Low Temperature Promotes Annexin V Expression in Newt Testis

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Low Temperature Promotes Annexin V Expression in Newt Testis

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ABSTRACT—We examined the effect of low temperatures on annexin V expression in newt testis. When newts were transferred to a low temperature (12°C), up-regulation of annexin V protein was observed in secondary spermatogonia. In primary spermatocytes, high levels of annexin V expression were observed at both 12°C and 22°C, but at 12°C the protein was localized in part of the cytoplasm of primary spermatocytes. These results indicate that in newt testis annexin V is a cold-sensitive protein, suggesting the possibility that annexin V might have a cold stress-related function in newt germ cells.

Key words: annexin V, low temperature, spermatogenesis, newt

INTRODUCTION

Annexins consist of a family of calcium-dependent phospholipid-binding proteins and distribute abundantly in various tissues of plants and animals (Smith and Moss, 1994). All the annexins contain a highly conserved calcium binding core domain and a variable N-terminal region (Raynal and Pollard, 1994). It has been suggested that annexins function in a broad range of physiological events including inhibition of phospholipase A2 (Davidson *et al*., 1987), anticoagulation (Romisch *et al*., 1990) and anti-inflammatory processes (Goulding and Guyre, 1992).

Previously, we isolated annexin V as a protein differentially expressed during newt spermatogenesis (Yamamoto *et al*., 1996). Newt annexin V is abundantly expressed in the cytoplasm of primary spermatocytes and round spermatids. In mammalian testis, several types of annexins are expressed. Annexins I and II are localized in the acrosome of spermatids, and annexin IV is associated with the endoplasmic reticulum (ER) in spermatids of ram testis (Feinberg *et al*., 1991). Annexin V was detected in Sertoli and Leydig cells of rat testis (Giambanco *et al*., 1991). However, the functions of annexins in testis are unknown.

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Recently, it was reported that annexin I is up-regulated in HeLa cells in response to heat shock (Rhee *et al*., 2000). In wheat, novel annexin expressions are induced by low temperatures during cold acclimation (Breton *et al*., 2000). These results suggest that the annexin family proteins may be new members of the stress protein family. Therefore, we examined a possibility that annexin V has stress-related proteins in newt germ cells. Since newts are poikilothermal animals and exposed to low temperatures during winter, we examined the effect of low temperatures on annexin V protein expression in newt testis and found that low temperatures promoted annexin V expression in secondary spermatogonia of newt testis.

MATERIALS AND METHODS

Animals

Adult male newts, *Cynops pyrrhogaster*, were purchased from a supplier (Hamamatsu Seibutsu Kyozai Ltd., Hamamatsu, Japan), kept at 22°C, and fed frozen Tubifex.

Western blot analysis

Protein extraction and Western blot analysis using a monoclonal antibody against recombinant newt annexin V were performed as described previously (Yamamoto *et al*., 1996). Ten micrograms of protein extracted from newt testis were separated by 12% SDS-PAGE, blotted onto PVDF membrane (Millipore), and treated with the anti-newt annexin V monoclonal antibody. The

bound antibody was detected using a goat anti-mouse IgG conjugated with HRP (Wako) and ECL system (Amersham).

Immunohistochemistry

Immunohistochemistry was performed using sections from newt testis fixed in Bouin's solution. The sections were treated with anti-annexin V monoclonal antibody at 4°C overnight, and then incubated with a goat anti-mouse IgG conjugated with HRP (Wako) at room temperature for 1 hr. The color reaction was developed using the Vectastein Elite ABC kit (Vector).

RESULTS AND DISCUSSION

To examine the possibility that annexin V has cold stress-related functions in testis, we transferred newts, with testes consisting of spermatogonia and primary spermatocytes, to low temperatures (12°C and 18°C), incubated them for 1 week and performed Western blot analysis. As shown in Fig. 1, an increased level of annexin expression was observed in testis from newt incubated at 12°C compared to those at 18°C and 22°C.

At 22°C, annexin V was expressed abundantly in cytoplasm of primary spermatocytes (Fig. 2A), but very weakly in secondary spermatogonia (Fig. 2A). After incubation at 18°C, the expression pattern of annexin V was consistent with that at 22°C (data not shown). In contrast, after incubation at 12°C, annexin V expression was up-regulated in secondary spermatogonia (Fig. 2B). This up-regulation was observed in all stages of secondary spermatogonia (data not shown). In addition, at 12°C a high level of expression was observed in primary spermatocytes, as was also observed at 18°C and 22°C. However, annexin V was localized in part of the cytoplasm at 12°C (arrowhead, Fig. 2B) in contrast to its uniform expression at 18°C and 22°C.

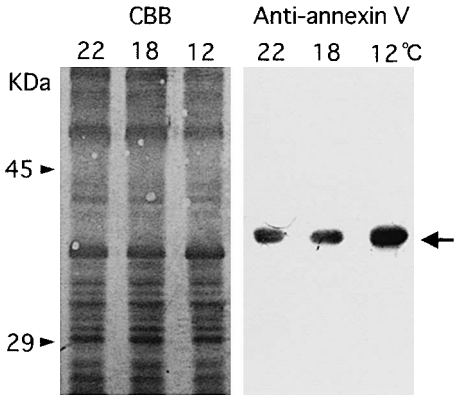

Fig. 1. Western blot analysis of annexin V in the testis of newt maintained at 22°C, 18°C, and 12°C for a week. An arrow shows the position of 35 kDa annexin V proteins.

In newt testis, low temperature (12°C) induces cell death of spermatogonia just before meiosis (Yazawa *et al*., 1999). Therefore, we examined the annexin V expression in degenerated spermatogonia. As shown in Fig. 2C, there was no significant difference between expression levels in living spermatogonia and that in degenerated spermatogonia (arrowhead), although up-regulation of annexin V expression was detected in both.

Recently it was reported that heat stress induces the expression of annexin I in HeLa cells (Rhee *et al*., 2000). In wheat, 39 kDa and 25 kDa annexins were induced in response to low temperatures during cold acclimation (Breton *et al*., 2000). These results suggest that several types of

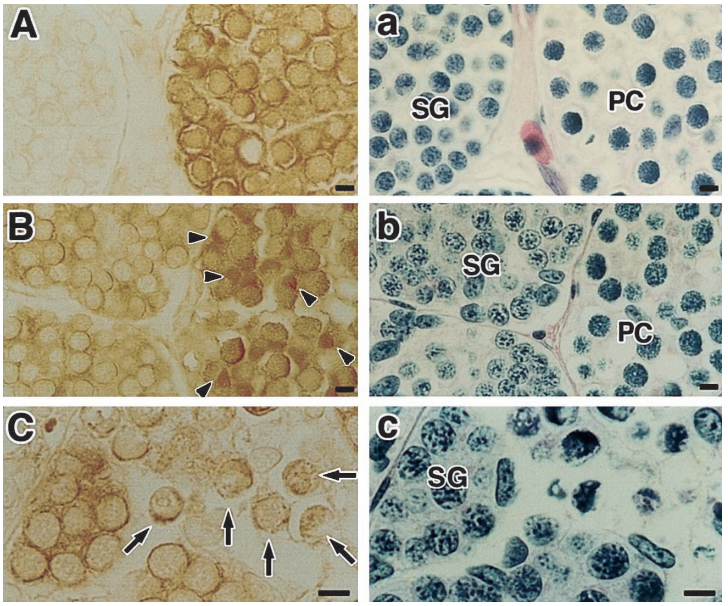

Fig. 2. Photomicrographs showing immunohistochemical localization of annexin V (A, B, C) in the testis of newt maintained at 22°C (A) and 12°C (B and C) for a week. The neighboring sections were stained with hematoxylin and eosin (a, b, c). SG, secondary spermatogonia; PC, primary spermaticytes. Arrowheads indicate the localized area within cytoplasm of primary spermatocytes where annexin V was highly expressed. Arrows indicate degenerated spematogonia. Scale bar, 10 μm.

annexins are temperature-sensitive proteins. Consistent with these results, newt annexin V expression was induced under low temperature conditions in secondary spermatogonia, and its localization changed in primary spermatocytes. Therefore, our present results suggest that annexin V may be involved in the cold stress response of germ cells, consistent with the idea that the annexin family proteins have stress-related functions.

In newt, active spermatogenesis occurs from early spring to late autumn. Subsequently, spermatogenesis stops, and does not proceed during winter when early spermatogonia are kept alive under low temperature stresses (Sáez *et al*., 1990, Yazawa *et al*., 2000, 2002). Therefore, it is postulated that increased annexin V induced by low temperatures may function for the maintenance of spermatogonia during the winter months.

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