Differential expression of cyclophilin isoforms during keratinocyte differentiation

Dominique CHATELLARD-GRUAZ, Jean-Hilaire SAURAT and Georges SIEGENTHALER*

Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14, Switzerland

Cyclophilin A, the major intracellular binding protein for the immunosuppressive drug cyclosporin A (CsA), was studied in human keratinocytes during differentiation both *in vivo* and *in vitro*. Analysis of cyclophilin by gel-filtration radiobinding-assay with tritiated CsA showed one specific radioactive peak at 17 kDa. By this technique, the levels of cyclophilin (mean 55.23 ± 8.43 pmol/mg protein) did not significantly differ during keratinocyte differentiation. When the protein extracts from calcium-induced differentiating keratinocytes and normal human skin were analysed by PAGE radiobinding-assay, two specific radioactive CsA-binding peaks were detected. The major peak

(R_f 0.13) was expressed in all samples (mean 47.32 ± 17.53 pmol/mg protein) whereas the minor peak (R_f 0.23) was dramatically decreased about 6-fold in abnormally differentiated skin (psoriasis) as well as in non-differentiated keratinocytes. At least six [3 H]CsA-binding isoforms with pI values ranging from 5.58 to 7.75 were detected by isoelectrofocusing autoradioblotting-assay in normal human skin; three of them immunoreacted with antibodies to cyclophilin. These results demonstrated the presence of several cyclophilin isoforms in human epidermal cells and an expression which correlated with the differentiation of human keratinocytes both *in vivo* and *in vitro*.

INTRODUCTION

Cyclosporin A (CsA), which is widely used as an immunosuppressive drug, also has biological effects on non-lymphoid cells including endothelial cells, kidney epithelial cells and keratinocytes [1–3]. Moreover, CsA has been reported to be effective in the treatment of severe psoriasis, a skin disease characterized by hyperproliferation and a defective differentiation of keratinocytes [4,5]. The antiproliferative effect of CsA on keratinocytes has been shown *in vivo* and *in vitro* [3,6,7] and might be relevant in part to its therapeutic effectiveness in psoriasis. Among the biological effects of CsA, the mechanisms leading to immunosuppression have been studied extensively. CsA inhibits T-cell activation and/or differentiation by preventing the activation of transcription factors (i.e. NF-AT) involved in interleukin-2 gene expression [8–10]. This effect appears to be mediated by CsA binding to a cytosolic receptor, termed cyclophilin [11]. It was reported that the cyclophilin–CsA complex could bind to calcineurin, a calcium- and calmodulin-dependent phosphatase, and inhibit its activity, thus implicating this phosphatase in events suppressed by CsA [12–14].

Cyclophilin A was isolated from the protein extract of calf thymus [15] and was subsequently shown to be an abundant, ubiquitous and highly conserved protein [16]. Two 17.8 kDa isoforms of cyclophilin A, a major one with a pI ranging between 8.1 and 8.6 and a minor one with a pI of 6.3–7.4, have been characterized in cytosol extracts from normal and neoplastic tissues [16]. Studies using both biochemical and molecular approaches have clearly shown the presence of several low-abundance CsA-binding proteins in human tissues and cells, including cytosolic, membrane-associated and secreted forms [17–22]. These cyclophilin isoforms (cyclophilin B [17], cyclophilin C [21], cyclophilin 3 [20] and cyclophilin 40 [22]) possess molecular masses varying from 17 kDa to 40 kDa. Whereas cyclophilin C appears to be expressed in a restricted number of tissues, other cyclophilin isoforms are expressed in a wide variety

of tissues and cell types and are not specific for T-cells [16–22]. All cyclophilin isoforms described to date possess a peptidyl-prolyl *cis-trans*-isomerase activity (PPIase) involved in folding or trafficking of several proteins [17,20–24]. Although the PPIase activity of cyclophilin is blocked by CsA, its inhibition does not seem to be sufficient for mediating the biological effects of CsA [25–27].

In order to study how CsA may act in human epidermal cells, we performed cyclophilin analysis with a modified PAGE radiobinding technique [28] and an isoelectrofocusing (IEF) radiobinding-assay on various stages of keratinocyte maturation. Thus CsA-binding proteins were studied in completely differentiating epidermis (normal human skin), in epidermis displaying an incomplete differentiation (psoriatic plaque), and in cultured human keratinocytes (differentiating and non-differentiated). We demonstrate the presence of several 17 kDa cyclophilin isoforms whose expression was significantly altered during keratinocyte differentiation. These results suggest that multiple cyclophilin isoforms might play a specific role in keratinocyte differentiation.

MATERIALS AND METHODS

Materials

CsA and [14 C]CsA (40.3 μ Ci/mg) (a gift from Dr. A. Stutz, Sandoz, Vienna, Austria) were dissolved in dimethyl sulphoxide (DMSO). Cyclophilin anti-serum was kindly provided by Professor R. E. Handschumacher (Yale University, New Haven, CT, U.S.A.). All other reagents and solvents were of the highest purity commercially available.

Human skin samples

Keratized (keratome set at 180 μ m) normal human skin (about 70 % epidermis) were obtained from the breast or the abdomen of four healthy women undergoing plastic surgery, as

Abbreviations used: CsA, cyclosporin A; IEF, isoelectrofocusing; PPIase, peptidyl-prolyl *cis-trans*-isomerase; DMSO, dimethyl sulphoxide; DTT, dithiothreitol.

* To whom correspondence should be addressed.

previously described [29]. Six adult patients with psoriasis vulgaris disease (three males and three females; between 16 and 56 years old, mean age 38 ± 5 years) not undergoing any treatment, gave their informed consent for keratome skin sampling. Keratome biopsies were obtained from involved stable plaques (referred to as lesional skin) located on buttocks (three cases) or thigh (three cases) with keratome set at $280 \mu\text{m}$ [29]. Normal and psoriatic specimens were quickly frozen in liquid nitrogen and stored at -80°C until use.

Culture of human keratinocytes

Human keratinocytes derived from epidermal sheet of newborn foreskin [30] were cultured in the presence of irradiated 3T3 cells according to a previously described method [31,32]. Keratinocytes were grown first at normal Ca^{2+} concentration (1.3 mM) until confluency and stratification, and then with low Ca^{2+} medium (0.03 mM) during 2 or 3 days [33]. In low Ca^{2+} medium, the differentiating cells were sloughed off in the medium while the non-differentiated cells remained attached to the culture dish. These cell populations, constituted 95% by keratinocytes, were separated from the culture as described previously [33]. The ability of cells to form cross-linked envelopes, the content of cellular retinoic acid binding-protein type II and the enzymic activity that transforms retinol to retinoic acid were taken as markers of cell differentiation. These markers were strongly expressed in the detached keratinocytes [33–35] which were termed 'differentiating' cells. The attached keratinocytes showing weak or absent expression of these markers were termed 'non-differentiated' cells [33–35].

Preparation of protein extracts

Lyophilized tissues and cells were homogenized with a tissue homogenizer (Polytron-PT7, Kinematica, Lucerne, Switzerland) in Tris/HCl 50 mM, pH 7.5, containing 25 mM NaCl/2 mM EDTA/1 mM dithiothreitol (DTT). The cocktail of proteinase inhibitor used contained 1 mM phenylmethanesulphonylfluoride, 25 mM tosyl-lysylchloromethane, 25 mM tosyl-phenylalanyl chloromethane, 0.7 $\mu\text{g}/\text{ml}$ aprotinin in Tris/HCl buffer. Supernatants were obtained after ultracentrifugation at $100\,000\text{ g}$ for 60 min at 4°C . Protein contents were determined by the method of Lowry et al. [36], with BSA as a standard.

Incubation with [^{14}C]CsA

Aliquots (100 μl) of protein extracts (300 μg) were incubated with [^{14}C]CsA (400 nM) for 1 h at 37°C and analysed by gel-filtration chromatography, PAGE and IEF techniques. Non-specific binding of [^{14}C]CsA was studied in the presence of a 100-fold excess of unlabelled CsA. The concentration of DMSO in the incubation solution never exceeded 4%.

Gel filtration

Samples incubated with [^{14}C]CsA were subjected to gel-filtration chromatography on a Sephadex G-75 column (1.0 cm $\times$ 15 cm) equilibrated with 100 mM Tris/HCl/50 mM NaCl/5 mM EDTA/2 mM DTT/20 mM NaN_3 , pH 7.4. Each fraction (0.3 ml) was mixed with 3 ml of Ultima Gold (Packard) and assayed for radioactivity in a liquid-scintillation counter. The amount of binding activity was expressed in pmol of [^{14}C]CsA bound per mg protein. Protein extracts (300 μg) of normal human skin without previous incubation with [^{14}C]CsA were also subjected to Sephadex G-75 filtration. Fractions 20–25 and 10–15 were separately pooled and concentrated to 100 μl using Centricon-3 microconcentrators with a 3 kDa cut-off (Amicon).

These concentrated fractions were analysed by SDS/PAGE or IEF and immunoblotting as described below.

PAGE radiobinding-assay

Protein extracts (300 μg) incubated with [^{14}C]CsA were subjected to non-denaturing PAGE on a 7.5% acrylamide slab gel (pH 8.8). PAGE analysis was performed according to published procedures [28] except that *N,N'*-methylenebisacrylamide in the running gel was adjusted to 0.6% instead of 1.2%. After electrophoresis, the gel was divided in lanes, cut into 2 mm bands and proteins were hydrolysed overnight in Solvable (Du Pont De Nemours, Bad Homburg, Germany). The resulting fractions were assayed for radioactivity as described above.

Immunoblotting

Proteins from epidermal cells and from the pooled and concentrated Sephadex G-75 protein fractions were first separated by SDS/PAGE (12%) or IEF and then blotted onto a nitrocellulose sheet. Proteins were denatured during 15 min in an aqueous solution containing 20% ethanol/10% acetic acid and then washed in isotonic PBS containing 0.05% Tween-20/0.05% NaN_3 . Immunoblots were visualized with horseradish peroxidase-conjugated sheep anti-(rabbit IgG), as previously described [28].

Protein elution from PAGE

Proteins separated by PAGE under non-denaturing conditions and showing electrophoretic mobility (R_f) of 0.13 and 0.23 were extracted from the gel by homogenizing the corresponding bands in PBS with a Polytron. The gel was removed by centrifugation at 700 g for 20 min and the supernatant was concentrated using Centricon-10 centrifugal microconcentrators with a 10 kDa cut-off. The separation of the major and minor CsA-binding forms was verified by PAGE radiobinding analysis and immunoblotting.

Isoelectrofocusing

IEF was performed using a horizontal PAGE device (Pharmacia, Uppsala, Sweden). The gel contained 5.8% acrylamide/13.3% saccharose/6.3% Ampholyte (Pharmacia), pH 3–10. Electrode solutions were: anode 0.04 M aspartic acid, cathode 1 M NaOH. IEF was performed during 2 h at 13°C at constant power (20 W). pH curves were obtained by excision of a series of segments from the gel (each 1 cm) followed by elution in H_2O for 30 min and measurement of each fraction by a conventional electrode.

Autoradioblotting

The separated protein bands obtained by IEF were transferred to nitrocellulose membranes [28]. Labelled proteins were visualized by direct autoradiography with Hyperfilm-MP (Amersham International).

Statistical analysis

Student's *t*-test was used to compare the amounts of cyclophilin in normal and involved psoriatic skin as well as in cultured differentiating and non-differentiated keratinocytes.

RESULTS

Gel-filtration chromatography analysis of cyclophilin

A typical elution profile of protein extract from normal human skin incubated with [^{14}C]CsA is shown in Figure 1. Similar

Figure 1 Sephadex G-75 column analysis for binding of [^{14}C]CsA to normal human skin extract

Protein extract (300 μg in 100 μl of buffer) was incubated with 400 nM [^{14}C]CsA for 1 h at 37 $^{\circ}\text{C}$, in the absence (▲) or presence (○) of a 100-fold molar excess of unlabelled CsA, and applied on a Sephadex G-75 column, which was eluted as described in Materials and methods. Fractions (300 μl) were assayed for radioactivity in 3 ml of scintillation liquid. Similar elution profiles were obtained with psoriatic skin and cultured keratinocytes.

Figure 2 SDS/PAGE immunoblot analysis of cyclophilin in human epidermal cells

Proteins were subjected to SDS/PAGE (12%) and then blotted onto nitrocellulose sheets. Cyclophilin isoforms were immunoreacted with rabbit cyclophilin A antiserum. Bound antibodies were visualized using horseradish peroxidase-labelled second step antibodies. Lane 1, concentrated fractions (20–25) of Sephadex G-75 column (see Figure 1); lane 2, protein extracts of normal human skin (300 μg); lane 3, concentrated fractions (10–15) of Sephadex G-75 column. Arrow indicates cyclophilin at 17 kDa.

elution profiles were obtained with protein extracts from lesional psoriatic skin and from differentiating and non-differentiated cultured human keratinocytes (data not shown). Two radioactive peaks were detected in fractions 22 and 28. The first peak (fraction 22) was specific since a 100-fold molar excess of unlabelled CsA abolished it totally (Figure 1). The second peak (fraction 28) corresponds to unbound [^{14}C]CsA. Saturation experiments showed that the binding was ligand concentration-dependent with maximal binding obtained at 0.4 μM of [^{14}C]CsA. Binding of [^{14}C]CsA was linear for between 50 μg and 350 μg of proteins.

Figure 3 PAGE analysis of [^{14}C]CsA binding to normal human skin

Protein extracts (300 μg) were incubated with [^{14}C]CsA in the absence (▲) or in the presence (○) of a 100-fold molar excess of unlabelled CsA, and subjected to PAGE analysis, as described in Materials and methods.

In order to demonstrate that the peak eluted at fraction 22 (Figure 1) corresponded to cyclophilin, fractions 20–25 were pooled and analysed by SDS/PAGE immunoblotting. An intense immunoreactive protein band at 17 kDa was observed (Figure 2, lane 1). Whole extracts of human epidermis (lane 2) showed a similar pattern. The additional high-molecular-mass immunoreactive protein bands observed in human epidermal extract were also seen in protein fractions eluted in fractions 10–15 during gel filtration (Figure 2, lane 3) but did not bind [^{14}C]CsA in non-denaturing gel-filtration chromatography (Figure 1). These high-molecular-mass bands seem therefore to correspond to non-specific immunoreactivity or to aggregation of denatured cyclophilin protein. The measurement by Sephadex G75 of cyclophilin amounts in normal human skin ($n = 4$), in lesional psoriatic skin ($n = 5$) as well as in human cultured non-differentiated ($n = 3$) and differentiating keratinocytes ($n = 3$) did not show any significant differences in CsA-binding activities (mean 55.23 ± 8.43 pmol/mg protein). Griffiths et al. [37] found similar results for normal human skin and psoriatic plaques with chromatography on a Sephadex LH-20 column.

PAGE analysis of cyclophilin

Supernatant of normal human skin incubated with [^{14}C]CsA and analysed by PAGE show two specific radioactive peaks which were completely abolished with an excess of CsA (Figure 3). Based on their total concentration, the first peak at R_f 0.13 (band 7) was termed 'major form', and the second peak at R_f 0.23 (band 12) 'minor form'. The occurrence of two peaks seems to be unrelated to protein degradation since similar results were obtained with supernatants containing a cocktail of proteinase inhibitors (data not shown). In order to characterize these two radioactive peaks, supernatants of normal human skin extracts were first resolved by PAGE under non-denaturing conditions. Proteins migrating at R_f 0.13 and 0.23 were separately extracted from the gel and analysed by SDS/PAGE immunoblotting. Both fractions showed an immunoreactive band at 17 kDa (data not shown), suggesting that major and minor forms correspond to members of the cyclophilin family.

The CsA-binding activities of the major and minor forms of tissue and cell extracts, as measured by PAGE, are presented in Table 1. The major cyclophilin form was found both *in vivo* and

Table 1 Cyclophilin isoforms analysis in human normal skin, psoriatic plaques and human cultured differentiating or non-differentiated keratinocytes

n = Number of experiments from samples of four normal epidermis, six lesional psoriatic skin, three non-differentiated cultured keratinocytes and three differentiating cultured keratinocytes; **P* < 0.01, ***P* < 0.02.

Specimen	<i>n</i> *	Cyclophilin isoforms activity (pmol CsA bound/mg protein)		Ratio (minor/major)
		Major	Minor	
Human skin				
Normal	7	54.96 ± 18.18	27.51 ± 17.25*	0.50
Psoriatic plaques	8	56.30 ± 24.54	4.78 ± 9.06*	0.08
Keratinocytes				
Non-differentiated	5	37.55 ± 16.16	1.94 ± 1.42**	0.05
Differentiating	6	40.47 ± 11.25	11.32 ± 6.96**	0.28

Figure 4 IEF-autoradioblotting and -immunoblotting analysis of cyclophilin isoforms

(a) Protein extracts were incubated with [¹⁴C]CsA and subjected to IEF-autoradioblotting analysis. Lane 1, proteins separated by PAGE at *R_F* = 0.13; lane 2, proteins separated by PAGE at *R_F* = 0.23; lane 3, protein extracts of normal human skin (250 µg); lane 4, protein extracts of normal human skin with a 10-fold excess of unlabelled CsA. (b) IEF-immunoblotting of protein extracts of normal human skin (400 µg). The arrowhead indicates the additional immunoreactive band at pI 5.78.

in vitro at similar levels in all samples studied. In contrast, the minor form was expressed at significantly lower levels in psoriatic plaques as compared with normal human skin (*P* < 0.01). When cultured keratinocytes were induced to differentiate and stratify in the presence of 1.2 mM extracellular calcium, the level of the minor form of cyclophilin dramatically increased about 5.8-fold (*P* < 0.02).

The total amounts of cyclophilin measured by gel filtration and the sum of the major and minor forms measured by PAGE in the various samples were approximately equal, suggesting that labelled CsA is not released during electrophoresis because of its high affinity for cyclophilin isoforms.

IEF analysis of major and minor cyclophilin forms

Proteins corresponding to the major and minor cyclophilin forms were extracted separately from PAGE gels and analysed by IEF for pI determination. CsA-binding proteins were detected by direct autoradioblotting. At least six radioactive bands were obtained from the major form (Figure 4a, lane 1), with pI values ranging from 5.58 to 7.75. Three radioactive bands were observed

in the minor form (lane 2), with pI values ranging from 5.58 to 6.66. The CsA-binding proteins at pI 5.89, 7.37 and 7.75 only belong to the major isoform because they were not detected in the minor form. The fact that the separation between the major and minor forms in PAGE was not entirely complete may explain the presence of identical isoform bands in both samples.

All radioactive bands detected in the major and the minor form were observed simultaneously when crude supernatant of normal human skin was studied by IEF-radiobinding assay (lane 3), ruling out the possibility that these bands are degradation products occurring during protein extraction from PAGE. Each radioactive band was almost abolished when IEF was performed with a 10-fold excess of unlabelled CsA, suggesting that these six radioactive bands specifically bind CsA (lane 4).

To correlate binding properties with immunoreactivity, the proteins of whole extracts of human epidermis were immunoblotted after separation by IEF (Figure 4b, lane 1). Four immunoreactive bands were observed, three of which corresponded to radioactive bands (pI 5.58, 6.66 and 7.75), suggesting that they represent CsA-binding isoforms. The CsA-binding proteins at pI 5.89, 6.54 and 7.37 were not detected by cyclophilin antiserum, possibly due to the fine epitope specificity of the latter. Another immunoreactive protein, which showed no binding of [¹⁴C]CsA (Figure 4a), was detected at pI 5.78 (indicated by the arrowhead in Figure 4b).

To determine if the presence of at least six CsA-binding proteins was specific to human epidermal cells we analysed cytosol extracts of normal human colon tissue. Analysis of this tissue by IEF-autoradioblotting showed the same pattern of radioactive bands as in normal human skin (data not shown), suggesting that these CsA-binding proteins were not restricted to human epidermal cells.

DISCUSSION

Cyclophilin isoforms have been found to occur in many different human tissues [17–22] by techniques that are however not appropriate for microscale analysis such as that necessary for skin biopsy samples. In the present study, we demonstrated that cyclophilin isoforms can be measured in tissues and cell culture with a new analytical procedure involving PAGE- or IEF-autoradioblotting techniques. The non-denaturing PAGE radiobinding-assay we developed for studies on retinoid-binding proteins [28] was modified to allow analysis of cyclophilins. We observed that cyclophilins migrate very slowly in PAGE, therefore, the concentration of the cross-linking agent *N,N'*-methylenebisacrylamide was decreased from 1.2% to 0.6% in order to facilitate the migration of cyclophilins by decreasing their interaction with the gel matrix.

Although analysis by gel filtration of cyclophilin levels showed no significant differences in epidermal samples, with the PAGE technique we were able to separate two cyclophilin isoforms (major and minor peak). The minor peak was higher in fully differentiated epithelium (normal skin) as compared with in an incompletely differentiated epithelium such as psoriatic plaque. Moreover, a similar difference was also observed with human cultured keratinocytes, as shown by an increase of minor isoform after differentiation by calcium. These results strongly favour the hypothesis that the state of keratinocyte differentiation is an important factor determining the regulation of minor cyclophilin isoforms both *in vivo* and *in vitro*.

Using IEF-autoradioblotting and IEF-immunoblotting, at least six different CsA-binding proteins and four immunoreactive bands respectively were separated. Cyclophilin has been analysed previously by sucrose density-gradient IEF in various normal

and neoplastic human tissues [16]. One major cyclophilin form (pI 8.1–8.6) and one minor form (pI 6.6–7.4) were constantly detected in all tissues. Human epidermal cells did not show cyclophilin species with pI values higher than 7.75, which could be explained by the various techniques used for detection. The detection of an immunoreactive form of cyclophilin not able to bind labelled CsA, suggests that this species might belong to the family of cyclophilin or cyclophilin-related proteins which might bind a ligand structurally different from CsA or which have lost their binding properties.

Whether the different isoforms we observed represent a post-translational modification or are processed from distinct gene products remains to be established. Southern-blot analysis of mammalian genomic DNA revealed the presence of at least 20 related genes or pseudogenes [38,39]. The two isoforms of cyclophilin A appear to be due to post-translational modification of a single precursor protein, while the previously described cyclophilin isoforms (cyclophilin B and cyclophilin C) could be the products of different genes [16,20,21].

We are particularly indebted to Professor R. E. Handschumacher for suggestions and improvement of the manuscript and for providing us with rabbit cyclophilin anti-serum. This investigation was partly supported by the Swiss National Science Foundation (grant no. 32-31338.91) to G.S.

REFERENCES

- Zoja, C., Furi, L., Ghilardi, F., Zilio, P., Benigni, A. and Remuzzi, G. (1986) *Lab. Invest.* **55**, 455–462
- Becker, G. M., Gandolfo, A. J. and Nagle, R. B. (1987) *Res. Commun. Chem. Path. Pharmacol.* **56**, 277–280
- Furie, M., Gaspari, A. A. and Katz, S. I. (1988) *J. Invest. Dermatol.* **90**, 796–800
- Ellis, C. N., Gorsulowsky, D. C., Hamilton, T. A., Billings, J. K., Brown, M. D., Heading, J. T., Cooper, K. D., Baadsgaard, O., Dvell, E. A., Annesley, T. M., Turcotte, J. G. and Voorhees, J. J. (1986) *JAMA, J. Am. Med. Assoc.* **256**, 3110–3116
- Griffiths, C. E. M., Powles, A. V., Leonard, J. N., Fry, L., Baker, B. S. and Valdimarsson, H. (1986) *Br. Med. J.* **293**, 731–732
- Dykes, P. J., Brust, J. and Marks, R. (1990) *Br. J. Dermatol.* **122**, 173–180
- Urabe, A., Kanitakis, J., Viac, J. and Thivolet, J. (1989) *J. Invest. Dermatol.* **92**, 755–757
- Elliot, J. F., Lin, Y., Mizel, S. B., Bleackley, R. C., Harnish, D. G. and Paetkau, V. (1984) *Science* **226**, 1439–1441
- Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, I. M., Lacy, E. and Crabtree, G. R. (1989) *Science* **246**, 1617–1620
- Flanagan, W. M., Corthesy, B., Bram, R. J. and Crabtree, G. R. (1991) *Nature (London)* **352**, 803–807
- Tropschug, M., Barthelmeß, I. B. and Neupert, W. (1989) *Nature (London)* **342**, 953–955
- Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I. and Schreiber, S. L. (1991) *Cell* **66**, 807–815
- O'Keefe, S. J., Tamura, J., Kincaid, R. L., Tocci, M. J. and O'Neil, E. A. (1992) *Nature (London)* **357**, 692–694
- Clipstone, N. A. and Crabtree, G. R. (1992) *Nature (London)* **357**, 695–697
- Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J. and Speicher, D. W. (1984) *Science* **226**, 544–547
- Koletsky, A. J., Harding, M. W. and Handschumacher, R. E. (1986) *J. Immunol.* **137**, 1054–1059
- Price, E. R., Zydowsky, L. D., Jin, M., Baker, C. H., McKeon, F. D. and Walsh, C. T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1903–1907
- Spik, G., Haendler, B., Delmest, O., Mariller, C., Chamoux, M., Maes, P., Tartar, A., Montreuil, J., Stedman, K., Kocher, H. P., Keller, R., Hiestand, P. C. and Movva, N. R. (1991) *J. Biol. Chem.* **266**, 10735–10738
- Hasel, K. W., Glass, J. R., Godbout, M. and Sutcliffe, J. G. (1991) *Mol. Cell. Biol.* **1**, 3484–3491
- Bergsma, D. J., Eder, C., Gross, M., Kersten, H., Sylvester, D., Appelbaum, E., Cusimano, D., Livi, G. P., McLaughlin, M. M., Kasyan, K., Prichett, W. P., Bossard, M. J., Brandt, M. and Levy, M. A. (1991) *J. Biol. Chem.* **266**, 23204–23214
- Friedman, J. and Weissman, I. (1991) *Cell* **66**, 799–806
- Kieffer, L. J., Thalhammer, T. and Handschumacher, R. E. (1992) *J. Biol. Chem.* **267**, 5503–5507
- Takahashi, N., Hayano, T. and Suzuki, M. (1989) *Nature (London)* **337**, 473–475
- Fischer, G., Wittman-Liebold, B., Lang, K., Kieffhaber, T. and Schmid, F. X. (1989) *Nature (London)* **337**, 476–478
- Bierer, B. E., Mattila, P. S., Standaert, R. F., Herzenberg, L. A., Burakoff, S. J., Crabtree, G. and Schreiber, S. L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9231–9235
- Bierer, B. E., Somers, P. K., Wandless, T. J., Burakoff, S. J. and Schreiber, S. L. (1990) *Science* **250**, 556–559
- Sigal, N. H., Dumont, F., Durette, P., Siekierka, J. J., Perterson, L., Rich, D. H., Dunlap, B. E., Staruch, M. J., Melino, M. R., Koprak, S. L., William, D., Witzel, B. and Pisano, J. M. (1991) *J. Exp. Med.* **173**, 619–628
- Siegenthaler, G. (1990) *Methods Enzymol.* **189**, 299–307
- Siegenthaler, G., Saurat, J.-H., Hotz, R., Camenzind, M. and Merot, Y. (1986) *J. Invest. Dermatol.* **86**, 42–45
- Kitano, Y. and Okada, N. (1983) *Br. J. Dermatol.* **108**, 555–560
- Rheinwald, J. G. and Green, H. (1975) *Cell* **6**, 331–343
- Rheinwald, J. G. and Green, H. (1975) *Cell* **6**, 317–330
- Siegenthaler, G., Saurat, J.-H. and Poncet, M. (1988) *Exp. Cell. Res.* **178**, 114–126
- Siegenthaler, G. and Saurat, J.-H. (1990) *Biochem. J.* **268**, 371–378
- Siegenthaler, G., Tomatis, I., Chatellard-Gruaz, D., Jaconi, S., Eriksson, U. and Saurat, J.-H. (1992) *Biochem. J.* **287**, 383–389
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Griffiths, C. E. M., Fisher, G. J., Harding, M. W., Elder, J. T. and Voorhees, J. J. (1990) *J. Invest. Dermatol.* **94**, 436–440
- Danielson, P., Forss-Petter, S., Brow, M., Calavetta, L., Douglass, J., Milner, R. and Sutcliffe, J. (1988) *DNA* **7**, 261–267
- Haendler, B., Hoefler-Warbinek, R. and Hofer, E. (1987) *EMBO J* **6**, 947–950