Spc) and elongating and condensing spermatids (EC) and submitted to extensive micrococcal nuclease digestion. Material released through the action of the enzyme was visualized after silver staining of a gel. The two indicated bands were cut on another gel and identified by mass spectrometry. Band 1 contained HSPA2 and HSPA1L/HSC70t, and band 2 contained disulfide isomerase A3 (PDA3). B, stage-specific intranuclear redistribution of HSPA2 during spermiogenesis. Suspension of spermatogenic cells was used to prepare nuclei that were then subjected to co-immunolocalization of HSPA2 and acetylated lysines (monoclonal antibody detecting essentially histones in the nucleus). Images were acquired using a confocal microscope. Four representative situations are shown with R, round spermatids; E, elongating spermatids; and C, condensing spermatids. White arrows indicate regions of HSPA2 accumulation. These experiments were performed on purified nuclear suspensions because the cytoplasmic HSPA2 signal interfered with a clear detection of nuclear HSPA2, which was the object of this study. Some of the remaining cytoplasmic HSPA2 can be observed in the C panel (arrow head). Scale bar represents 2 μm.

We then examined the isoelectric points (pI) of all the proteins found soluble in H2SO4 and plotted the pI values for each of these proteins along a pI scale (Fig. 1). This representation revealed the presence of two distinct groups of proteins. The first group contained, as expected, basic and mostly DNA-packaging structural proteins (Fig. 1, black arrows). Surprisingly, the other group was mostly composed of acidic proteins with a pI between 4.5 and 6 (Fig. 1, gray arrows). An analysis of the nature of these acidic proteins revealed that known chaperones are among them. Based on these data, we hypothesized that a tight association between these acidic proteins, mainly the chaperones, and their basic partners may have induced their solubility in H2SO4. Accordingly, we had here an interesting opportunity to uncover new functions for some of these chaperones in spermatids by identifying their partners.

Stage-specific HSPA2 Acid Solubilization—Among the identified chaperones, HSPA2, a testis-specific member of the HSP70 family (15), appeared to us as an interesting candidate chaperone. Indeed, the data presented above provided an excellent opportunity to investigate its functions in post-meiotic cells where its role had remained obscure.

Spermatogenic cells were fractionated, and pools of cells enriched in spermatocytes (Spc), round spermatids (R), a mix of round and elongated spermatids (RE), and a mix of elongating and condensing spermatids (EC) were obtained and used to confirm that HSPA2 is expressed in meiotic as well as in post-meiotic cells (Fig. 2A). Our proteomic data suggested that HSPA2 becomes acid soluble because of its tight association with DNA-packaging basic proteins. To test this hypothesis, fractionated spermatogenic cells obtained as above were submitted to acid extraction. Fig. 2B shows the results of two independent experiments. Very interestingly, a fraction of HSPA2 becomes acid soluble only at late stages of spermiogenesis, after the accumulation of transition protein 2 (TP2). In the same extracts we did not find any evidence for the selective solubility of HSPA1B/HSP70 (Fig. 2B, Exp 2, and not shown). Because of high sequence identity between HSPA2 and HSPA1B, this experiment ruled out the possibility that the stage-specific acid solubility of HSPA2 could be due to nonspecific binding of basic proteins to HSPA2 during the extraction procedure.

HSPA2 Function Switch during Spermiogenesis—Our data suggested that the function of HSPA2 changes during spermiogenesis and that it becomes specifically associated with spermatid-specific DNA-packaging proteins during the late stages of spermiogenesis. According to this hypothesis, we predicted a recruitment of HSPA2 to DNA-organizing structures as spermiogenesis proceeds. Proteins preferentially released by micrococcal nuclease from post-meiotic cell nuclei (EC) compared with a spermatocyte pool (Spc) were identified by a proteomic approach. A silver stain analysis of these proteins showed that at least two major proteins are preferentially released from the nuclei of spermatids compared with spermatocytes (Fig. 3A, arrows). Interestingly, in band 1, in addition to HSPA2, mass spectrometry identified HSPA1L/HSC70t, another testis-specific member of the HSP/HSC70 family. Band 2 contained disulfide isomerase A3 (PDA3).

We also tried to visualize the stage-specific HSPA2 redistribution in situ. A wave of histone acetylation is known to occur prior to histone replacement during spermatid elongation (2, 3). To have an insight into the relationship between HSPA2 localization and histone replacement, both histone acetylation and HSPA2 were detected in spermatogenic cells using, respectively, a monoclonal mouse anti-acetyl-lysine antibody (16) and a rabbit polyclonal antibody specific for HSPA2. Fig. 3B shows...
examples of three critical periods during spermiogenesis as a function of changes occurring in global histone acetylation. In round spermatids (R), HSPA2 is almost homogenously distributed in the nucleus, and, as previously shown, histones are found underacetylated in these cells (17). Interestingly, the wave of histone acetylation observed at stage VIII spermatids (17) is associated with a dramatic redistribution of HSPA2, which becomes concentrated in a cap-like structure penetrating into the inner side of the nucleus. Co-localization with 4',6-diamidino-2-phenylindole DNA staining clearly shows that these regions are DNA-containing subacrosomal domains and, interestingly, also correspond to the domain previously shown to accumulate the spermatid-specific linker H1t2 at stage VIII (14). Moreover, the absence of hyperacetylated histones and accumulation of H1t2 in this region indicate that this particular zone is probably one of the first concerned by the replacement of somatic-type histones in the nucleus of condensing spermatids. Fig. 3B also shows an example of a more advanced spermatid (C), where most of the histone replacements have already occurred. It is interesting to note that the global disappearance of histone acetylation nicely matches the extension of HSPA2 localization into various regions and its concentration in the nucleus.

**Acid-resistant Association between TP1/TP2 and HSPA2**—To visualize the binding of proteins to HSPA2 in condensing spermatids, which supposedly mediates the latter’s acid solubility, acid extracts from round and elongating/condensing pools of fractionated spermatogenic cells were used to immunoprecipitate HSPA2. The HSPA2 antibody did not immunoprecipitate any histone, showing that if there is any HSPA2-histone interaction, it does not resist acid extraction (Fig. 4A, lane 3). The silver-stained profile of the immunoprecipitated proteins, as well as other data shown here (mainly the timing of TP accumulation and HSPA2 acid solubility) suggest that HSPA2 can form an acid-resistant complex with TP1 and TP2. To confirm this hypothesis, acid extracts from condensing spermatids were immunoprecipitated either with an irrelevant antibody (Fig. 4B, Ir) or the anti-HSPA2. Western blots confirmed the existence of a specific acid-resistant complex between TP1/TP2 and HSPA2 in elongating and condensing spermatids. It is interesting to mention that HSPA2 immunoprecipitation, after sonication of elongating-condensing spermatids in a buffer containing increasing amounts of salt, also allowed pull down of DNA fragments containing TP1 and TP2 (not shown). This confirms the association of HSPA2 with genomic regions where TP1/2 assembly takes place.

**DISCUSSION**

HSPA2, although sharing important sequence identity with other members of the HSP70 family, shows unique functions in spermatogenic cells. Indeed, the knock-out approach revealed the occurrence of specific meiotic defects in male germ cells despite the presence of another testis-specific member, HSPA1L/HSP70t, and the somatic type HSP70 in these cells (8). Male germ cells lacking HSPA2 arrest in prophase of meiosis I and pachytene spermatocytes undergo massive apoptosis (8). In these cells, HSPA2 was shown to be associated with the synaptonemal complex and to play a role in desynapsis (7–9).
the activity of a chaperone, HSPA2, evolves in a differentiation-dependent manner. The detailed analysis of the nuclear distribution of HSPA2 in spermatids with respect to histone acetylation confirms the existence of a specific nuclear subacrosomal compartment in elongating spermatids where transitions in genome-reorganizing structures seem to start. Interestingly, Davidson and co-workers (14) had already noticed that H1t2, a new spermatid-specific linker histone, accumulates in a highly polar manner at the apical pole of stage V-VIII spermatids under the acrosome compartment before its spreading within the nucleus in stage X spermatids. We observed a specific accumulation of HSPA2 in the same region at approximately the same stage (VIII). Moreover, this particular mobilization of HSPA2 perfectly correlates with the disappearance of histone acetylation. Like H1t2 (14), HSPA2 accumulation spreads to various nuclear regions at later stages. Because histone acetylation is thought to be linked to their removal (2, 3), our data on the intranuclear distribution of HSPA2 strongly suggest that its spreading within the nucleus follows histone removal and the assembly of new spermatid-specific structures. This hypothesis is further supported by the fact that, at these stages, specific acid-resistant complexes are formed containing HSPA2 and TP1 and TP2.

Based on all these data we propose here that, after the completion of meiosis, HSPA2 acquires a new function as a chaperone of spermatid-specific DNA packaging proteins and hence could be considered as the first identified factor controlling the histone to TP transition.

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