PROTEINS OF THE MEMBRANE SKELETON IN RAT SERTOLI CELLS

E. ZIPARO*, B. M. ZANI, A. FILIPPINI, M. STEFANINI
Institute of Histology and General Embryology, 'La Sapienza' University, Rome, Italy
AND V. T. MARCHESI
Department of Pathology, Yale University, New Haven, Connecticut, USA

SUMMARY

Analogues of the α, β and γ subunits of human spectrin and erythroid protein 4.1 have been detected in rat Sertoli cell primary cultures. Immunofluorescence of permeabilized cells showed that erythroid type spectrin, protein 4.1 and actin co-distribute within the cells as filamentous structures. Fodrin-like molecules were distributed in a diffuse manner, mostly associated with the plasma membrane. Immunoprecipitation and immunoblotting experiments indicated that the polypeptides present in rat Sertoli cells are immunologically related and display molecular weights similar to their analogues in the human erythroid and non-erythroid membrane skeleton.

INTRODUCTION

The biological signals that are involved in hormone-specific responses and growth regulation act on cells through the binding of ligands to specific surface membrane receptors. Activation of these receptors, which seem to be integral membrane proteins of the trans-membrane type, evokes a variety of metabolic responses. It is believed that such activated receptors exert their effects in part by modifying the state of the cytoskeleton, yet the precise changes in the cytoskeleton that occur under these conditions and the mechanisms involved are essentially unknown.

The cytoskeletons of most cells are composed of microtubules, microfilaments and intermediate filaments, the latter class comprising filaments of vimentin, cytokeratins and a number of other similar proteins that are characteristic of different cell types. Recently it has become evident, largely as a result of studies of red cell membranes (Branton et al. 1981), that the cytoskeleton attaches to the surrounding surface membrane by interacting with another distinct set of proteins that are concentrated just beneath the lipid bilayer of the membrane. These proteins, often referred to as the membrane skeleton, provide a supporting network for the membrane and are attached to it by associating with internal segments of trans-membrane glycoproteins. Stimulation of the external segments of these same glycoproteins

*Author for correspondence at: Istituto di Istologia ed Embriologia generale, Via A. Scarpa, 14, 00161 Rome, Italy.

Key words: Sertoli cells, membrane, skeleton.
by the attachment of specific ligands has been shown to modify the state of the membrane skeleton (Marchesi, 1985). These observations have led to the hypothesis that the physiological stimulation of cells by external ligands such as hormones or growth factors involves a coupled response that is mediated by surface receptors that are linked to both the membrane skeleton and the cytoskeleton.

Cultured Sertoli cells retain their capacity to respond to specific hormone stimulation by a series of metabolic responses and by undergoing dramatic changes in cell shape when exposed to follicle-stimulating hormone (FSH) (for a review, see Stefanini et al. 1985). These effects are believed to be mediated by FSH receptors that might be functionally linked to the cytoskeleton. Since the proteins that make up the cytoskeleton of Sertoli cells have not been studied in great detail, little is known about their molecular properties, and information about the components of the membrane skeleton is scarce (Borland et al. 1985).

The principal supporting element of the red cell membrane skeleton is spectrin (Marchesi, 1983), which is linked non-covalently to an oligomeric form of actin (Brenner & Korn, 1979). The association between spectrin and actin is greatly enhanced by protein 4.1, which is itself linked to the cytoplasmic segments of glycophorin and band 3 (Anderson & Lovrien, 1984; Pasternak et al. 1985). These coupled associations of glycophorin to spectrin–actin via protein 4.1 and to band 3 via ankyrin (Bennet & Stenbuck, 1980) represent the first clearly defined links between trans-membrane glycoproteins and the membrane skeleton and serve as a model that can be used to evaluate the presence of similar macromolecular complexes in other cells.

The results described below show that immunologically defined analogues of the proteins of the red cell membrane also exist in cultured rat Sertoli cells. Since these Sertoli cells contain most of the major components it is conceivable that functional complexes exist in these cells that are comparable to those of the red cell membrane skeleton.

**MATERIALS AND METHODS**

*Culture conditions and cell labelling*

Cultures of Sertoli cells, from 20-day-old Wistar rats were prepared as described (Galdieri et al. 1981).

For immunoprecipitation experiments cells in culture were labelled for 18 h with $^{35}$S-methionine (sp. act. 1000 Ci mmol$^{-1}$) 20 μCi ml$^{-1}$ (NEN).

*Preparation of antibodies*

Monoclonal and polyclonal antibodies were prepared and characterized using established procedures. Mouse monoclonal antibodies to human erythrocyte spectrin were prepared as described (Yurchenko et al. 1982; Harris et al. 1986) and their specificity was determined by quantitative radioimmunoassays and immunoblotting using two-dimensional isoelectric focusing and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Antisera to fodrin and protein 4.1 were prepared in rabbits by immunizing them with purified human brain fodrin (Harris et al. 1985) or human erythrocyte protein 4.1 (Leto & Marchesi, 1984) and their specificity was determined as described above.
Immunofluorescence

Cells, grown on glass coverslips, were fixed and permeabilized by incubation for 10 min at room temperature in phosphate-buffered saline, pH 7-4 (PBS) containing 3-7 % paraformaldehyde and 0-2 % Triton X-100, followed by repeated washings with 25 mM-Tris·HCl, pH 8-1, 144 mM-NaCl and with PBS, 0-2 % foetal calf serum (FCS). Coverslips were incubated with specific antibodies diluted in PBS, 0-2 % FCS for 45 min at room temperature, extensively washed in PBS/FCS and stained with rabbit anti-mouse immunoglobulin G (IgG) or goat anti-rabbit IgG labelled with fluorescein (Kirkegaard & Perry Lab. Inc.). Polymeric actin was labelled with rhodamine-labelled phalloidin (Sigma).

Immunoprecipitation

Cellular monolayers were scraped with a rubber policeman and solubilized in buffer A (10 mM-Tris·HCl, pH 8-0, 5 mM-EDTA, 1 mM-dithiothreitol, 1 % SDS, 2 mM-phenylmethylsulphonyl fluoride (PMSF), 1 % Aprotinin), boiled for 2 min, sonicated and centrifuged at 12000 g. The supernatants were then diluted 10 times in buffer B (20 mM-Tris·HCl, pH 8-0, 130 mM-NaCl, 5 mM-EDTA, 1 % Triton X-100, 0-1 % SDS, with protease inhibitors as in buffer A). Samples were incubated with antibodies followed by Sepharose–protein A beads. The beads were washed several times by centrifugation in buffer B and then boiled for 10 min in Laemmli sample buffer. The solubilized proteins were analysed by SDS–PAGE (Laemmli, 1970) and fluorography.

Immunoblotting

Cellular monolayers were solubilized in Laemmli sample buffer and separated by 12 % polyacrylamide–SDS electrophoresis. The proteins were then transferred to a nitrocellulose sheet and incubated with antibodies and 125I-labelled protein A following the procedure of Towbin et al. (1979).

RESULTS

Monoclonal antibodies to α and β subunits of human erythroid spectrin have been used in indirect immunofluorescence experiments on monolayers of rat Sertoli cells cultured in vitro.

Using both antibodies, the fluorescence was organized along the plasma membrane and in fibres that crossed the cells along a longitudinal axis in a parallel way (Fig. 1A,B). Similar results were obtained when polyclonal antibodies directed to the $80\times10^3 M_r$ peptide of the α-chain of spectrin were used. Both the monoclonal and polyclonal antibodies directed to the $80\times10^3 M_r$ peptide of the α-chain of spectrin also diffusely stained the perinuclear region at one pole of the cells. The fluorescent fibres showed a parallel organization, which seemed to follow that of actin fibres as demonstrated by double immunofluorescence experiments with rhodamine-labelled phalloidin (Fig. 1C,D).

We have also decorated the cells with polyclonal antibodies that recognize both subunits of human brain spectrin (fodrin). These immunoglobulins mainly stained the cellular edges (Fig. 1E). Affinity-purified antibodies to proteins 2.1 and 4.1 were also used. Whereas the former did not show any appreciable staining of the cells above the background fluorescence, the latter decorated filamentous structures very similar to those obtained when anti-α and -β spectrin antibodies were used (Fig. 1F).

Immunoprecipitation experiments were performed to characterize the components of Sertoli cells to which antibodies bound. Monoclonal antibodies to α and
Fig. 1. Immunofluorescence micrographs of Sertoli cells treated with: A, monoclonal antibodies to the \( \alpha \) subunit of human erythroid spectrin; B, monoclonal antibodies to the \( \beta \) subunit of human erythroid spectrin; C, polyclonal antibodies to the \( 80 \times 10^3 M_r \) peptide of human erythroid \( \alpha \) spectrin; D, rhodamine-labelled phalloidin for polymeric actin (same cells as in C); E, polyclonal antibodies to human brain spectrin (fodrin); F, polyclonal antibodies to human erythroid protein 4.1. A, \( \times 2700 \); B, \( \times 2200 \); C, D, \( \times 1700 \); E, \( \times 640 \); F, \( \times 970 \).
β subunits of human erythroid spectrin immunoprecipitated polypeptides from rat Sertoli cells with \( M_r \) values similar to their red cell analogues (Fig. 2A,B). Different results were obtained with polyclonal antibodies to human fodrin, when polypeptide analogues to the \( \alpha \) and \( \gamma \) subunits of spectrin were immunoprecipitated (Fig. 2C). These results were also confirmed by immunoblotting experiments in which affinity-purified polyclonal antibodies to human erythrocyte protein 4.1 were also used. These experiments demonstrated the presence in Sertoli cells of polypeptides related to the \( \alpha \), \( \beta \) and \( \gamma \) subunits of spectrin (data not shown), as well as of polypeptides immunologically related and with \( M_r \) values similar to protein 4.1 of the human red cell (Fig. 3).

When immunoprecipitations were performed using monoclonal antibodies to the \( \beta \)-chain, a prominent band with \( M_r \) around \( 58 \times 10^3 \) was present together with the band corresponding to \( \beta \) spectrin (Fig. 2B). The \( 58 \times 10^3 \) \( M_r \) band was not evident when the immunoprecipitation was performed using a monoclonal antibody to the \( \alpha \)-chain or polyclonal antibodies to fodrin (Fig. 2A,C). We then immunoprecipitated Sertoli cell spectrin using a polyclonal antibody to human erythroid spectrin and the main band that appeared was one corresponding to the \( M_r \) of the \( \alpha \) subunit. In this case, however, a band of \( M_r 58 \times 10^3 \) appeared also. This band co-migrated with vimentin when this protein was immunoprecipitated with a specific polyclonal antibody.

---

![Fig. 2](image-url)  
**Fig. 2.** Fluorography of PAGE of immunocomplexes recovered after treatment of \([^{35}S]\)methionine-labelled Sertoli cells with: A, monoclonal antibodies to the \( \alpha \) subunit of human erythroid spectrin; B, monoclonal antibodies to the \( \beta \) subunit of human erythroid spectrin; C, polyclonal antibodies to human brain spectrin. \( M_r (\times 10^3) \) are given at the left in Figs 2, 4, and at the right in Fig. 3.
Fig. 3. Detection of a protein 4.1-like polypeptide in rat Sertoli cells. Cells were processed for SDS–PAGE and then immunoblotted with antibodies specific for human protein 4.1 and with $^{125}$I-labelled protein A. The resulting autoradiogram shows that a polypeptide of $M_r$ 78 000 (arrowhead) reacted with the antibodies.

Fig. 4. Fluorography of PAGE of immunocomplexes recovered after treatment of $[^{35}S]$methionine-labelled Sertoli cells with: A, polyclonal antibodies to vimentin; and B, polyclonal antibodies to human erythroid spectrin.
Sertoli membrane skeleton

Fig. 5. Immunofluorescence micrograph of Sertoli cells treated with polyclonal antibodies to hamster vimentin. ×640.

antibody (Fig. 4). Polyclonal antibodies to vimentin were also tested in immunofluorescence experiments showing the distribution typical of this kind of intermediate filament (Fig. 5).

DISCUSSION

Immunochemical evidence for the presence of membrane skeletal proteins of red cells has been found in a wide variety of non-erythroid cell types (Mangeat & Burridge, 1984; Levine & Willard, 1981; Repasky et al. 1982; Glenney & Glenney, 1983) and thus their presence in Sertoli cells is not unexpected. However, it has been difficult to appreciate the significance of these observations without some positive indication that these proteins function in some skeletal capacity that is comparable to their role in red cells. Several lines of evidence support the idea that the analogues that we have detected in Sertoli cells may play such a role.

The four proteins that make up the bulk of the red cell membrane skeleton remain insoluble when red cell ghost membranes are treated with Triton X-100, and the Sertoli cell analogues share this property. Copies of each of three components (actin, spectrin and protein 4.1) are present in all the cells, and most of the material seems to be arranged in a fibrillar network.

The identity of these Triton-insoluble proteins as red cell analogues is based on both their capacity to react with specific antibodies raised against red cell components and their subunit size as determined by SDS–PAGE. Two sets of monoclonal antibodies directed against human erythrocyte spectrin were used to identify spectrin-like material in these cells, and the specificity of each antibody was determined by quantitative radioimmunoassays and immunoblotting techniques. Antibodies directed against the spectrin α subunit reacted solely with a peptide derived from the amino-terminal end of this subunit, while the antibodies raised
against the β subunit reacted only with peptides from the carboxy-terminal region of this polypeptide chain. Neither antibody reacted with any other domain of spectrin nor with any other component of the red cell membrane.

The distribution of spectrin-like proteins in Sertoli cells as Triton-insoluble filaments that traverse the length of the cells, following the actin bundles, was clearly evident when monoclonal antibodies directed against both α and β subunits of spectrin were used to stain the cells. The fibrillar distribution was also obtained with anti-4.1 sera, but to a lesser extent. When anti-fodrin antibodies were used the labelling indicated that fodrin was primarily located at the plasma membrane.

The distribution of anti-spectrin reactivity in the form of extended filaments could be explained either by the presence of spectrin-like molecules that have polymerized in an end-to-end fashion to create long filaments, or by the attachment of spectrin-like molecules to pre-existing filamentous structures composed of some other protein or proteins. Since Sertoli cells have a rich array of filamentous actin and spectrin-like proteins might be expected to bind to actin, this latter model is perhaps the most likely. The presence of 4.1-like protein at the same sites is another point in favour of this interpretation, since red cell protein 4.1 greatly augments the association of red cell spectrin to F-actin. The possibility that these spectrin-like proteins are associated with other cytoplasmic proteins cannot be ruled out, considering the co-precipitation, in our experiments, of vimentin with spectrin and the data that were published recently on interactions between vimentin and protein 2.1 (Mangeat & Burridge, 1984; Georgatos & Marchesi, 1985; Georgatos et al. 1985).

Spectrin-like molecules have been described in many cell types and in most of the cases the sizes of the subunits are comparable to those of fodrin (α/γ) (Nelson & Lazarides, 1983, 1984; Lazarides et al. 1984). Studies on avian cells have demonstrated the sequential or simultaneous expression of all three subunits of spectrin (α, β and γ) only in muscle cells (Nelson & Lazarides, 1983) and neurones (Lazarides et al. 1984), where different isoforms of ankyrin are also found (Nelson & Lazarides, 1984). Our findings provide the first evidence of the simultaneous presence of the three subunits in a mammalian cell. The α/β and α/γ dimers in neurones are arrayed in different parts of the cytoplasm (Lazarides et al. 1984). In non-erythroid chicken cells the α subunit has been reported to be highly homologous to erythroid α spectrin (Repasky et al. 1982). Our studies with Sertoli cells cultured in vitro indicate that antibodies directed against subunits both of fodrin and spectrin do not co-distribute. It is conceivable that in vivo the complex and polarized structure of Sertoli cells might lead to a differential distribution of the two spectrin forms. These data could suggest that our monoclonal antibodies against α erythroid spectrin and polyclonals against α fodrin recognize sequences specific for each of the two isoforms that in our cells display a different intracellular localization, probably due to the association with the β and γ subunits, respectively. In fact, the 80X 10^3 M^−1, peptide of the α erythroid subunit to which the antibodies are directed does not seem to be shared by the α subunit of fodrin (Morrow, personal communication). On the other hand, in certain neurones the β and γ subunits are expressed in distinct membrane domains, probably associated with specific ankyrin isoforms (Lazarides et al. 1985).
et al. 1984; Nelson & Lazarides, 1984; Moon et al. 1985; Nelson & Lazarides, 1985). Some of our data are in disagreement with a recent report (Borland et al. 1985) in which the absence of the $\alpha/\beta$ form is described and a different pattern of intracellular distribution of spectrin-like proteins in Sertoli cells is shown. We believe that the discrepancy between the data might be due to the different specificities of the antibodies used.

Polypeptides of lower molecular weight that react with anti-spectrin antibodies are often encountered in non-erythroid cells, but it has been difficult to decide whether they represent partially degraded forms of higher molecular weight polypeptides that are preparative artifacts or gene products of modified spectrin genes.

In contrast to what has been reported from experiments on birds (Granger & Lazarides, 1984), rat Sertoli cells seem to express a form of protein 4.1 that displays a molecular weight comparable to its erythroid analogue and its distribution suggests an association with spectrin, whereas we have not been able to detect any polypeptide immunologically related to the human erythroid form of ankyrin. We do not know whether ankyrin is lacking in our cell type or, more probably, whether our antibodies recognize only erythroid sequences not shared by the Sertoli cell analogue.

The main conclusions of our work are: (1) rat Sertoli cells express polypeptides related to the $\alpha$, $\beta$ and $\gamma$ subunits of human spectrin and fodrin and to protein 4.1; (2) spectrin-like and fodrin-like proteins show two distinct intracellular distributions; (3) erythroid spectrin-like subunits and protein 4.1 analogue co-distribute within the cells with each other and with actin; (4) there might be some form of association between spectrin and vimentin in Sertoli cells.

We think that the discovery in a hormone-responsive mammalian cell of virtually the complete set of known proteins of the membrane skeleton offers a promising model for the study of the functional role of such structure in mediating transmembrane signalling.

This research was supported by grants from MPI and 'La Sapienza' University to E.Z. and to B.M.Z.

REFERENCES


(Received 15 May 1986 – Accepted 18 July 1986)