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CELL SURFACE PROTEINS IN THE EARLY EMBRYOGENESIS OF *PLEURODELES WALTLII*

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Surface proteins in the first embryonic stages (8–32 cells, morula, blastula, early and late gastrula) of *Pleurodeles waltlii* were selectively labelled by ^{125}I using lactoperoxidase and glucose/glucose oxidase. Iodination was effected either on non-dissociated embryos or after their dissociation with EDTA. On the outer surface of non-dissociated embryos the two-dimensional electrophoresis revealed only three groups of ^{125}I-labelled proteins which did not change during all studied stages. Quite different results were obtained with the cells of dissociated embryos. In addition to the iodinated proteins of the embryonic outer surface seven major iodinated proteins were identified. These proteins originate from the regions of cell-cell contacts in intact embryo. Their two-dimensional pattern in dissociated cells changes between stages 8–32 cells and morula. The next important difference was observed during gastrulation, which corresponds in *Pleurodeles waltlii* to the first morphogenetic movements. Therefore the outside and inside cell surfaces of embryo are different already at stage 8–32 cells (and probably earlier), before the first step of morphogenesis. The changes of cell surface proteins at early embryonal development take place inside the embryo, in the regions of cell-cell interactions.

Introduction

Cell surface has been suggested as important factor in cell differentiation and morphogenesis [1–3].

This hypothesis was experimentally corroborated by the data obtained by immunological techniques [4] as well as by the study of glycoconjugates expressed on the cell surface [5].

Also results obtained with teratocarcinoma cell lines have shown that well pronounced quantitative and qualitative changes of surface proteins take place in the very beginning of cell differentiation [6]. Nevertheless embryonal carcinoma cells aside of their embryonic properties possess also the properties of tumor cells. Therefore, we looked for a more suitable model for the study of cell surface proteins during the first stages of embryonal development. For the present study, we have chosen *Pleurodeles waltlii.*

As the cleavage of the fertilized amphibian egg proceeds, the overall volume of the embryo does not change, the surface of the external cells remains surrounded by the vitelline membrane. The first morphogenetic movements take place during the process of gastrulation [7].

The data supplied by electron microscopy have shown that at the onset of gastrulation the cell surface properties are modified [8–10]. The previous work revealed that cell surface glycoproteins involved in morphogenetic movements of gastrulation have concanavalin A-binding sites [11]. More-

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over it has been shown that dissociated cells obtained at the gastrula stages are also characterised by changes of surface charges [12] and changes of affinities for different lectins [13–15]. Finally, comparison between two-dimensional pattern derived from fertilized eggs and gastrula stages have shown changes in total protein synthesis [16].

In this paper, we compare the pattern of surface proteins of non-dissociated and dissociated embryos during early development of *Pleurodeles waltii*.

Materials and Methods

Embryos. Fertilized eggs were obtained from *Pleurodeles waltlii* maintained at the laboratory. The embryos were staged according to the instruction of Gallien and Durocher [17]. For each set of experiments twenty embryos from a single batch were selected. Eggs were mechanically dejellied, washed twice with Steinberg buffer pH 7.4 (0.2 M NaCl; 3.7 mM KCl; 19 mM $Ca(NO_3)_2$; 50 mM $MgSO_4$; 68 mM Tris-HCl; 10 mg/l sodium benzyl penicillin and streptomycin sulphate). Vitelline membrane was removed with forceps. Embryos and dissociated eggs were used. For dissociation, embryos were incubated during 15 to 30 min at room temperature in (Ca^{2+}, Mg^{2+})-free Steinberg solution which was 1 mM in EDTA, at pH 7.8.

Iodination. The lactoperoxidase glucose/glucose -oxidase procedure of iodination was used [18], with the following modifications: intact or dissociated embryos were washed and suspended in 2.5 ml of Steinberg buffer which was 5 mM in glucose. Then 5 μg of glucose-oxidase (228 units/mg, Sigma), 50 μg of lactoperoxidase (67 units/mg, Sigma) and 1 mCi of carrier free $Na^{125}I$ (100 Ci/l, Amersham) were added. The iodination was performed at room temperature for 30 min with constant agitation. Then eggs or cells were washed in 15 ml of Steinberg buffer made 150 mM in unlabelled KI.

Sample solubilization. The samples were dissolved in 150 μl of lysis buffer containing 9.5 M urea, 2% (w/v) Nonidet P 40, 2% (v/v) ampholine pH range 2–11 (Servalyt) and 5% (v/v) 2-mercaptoethanol. The samples were frozen rapidly and kept at −20°C.

Gel electrophoresis. (a) First dimension separation by isoelectric focusing. Gels 0.25 × 10.5 cm were prepared according to O'Farrell [19] using ampholines pH range 2–11. A sample was loaded into each gel and the gels were run at 300 V overnight. Then the gels were equilibrated for 30 min in 10 ml of buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% glycerol, 5% SDS, 5% 2-mercaptoethanol and 0.1% Bromophenol blue. For pH measurement a control gel was cut into 0.5 cm pieces which were incubated with 1 ml of distilled water for 10 min. Then pH was measured with Metrohm Herisau pH meter.

(b) Second dimension, separation on SDS polyacrylamide gels. Polyacrylamide gels were prepared according to O'Farrell [19]. Molecular weight marker proteins (galactosidase 130000; phosphorylase 96000; bovine serum albumin 69000; aldolase (subunit) 40000 and chymotrypsinogen 25000) were applied on the gel. Electrophoresis was performed at a constant current of 40 mA/gel.

Co-electrophoresis. Fibronectin (fibronectin bovine, IBF), actin and tubulin (from embryonal mouse brain, gift of P. Desnoulet). Each protein (50 μg) was electrophoresed with iodinated sample.

Staining, autoradiography. Gels were stained with Coomassie brilliant blue and destained in 45% methanol/10% acetic acid. The dried gels were exposed with Kodak X ray film.

Histoautoradiography. Blastulae were iodinated as described above. After labelling procedure, embryos were fixed with Smith fixator and embedded in paraffin. Thin sections (6 μm) were coated with Ilford L_4 emulsion, exposed at 4°C for 10 days, developped (D 19 Kodak) and stained with pyronin-methyl green.

Results

Histoautoradiography of iodinated embryos

Histoautoradiography served as control of the localisation of labelled proteins.

Embryos at the stage of late blastula were liberated from the vitelline membrane and submitted to lactoperoxidase iodination. The results of Fig. 1 show that ^{125}I labelling was found on embryo surface only; it was more concentrated in the region of the presumptive ectodermal cells located at the animal pole, than on the surface of

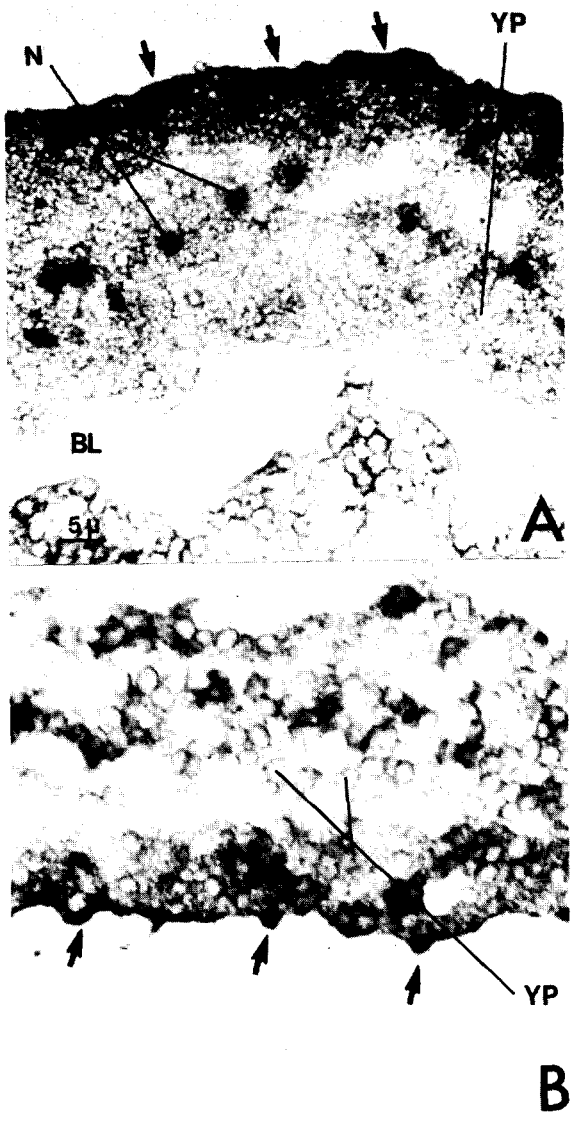

Fig. 1. Histoautoradiography of iodinated blastula. Arrows indicate the localisation of ^{125}I-labelling. (A) Presumptive ectodermal cells (animal pole). (B) Presumptive endodermal cells (vegetal pole). BL, blastocoele; N, nuclei; YP, yolk platelets. ^{125}I-labelling was developed as black grains.

presumptive endodermal cells situated at the vegetal pole. This could be explained by the difference in the cell size at the two poles. No labelling was detected on yolk platelets which contain phosvitin and lipovitellin. 3% and 0.1% of tyrosine were found in phosvitin and lipovitellin, respectively [20]. As radioiodination is oriented substantially on tyrosine residues of proteins, in the case of cell lysis even traces of these iodinated proteins would be easily detected as the major iodinated spots. Therefore the intensity of eventual labelling of vitellus permits to check the degree of cell lysis.

An other control for the specificity of enzymic iodination was the incubation of embryos under the same experimental conditions but in the absence of lactoperoxidase and glucose oxidase. No traces of ^{125}I-labelled proteins were found on autoradiograms.

Therefore, the results obtained by histoautoradiography of iodinated embryos confirmed that the lactoperoxidase iodination procedure permits to label specifically external membrane proteins in *Pleurodeles waltlii*.

Major iodinated surface proteins of dissociated cells

The cell surface proteins of dissociated embryos were examined at the following stages: 8–32 cells, morula, blastula, early gastrula, mid-gastrula, late gastrula and first stage of neurulation.

The experiments were performed four or five times for each stage. The reproducible results have shown ten major proteins (Fig. 2).

The first changes were found between stage 8–32 cells and morula (Fig. 2A, B): the quantity of labelled proteins 2 and 6 increase and proteins 8, 9 decrease. No change was observed between morula and blastula (Fig. 2B, C).

A comparison of Fig. 2D and 2E shows that proteins 4, 5 and 6 decrease during gastrulation. These results are summarized in Table I. In order to localise in our autoradiograms the position of certain known proteins we have co-electrophoresed the samples with either fibronectin, actin or tubulin. By this way, we have shown that protein 4 was co-electrophoresed with tubulin, protein 5 with actin and protein 10 with fibronectin.

Major iodinated proteins of non-dissociated embryos

In order to identify the proteins situated on the external surface of alive embryos, the same stages of development were examined.

In contrast to variety of cell surface proteins observed in different stages on dissociated embryos (Fig. 2A, B, C, D, E) three groups of proteins were found on the surface of non-dissociated em-

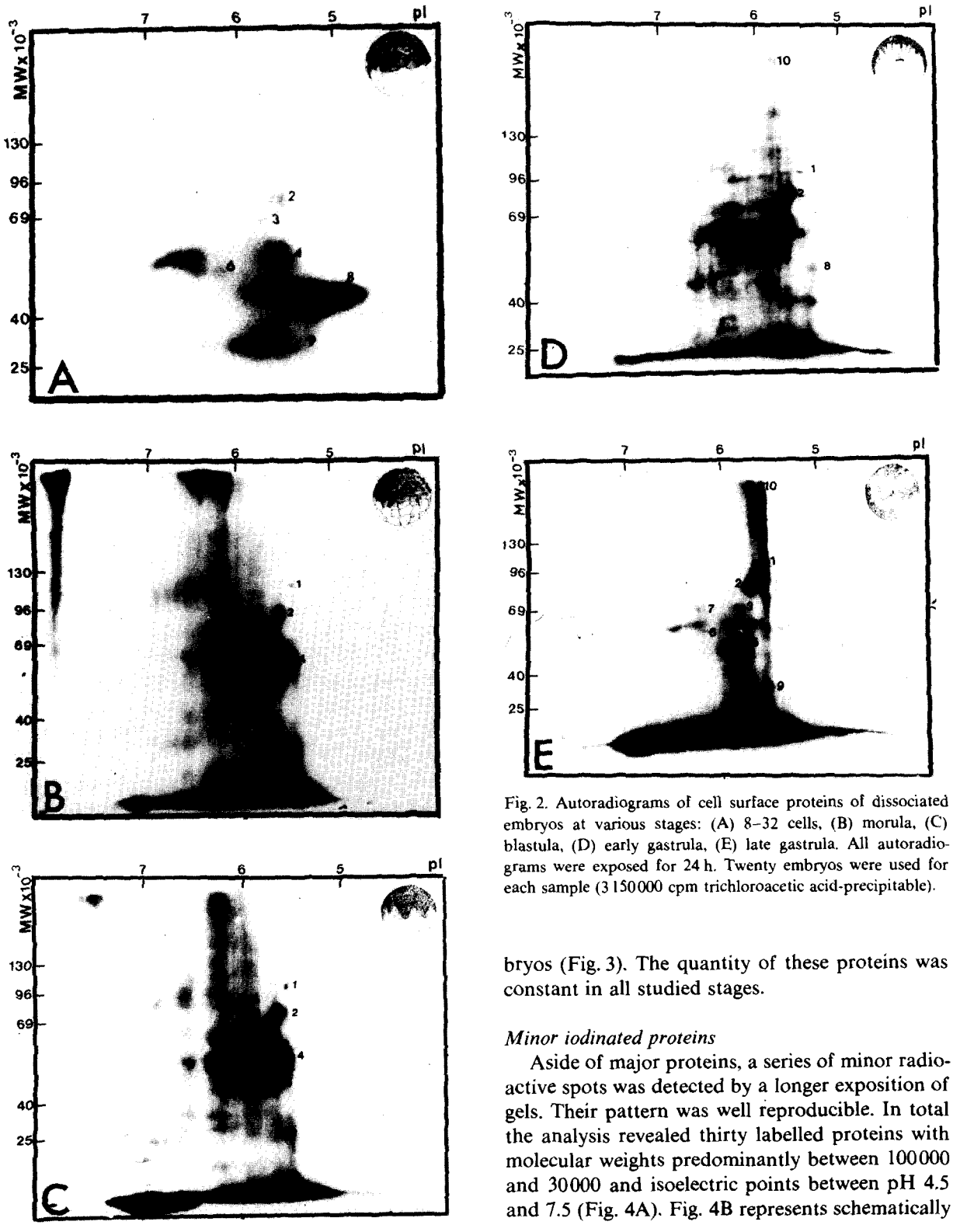

Fig. 2. Autoradiograms of cell surface proteins of dissociated embryos at various stages: (A) 8–32 cells, (B) morula, (C) blastula, (D) early gastrula, (E) late gastrula. All autoradiograms were exposed for 24 h. Twenty embryos were used for each sample (3 150000 cpm trichloroacetic acid-precipitable).

bryos (Fig. 3). The quantity of these proteins was constant in all studied stages.

Minor iodinated proteins

Aside of major proteins, a series of minor radioactive spots was detected by a longer exposition of gels. Their pattern was well reproducible. In total the analysis revealed thirty labelled proteins with molecular weights predominantly between 100000 and 30000 and isoelectric points between pH 4.5 and 7.5 (Fig. 4A). Fig. 4B represents schematically

TABLE I

MAJOR CELL SURFACE PROTEINS LABELLED ON DISSOCIATED EMBRYOS

Protein No.	Molecular weight ($\times 10^{-3}$)	Iso-electric point	Cleavage 8–32 cells→ morula→ blastula	Gastrulation early→ late gastrula
2	80	5.6	increases	increases
4	56	5.6	constant	decreases
5	45	5.8	constant	decreases
6	52	6.3	increases	decreases
8	40–50	5.0–6.0	decreases	constant
9	30	5.6–6.3	decreases	constant
10	220	5.7	–	appears

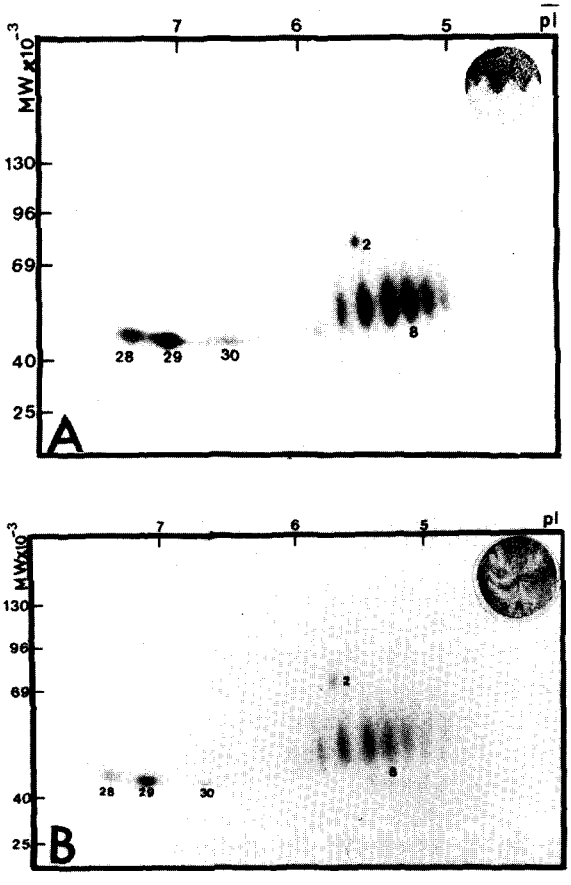

Fig. 3. Autoradiograms of cell surface proteins of non-dissociated embryos. (A) Blastula (4 days exposition). (B) Late gastrula (2 days exposition). Twenty embryos were used for each sample (311 000 cpm trichloroacetic acid-precipitable).

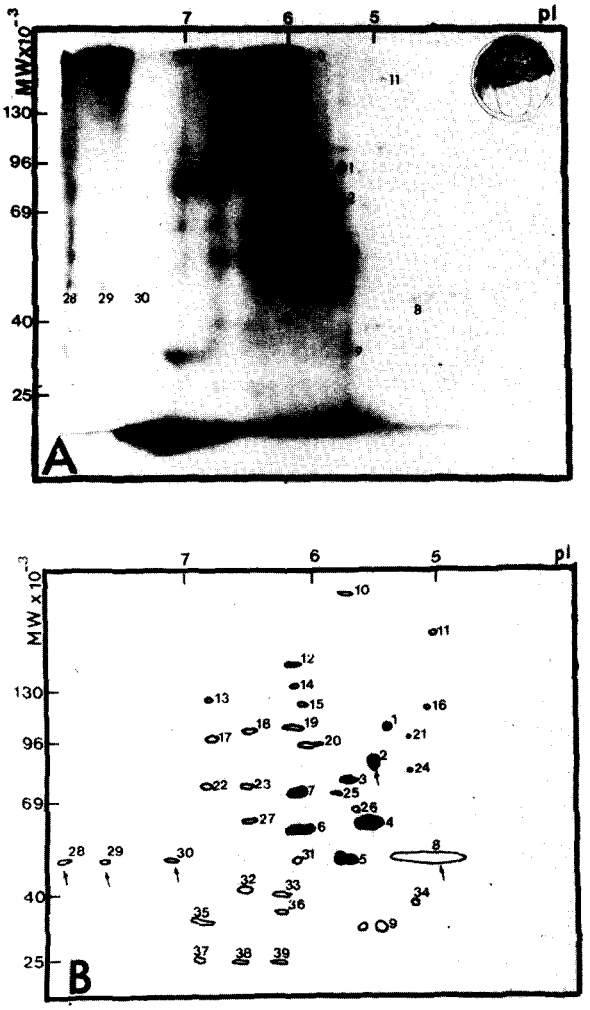

Fig. 4. (A) Total iodinated proteins at the gastrula stage. Various lengths of photographic exposure were used to show minor proteins. (B) Schematic representation of total iodinated proteins at the various stages studied. Arrows indicate cell surface proteins of non-dissociated embryos only. ○, minor proteins; ●, major cell surface proteins found on dissociated embryos.

all iodinated proteins which were found. No significant changes were observed in the pattern of minor surface proteins during different stages of early embryonal development.

A comparison of iodinated and Coomassie blue stained proteins

Whereas the radioautography reveals only the cell surface proteins, staining by Coomassie blue shows the total proteins present in the cells. Fig. 5

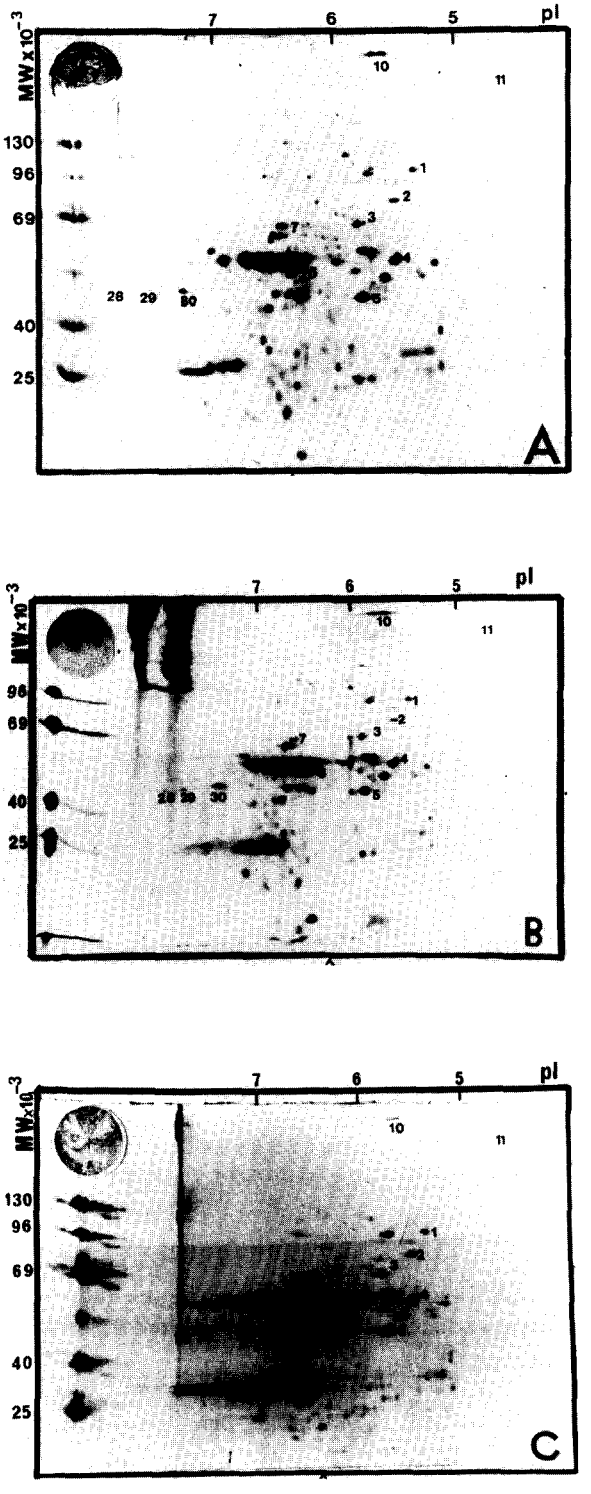

Fig. 5. Coomassie blue staining of total protein from (A) 8–32 cells, (B) blastula, (C) late gastrula.

shows the proteins stained by Coomassie blue. No significant changes in total cell proteins could be found in any of the analysed stages. From a comparison of Fig. 2, 4A and 5, it is evident that in our experimental conditions iodination was highly selective.

Discussion

A choice of *Pleurodeles waltlii* for the study of cell surface proteins in the beginning of embryonal development gave important advantages. Firstly the abundance of embryos at well controlled stages permitted to repeat the experiments for each stage several times. Secondly the embryonal development took place in natural conditions.

In order to see whether the cell surface proteins situated on the outer surface of an embryo are different from those localized in the inner part of an embryo we have iodinated both the intact embryo and the cells after its dissociation.

In the case of non-dissociated embryos the cell-cell contacts are so tight that lactoperoxidase and glucose oxidase (mol. wt. 80000 and 105000, respectively) do not penetrate between the cells during labeling procedure. As can be seen from Fig. 1 ^{125}I labelling is entirely localized on the external embryonic surface.

The results of Fig. 3 have shown that there are neither quantitative nor qualitative changes on the external surface of the cells situated on the outside of the embryo. This surface contains only three groups of proteins available for iodination and these proteins do not change up to gastrula.

On the contrary, the experiments performed on free cells after dissociation of the embryo reveal an important number of labelled proteins (Fig. 4B). Their quantity changes with the different stages (Table I). The difference between proteins originated from the surface of embryo and the total number of iodinated proteins after dissociation, indicates the proteins occurring in the regions of cell-cell contacts.

A comparison of Fig. 2A and 3A shows that at stage 8–32 cells the proteins of external embryonic surface 2 and 8 represent an important part of all proteins labelled after dissociation. In later stages the relative quantity of protein 8 decreases.

The first significant change in pattern of ^{125}I-

labelled proteins in dissociated embryos was observed between stage 8–32 cells and morula (Fig. 2A and B). This can be partially explained by the decrease of the ratio between external embryonal surface and total cell surface which resulted from cell division. In the later stages after following cell divisions, this ratio changes in favour of total cell surface. The proteins originated from external embryonic surface (in the stages morula to gastrula) were well detectible after four days exposition. In these conditions, the major iodinated proteins were overexposed. This explains why the proteins of external embryonic surface (28–30 and 8) are not seen after 24 h exposition on the Figs. 2B, C, D, E. An exception represents protein 2 which occurs on external embryonic surface and which is also one of the major proteins labelled on the cells of dissociated embryos. No changes were found between morula and blastula (Fig. 2B and C). Although no significant changes in the pattern of major surface proteins were observed between blastula and early gastrula (beginning of gastrulation), however a certain increase of proteins of lower molecular weights (40000 and less) must be mentioned.

The next significant change in surface proteins was observed during gastrulation, between early and late gastrula. This stage in *Pleurodeles waltlii* corresponds to the first morphogenetic movements. Figs. 2C and 2D show an increase of labelled protein 2 and a decrease of proteins 4, 5, 6 as compared to other major surface proteins. On the contrary no significant changes were observed in the intensity of Coomassie blue staining for the major surface proteins between any of the stages compared. Therefore the changes in the intensity of their iodination reflect either their displacement towards the membrane surface or the changes of their conformation which render them more available for lactoperoxidase iodination.

In order to identify the position of fibronectin, tubulin and actin in the two-dimensional electrophoretograms we have co-electrophoresed each of these proteins with our iodinated samples. Fibronectin was found in the position of protein 10 (Fig. 4B). A Coomassie stained spot in this position was found in all studied stages (Fig. 5) but iodinated spot in the position of fibronectin was detected during gastrulation only. This observation deserves a comparison with previous analogous studies. The first appearence of fibronectin as matrix glycoprotein in mouse embryos was observed in 3-day blastocyst [21,22]. It is known that in early mouse development the first morphogenetic changes take place at this stage.

As concerns tubulin and actin, they co-migrated with iodinated proteins 4 and 5, respectively. Actually, the accumulated data indicate that the organization of actin changes during the first morphological step of differentiation, from non-organized state in surface ruffles in undifferentiated cells to actin cables in differentiated cells [23]. However, no evidence existed of its occurrence on membrane surface. As concerns tubulin, studies show that tubulin-like polypeptides in cervical ganglia cells are exposed on external surface of the cells [24]. It is evident, however, that an identical electrophoretic mobility even in two-dimensional electrophoresis is not sufficient to conclude on the identity of proteins.

Our results show that a difference exists between proteins exposed on the surface of the embryo and those occurring in the region of cell-cell contacts, already at the stage 8–32 cells and probably earlier.

This is not in contradiction with results obtained with embryonal carcinoma cells [25]: the authors suggest that during the formation of embryoid body the cells within the centre are unchanged, whereas the outside cell develop irreversibly as endoderm. In other study Lovell-Badge and Evans [26] have reported that most observed changes in protein synthesis during differentiation of embryonal carcinoma cells take place before the endoderm was morphologically discerned.

Moreover the results of this work have shown that whereas the protein pattern of the outside surface of embryo does not change between the stage 8–32 cells and late gastrula, the cell surface proteins of inside part change. The most important differences were found between stage 8–32 cells and morula and then during gastrulation, which corresponds in *Pleurodeles waltlii* to the first morphogenetic movements.

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