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Plasma membrane transglutaminase and cytosolic transglutaminase form distinct envelope-like structures in transformed human keratinocytes

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Cross-linked envelope formation in the transformed human keratinocyte line SV-K14 requires treatment of the cells with a Ca^{2+} ionophore. Depending on the culture conditions, different extracellular Ca^{2+} concentrations are necessary to trigger the process which is catalyzed by the enzyme transglutaminase. Confluent cells grown in the presence of serum express only the cytosoluble form of the enzyme and need 5 mM Ca^{2+} for optimum protein cross-linking, whereas serum-starved cells which additionally contain the plasma membrane associated form of the enzyme require only 1 mM Ca^{2+}. The envelope-like structures thus synthesized are morphologically and biochemically distinct.

Transglutaminase; Keratinocyte; Protein cross-linking; Pseudo-envelope

1. INTRODUCTION

The formation of a cornified envelope at the inner periphery of the plasma membrane is one of the final events in the terminal differentiation of epidermal keratinocytes [1]. This process requires the enzyme transglutaminase (EC 2.3.2.13), an elevated level of intracellular Ca^{2+}, and the presence of substrate proteins which are cross-linked by the enzyme via γ-glutamyl-ϵ-lysine isopeptide bonds [2,3].

In cultured human keratinocytes, transglutaminase exists in two different forms, which are probably distinct gene products. One of these forms is cytosoluble (TG_c), the other associated with the plasma membrane (TG_m) [4]. The involvement of the membrane-associated enzyme in envelope formation has been demonstrated [5–7], whereas the role of TG_c is still under discussion. Most recently, participation of TG_c in apoptosis, the process of programmed cell death, has been suggested [8].

Envelope formation is used as a marker to assess keratinocyte differentiation. Cultured epidermal cells, especially those of transformed lines which have an impaired ability to synthesize envelopes spontaneously, are usually treated with a Ca^{2+} ionophore in order to artificially increase the intracellular Ca^{2+} level. Cells that form envelopes under these conditions are called 'envelope-competent' [9] and have been shown to possess membrane-associated transglutaminase activity [6,10].

In the present paper, we demonstrate that even non-competent transformed human keratinocytes, which are devoid of TG_m, can synthesize a rigid, envelope-like structure ('pseudo-envelopes') provided enough Ca^{2+} is supplied. The morphology of both types of envelopes is compared and their

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Abbreviations: DMEM, Dulbecco's modification of Eagle's medium containing 5.5 mM glucose and 4 mM glutamine; DTE, dithioerythritol; F12, Ham's medium F12; NHK, normal human keratinocytes; PBS, phosphate buffered saline; TG_c, cytosolic transglutaminase; TG_m, plasma membrane transglutaminase; Tris, tris(hydroxymethyl)aminomethane

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composition analysed by cyanogen bromide peptide mapping.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Culture media and fetal calf serum were obtained from Flow Laboratories and Gibco. Calcium ionophore A23187 and deoxyribonuclease I were purchased from Boehringer Mannheim. Cyanogen bromide was obtained from Sigma. Products for gel electrophoresis were from Bio-Rad, Sigma and Serva.

2.2. *Cells and culture conditions*

The SV-40 transformed human foreskin keratinocyte line SV-K14 (a kind gift from Drs B. Lane and J. Taylor-Papadimitriou, Imperial Cancer Research Fund, London) was used after about 20 passages. The cells were grown at 37°C (5% CO_2, humidified atmosphere) in DMEM and F12 (1:1) containing 100000 units penicillin, 100 mg streptomycin and 250 mg amphotericin B per liter of medium, which was supplemented with 5% (v/v) fetal calf serum. Envelope-competence was achieved by culturing confluent cells for 4 days in serum-free medium, with a medium change after two days.

2.3. *Trypsination of the cells*

Cells were washed twice with PBS before being treated for 8 min at 37°C with PBS containing 0.05% (w/v) trypsin and 0.02% (w/v) EDTA. The trypsination was stopped by the addition of fetal calf serum to give a final concentration of 20% (v/v).

2.4. *Induction of cornified envelope formation*

The procedure of Cline and Rice [9] was used with some modifications. 4×10^5 envelope-competent and confluent cells were incubated in 0.5 ml Ca^{2+} free DMEM/F12 containing 10 μg of the calcium ionophore A23187. To study the Ca^{2+} dependency of envelope formation, the incubation medium was supplemented with increasing concentrations of $CaCl_2$. After 3 h, 50 μl of 20% (w/v) SDS containing 1 mg DTE was added. The suspension was heated for 5 min at 100°C. After cooling, 10 μl of a deoxyribonuclease I solution (1 mg/ml) was added to prevent aggregation of the envelopes. Envelopes were counted with a hemocytometer chamber (five independent determinations for each sample).

2.5. *Purification of cornified envelopes*

Cornified envelopes were purified by boiling the samples under vigorous agitation for 10 min in a reaction mixture consisting of 2% (w/v) SDS and 0.1% (w/v) DTE. All of the envelopes were sedimented by centrifugation (for 5 min at 3500 × g). They were resuspended in fresh reaction mixture. Boiling and centrifugation were repeated four times.

2.6. *Cyanogen bromide cleavage of cornified envelopes [11]*

The protein content of the envelope preparations was determined by the Lowry procedure after digestion of an aliquot with proteinase K (50 μg/ml). The envelopes were suspended in 70% (w/w) formic acid, and 100 mg CNBr was added per mg envelope protein. After 24 h at room temperature the reaction was complete, and the mixture was diluted 1:5 with distilled water. After lyophilization, the peptides were suspended in Laemmli sample buffer [12].

2.7. *Electrophoresis*

SDS-polyacrylamide gel electrophoresis was carried out under reducing conditions as described [12]. A 15% gel was used, and 30 μg of envelope protein was routinely applied on each slot. The gels were silver stained according to the method of Ansorge described in [13].

3. RESULTS AND DISCUSSION

The transfer to serum-free medium of confluent SV-K14 cultures, grown in the presence of 5% fetal calf serum, results in the expression of substantial amounts of plasma membrane-bound transglutaminase (TG_m) [6]. After about 4 days the cells are envelope-competent. When these cells are treated with the Ca^{2+} ionophore A23187 in the presence of increasing extracellular calcium concentrations the cells synthesize a cornified envelope (fig.1). At about 1 mM Ca^{2+}, 100% of the cells

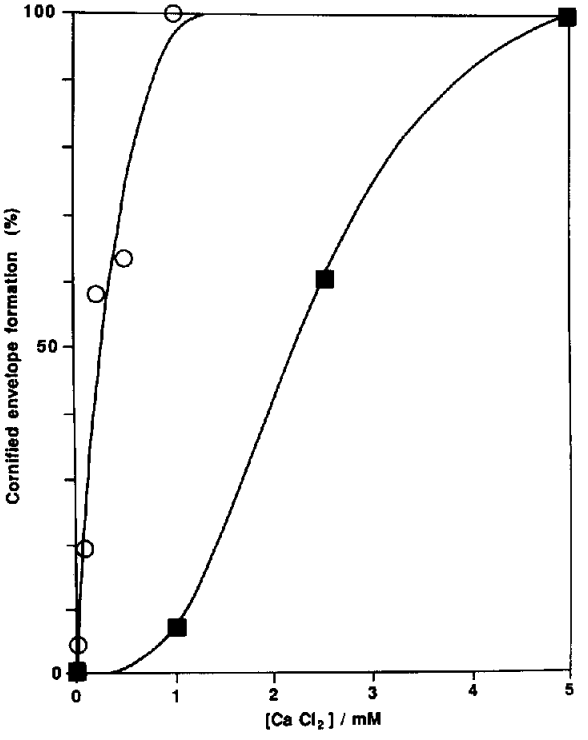

Fig.1. Ca^{2+} ionophore induced envelope formation in envelope-competent SV-K14 cells (○) and in confluent, non-competent cells (■) depending on the extracellular Ca^{2+} concentration.

form an envelope. Confluent but non-competent cells treated the same way synthesize, if any, very few envelopes. However, increasing the extracellular Ca^{2+} concentration to 5 mM gives rise to the formation of envelope-like structures even though the cells are not envelope-competent and contain hardly any TG_m. Thus, these pseudo-envelopes can be considered to result from protein cross-linking catalyzed by TG_c. In the presence of 1 mM cystamine, a transglutaminase inhibitor, pseudo-envelope formation is completely suppressed (not shown). The higher Ca^{2+} concentration necessary for TG_c (compared to TG_m) to form envelope-like structures in living cells corresponds to its higher Ca^{2+} requirement in cell-free extracts when the coupling of putrescine to *N,N*-dimethylcasein is measured [4].

The envelope-like structures synthesized by TG_m and TG_c exhibit under Nomarski contrast a different morphology (fig.2A to be compared with fig.2B). 'TG_m envelopes' are very irregularly shaped and appear thin and fragile, whereas 'TG_c pseudo-envelopes' are round and appear to be much thicker and more rigid.

The morphological differences between the two structures are reflected in the peptide patterns obtained after cyanogen bromide cleavage and subsequent SDS-polyacrylamide electrophoresis (fig.3). The peptide patterns of TG_m envelopes produced by competent SV-K14 cells are defined and similar but not identical to those obtained from normal human keratinocytes cultured essentially as described by Rheinwald and Green [14] (fig.3A, lane 2 to be compared with lane 3). The pattern of TG_c pseudo-envelopes, however, remains, over a wide range of dilutions, a continuous unresolved

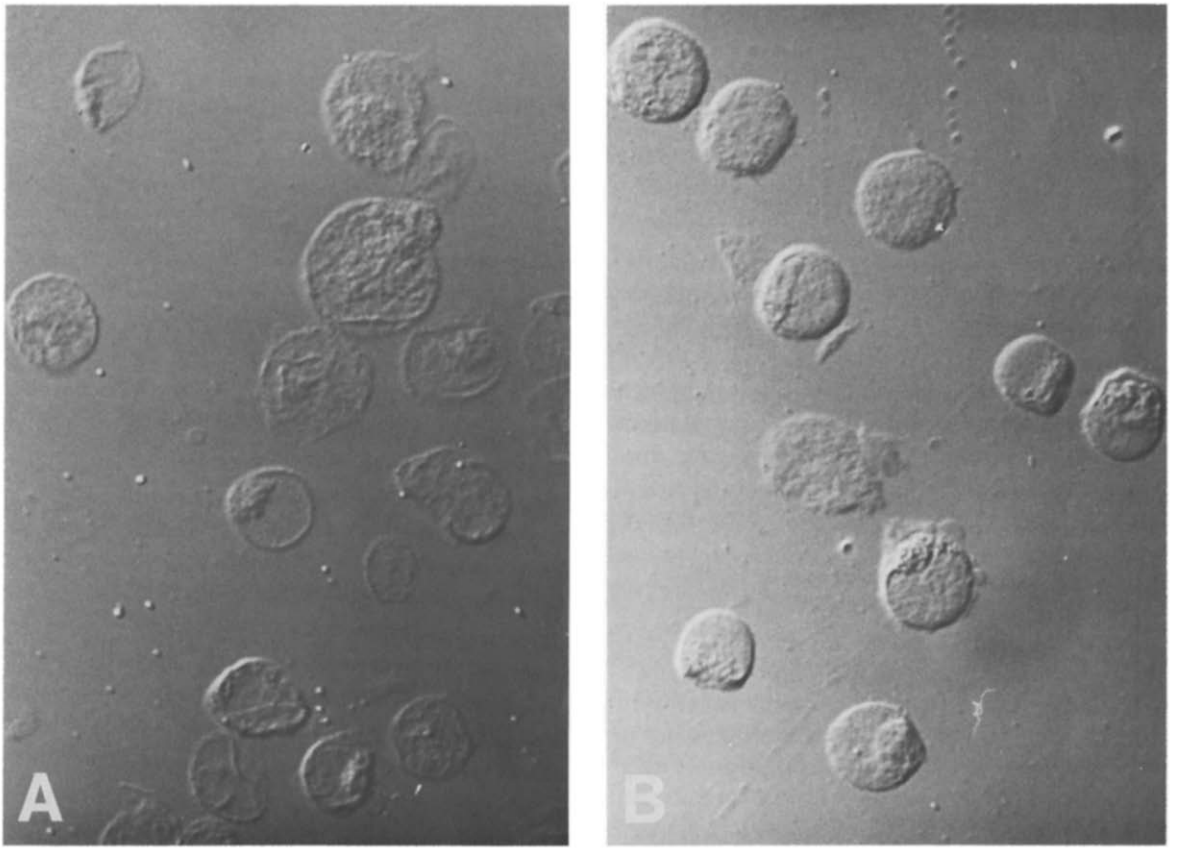

Fig.2. Nomarski contrast microscopy of cornified envelopes synthesized in envelope-competent SV-K14 cells (A) and pseudo-envelopes obtained after activation of TG_c in confluent SV-K14 cells (B).

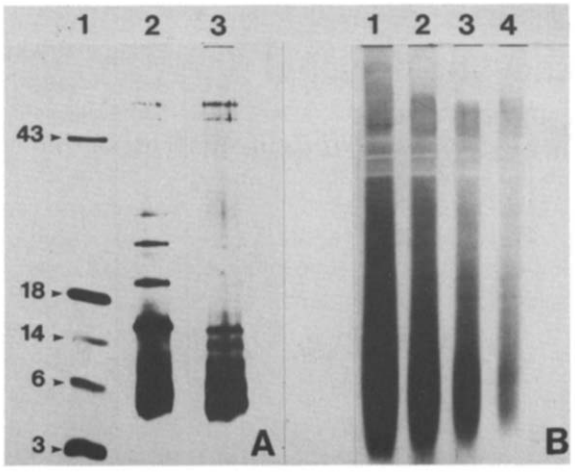

Fig.3. (A) Cyanogen bromide peptide maps of cornified envelopes formed, after ionophore treatment, in normal human keratinocytes (lane 2) and competent SV-K14 cells (lane 3). Lane 1 gives the position of molecular mass marker proteins. (B) Cyanogen bromide peptide maps of pseudo-envelopes synthesized in confluent SV-K14 cells. 30, 15, 7.5 and 3.75 μg of envelope protein were applied on slots 1–4, respectively. Essentially the same pattern is obtained when competent cells have been exposed to 5 mM Ca^{2+} in the presence of A23187.

smear (fig.3B, lanes 1–4). When competent cells which express both TG_c and TG_m are treated with the ionophore in the presence of high Ca^{2+} (5 mM), they synthesize the same pseudo-envelopes as confluent cells giving rise to the same unresolvable peptide smear after electrophoretic separation.

The striking differences in the morphology and the peptide patterns could be explained by a wider substrate specificity for the cytosolic enzyme. Furthermore, it is possible that the fixation of TG_m at the inner surface of the plasma membrane restricts protein cross-linking to two dimensions by this form of the enzyme giving rise to an envelope in the strict sense, whereas the cytosolic distribution of TG_c allows cross-linking in three dimensions resulting in envelope-like protein conglomerates.

Our finding that TG_c can be stimulated to synthesize an envelope-like structure is interesting in view of an earlier publication by Simon and Green [15] and a recent report by Thacher and Levitt [16]. Simon and Green [17] describe the synthesis of small but microscopically visible insoluble cross-linked fragments upon activation of transglutaminase in fibroblasts which obviously contain no or only very little of the particulate transglutaminase. Thacher and Levitt show that human lung cancer cell lines in culture can be stimulated by treatment with a Ca^{2+} ionophore to form 'cross-linked envelopes' even though they do not express any TG_m. Since our results demonstrate that TG_c can be stimulated to cross-link cellular proteins to pseudo-envelopes, the concept of 'envelope-competence' should be used with care and only applied to cells in which envelope formation is correlated with TG_m activity.

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