A role for phosphorylation in the dynamics of keratin intermediate filaments

Jesús M. Paramio[1)]
Cell and Molecular Biology Program, CIEMAT (IMA), Madrid/Spain

Received April 3, 1998
Received in revised version September 11, 1998
Accepted October 26, 1998

Keratin dynamics – protein kinases – protein phosphorylation – transfection – cell hybrids

Keratins undergo highly dynamic events in the epithelial cells that express them. These dynamic changes have been associated with important cell processes. We have studied the possible role of keratin phosphorylation-dephosphorylation processes in the control of these dynamic events. Drugs that affect the protein phosphorylation metabolism (activators or inhibitors of protein kinases or protein phosphatases) have been used in two different dynamic experimental systems. First, the behaviour of keratins after the formation of cell heterokaryons, and second, the assembly of a newly synthesised keratin after transfection into the pre-existing keratin cytoskeleton. The main difference between these two systems stems on the alteration of the amount of keratin polypeptides present in the cells, since in heterokaryons this amount was unaltered whilst in transfection experiments there is an increase due to the presence of the transfected protein. We observed in both systems that the inhibition of protein kinases led to a delayed dynamic behaviour of the keratin polypeptides. On the contrary, the inhibition of protein phosphatases by okadaic acid or the activation of protein kinases by phorbol esters promoted a substantial increase in the kinetics of these processes. Biochemical studies demonstrate that this behavioural changes can be correlated with changes in the phosphorylation state of the keratin polypeptides. As a whole, present results indicate that the highly dynamic properties of the keratin polypeptides can be modulated by phosphorylation.

Introduction

Eukaryotic cells contain three separate cytoskeletal systems: microtubules, microfilaments and intermediate filaments (IF). In epithelial cells the IF cytoskeletal network is composed by heteropolymers of the type I and type II IF proteins, acidic and neutral basic keratins (for recent reviews see [4, 11, 18, 41, 45]). These proteins form a dense network that radiates from the nuclear surface to the cell surface where they interact with desmosomes [20]. The heteropolymeric nature of keratin IF, containing equimolar amounts of type I and type II polypeptides, reflects the requirement of heterodimers in assembly [12, 33]. Moreover, the expression of the different members of the type I and type II keratins appears to be tissue- and differentiation-specific, in such a way that any epithelial cell can be characterised by the specific combination of keratins which it expresses [35, 46]. However, in vitro reconstitution experiments revealed that any equimolar combination of type I and type II polypeptides lead to the formation of IF [22].

Due to their highly insoluble biochemical behaviour, the IF proteins in general, and the keratins in particular, have been considered the least dynamic protein constituents of the cell cytoskeleton. However, there is a large and a still growing body of evidence indicating that these proteins undergo highly dynamic interchange processes (for reviews see [20, 42, 44, 45]). In fact, transfection [2, 3, 26, 39], microinjection [33, 34] and heterokaryon formation [38] experiments have suggested that these proteins exist as soluble unpolymerized forms in a dynamic equilibrium with insoluble, polymerised filamentous forms. This soluble pool has been recently characterised [7, 38]. Moreover, during certain cellular events in vivo, the IF cytoskeleton is reorganised. This is particularly evident during mitosis affecting the nuclear lamins [40], although similar changes have been described in certain cases for cytoplasmic IF [15, 30]. Processes of phosphorylation-dephosphorylation have been implicated in the assembly-disassembly of these IF structures [8–10, 40, 47]. In fact, these processes have been suggested to modify the structural properties of some of these proteins [14, 27] (for a review see [42]). Moreover, certain isotypes of protein kinase C appear to be closely associated with

[1)] Dr. Jesús M. Paramio, Cell and Molecular Biology Program, CIEMAT (IMA), Av. Complutense 22, E-28040 Madrid/Spain, e-mail: chus@ciemat.es, Fax: ++913466393.

0171-9335/99/78/01-33 $12.00/0

and involved in the phosphorylation of the keratins in cultured epithelial cells [37], although phosphorylation does not seem to play any obvious role in generating the soluble pool of subunits in simple epithelial cells [7].

In a recent study we have observed that different keratin polypeptides displayed a different dynamic behaviour related to their relative amounts in the soluble pool [38]. In fact, we fused two different types of epithelial cells and studied by immunofluorescence how the keratins from the parental cells recombine and copolymerise to form the heterokaryon cytoskeleton. In this process, the parental cytoskeletons undergo a depolymerisation event most apparent in the region adjacent to the fusion area. Afterwards the depolymerised subunits spread throughout the heterokaryon and copolymerise into the hybrid cytoskeleton in a very fast process, occurring in 4 to 9 hours, but depending on the keratin under study, K5, K8 and K18 being faster than K14, which requires 16 to 24 hours. We also analysed the involvement of phosphorylation in such differential dynamics, and we observed that this process was apparently not involved [38]. However, we could not rule out the possibility that phosphorylation can indeed play a functional role in keratin dynamics modifying the overall solubility of the keratin polypeptides. The studies on the role of protein phosphorylation in different biological processes, implicate in most cases the inhibition or activation of the cellular systems governing these processes. In this regard, a commonly used approach is to inhibit different protein kinase or phosphatase activities using various drugs (see for instance, [14, 27]). In this work, we have studied the possible role of phosphorylation and dephosphorylation on the dynamics of keratin polypeptides using a similar approach by analysing the changes, due to alterations in protein kinases and phosphatase activities, in two previously studied different dynamic situations: *a)* reorganisation and copolymerization events occurring after heterokaryon formation [38], and *b)* the assembly of a newly synthesised keratin in the pre-existing keratin cytoskeleton after transfection [39]. The main difference between these two experimental approaches consists in the fact that the first represents the study of reorganisation of keratins without alteration of the total amount present in the cells, whilst the second represents a forced disruption of the pre-existing equilibrium between the soluble and insoluble forms due to the synthesis of the transfected keratin. In both systems our results demonstrate that the phosphorylation-dephosphorylation events play a crucial role on the dynamic behaviour of the keratin polypeptides in the cells.

Materials and methods

Chemicals

To inhibit protein kinases staurosporine (Sigma, St. Louis, MO/USA) and 6-dimethylaminopurine (6-DMAP; Sigma) were used and added to the cultures in a final concentration of 0.1 μM and 1 mM respectively. Okadaic Acid (Boehringer Mannheim, Mannheim, Germany) was used at 1 μM, and phorbol-12-myristate-13 acetate (TPA) (Sigma) was used at 10 nM. Calf intestine alkaline phosphatase was obtained from Boehringer Mannheim, and was used following the manufacturer's recommendations.

Cell culture and fusion

PtK2 and BMGE+H cells were cultured as described [39]. BMGE+HxPtK2 cell hybrids were generated by polyethyleneglycol 1500 (Boehringer Mannheim, Mannheim, Germany) treatment as previously described [38]. During the fusion experiments 50 μM cycloheximide (Boehringer Mannheim, Mannheim, Germany) was used to inhibit protein synthesis.

Transfection

pCMVk10 and the transfection procedures have been described elsewhere [39]. The expression of the transfected keratin in synthesis and chase experiments was essentially as described previously for immunofluorescence analysis [39]. Immunoprecipitation analysis of phosphorylated forms of acidic keratins were performed essentially as described [7] using AE1 monoclonal antibody (ICN, Costa Mesa, CA/USA) after labelling the cells for 1 hour with 500 μCi/ml of inorganic [^{32}P] (Amersham, Roosendaal, The Netherlands) in phosphate-deficient medium (Sigma, St. Louis, Mo/USA).

Immunofluorescence

Cells grown on glass coverslips were fixed and processed for immunofluorescence staining as described [39, 38]. Primary antibodies were against K5 (1:500 dilution of a rabbit antiserum kindly provided by Dr. D. Roop, Baylor College, Houston, T/USA) and undiluted supernatant from LE61 hybridoma reacting with K18 (kindly provided by Dr. E. B. Lane, CRC Labs, Univ. Dundee, Dundee, UK). K8.60 (Sigma) diluted to 1:40 was used to detect transfected K10. TROMA 1 (1:10 dilution) was used to stain the endogenous PtK2 keratin cytoskeleton. Fluorochrome-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA/USA) and used as described elsewhere [38, 39]. The results were obtained by counting at least three independent experiments scoring at least 100 transfected cells or heterokaryons on each, and were represented as mean ± SD.

In vitro phosphorylation

Cells were cultured in the presence or absence of the different inhibitors for 16 h. At this time, cells were washed, and phosphate-deficient medium with or without inhibitors and supplemented with 500 μCi of [^{32}P]O_4Na_2 (10 mCi/ml; Amersham, Roosendaal, The Netherlands) was added. Cells were incubated under these conditions for one hour. Afterwards, cells were exhaustively washed, and cytoskeletal fractions were obtained as described [1]. Analysis of the cytoskeletal fractions was performed using polyacrylamide gel electrophoresis in the presence of SDS or using 2-D gel electrophoresis (NEpHGE+SDS PAGE) as described by [1]. Immunoprecipitation was performed as described [7] using either rabbit anti-K5 antiserum or AE1 monoclonal antibody. To detect transfected K10 a 1:10000 dilution of the K8.60 monoclonal antibody was used in Western blots as described [39]. To analyse the half-life of keratin polypeptides in the cytoskeletal fraction of BMGE+H cells, a pulse-chase experiment was performed. Briefly, cells were cultured in Met-deficient DMEM medium supplemented with hormones (see above), 20% dialysed FCS and 100 μCi/ml of [^{35}S]Met for 1 h. Afterwards cells were washed with PBS, and non-radioactive medium was added. Cells were cultured under this conditions for different times. Afterwards, keratin-enriched cytoskeletal fractions were obtained and analysed in 9% SDS-PAGE according to the method of [1], fluorography of the obtained gels was performed using En^{3}Hance (DuPont, NEN, Boston, MA/USA).

Results

The possible involvement of phosphorylation-dephosphorylation events in the dynamic behaviour of keratin polypeptides has been studied. To this end, drugs that inhibit or increase the rate of protein phosphorylation in cultured cells were used in two different experimental approaches. On the one hand an approach similar to the nearly steady state equilibrium using cell heterokaryons [38], and, on the other hand, one related to the dynamic incorporation of a newly synthesised keratin into the pre-existing keratin cytoskeleton upon transfection [2, 3, 39].

Keratin polypeptides undergo a high phosphorylation turnover

Due to the highly dynamic exchange processes that keratin polypeptides exhibit in living cells, the possible association of phosphorylation and dephosphorylation with these events implies that these polypeptides must undergo a high phosphorylation turnover. This possibility has been explored by pulse-chase experiments using BMGE+H cells. Cells were cultured for one hour in the presence of ^{32}P and afterwards the radioactive medium was discarded and replaced by non-radioactive medium. Under these conditions, cells were cultured for different periods after which cytoskeletal fractions were obtained and analysed by SDS-PAGE, and the gels were autoradiographed. Fig. 1a represents Coomassie staining of the gels and Fig. 1a′ the corresponding autoradiographs. As can be observed, immediately after labelling (t = 0 on Fig. 1a and a′) the amount of ^{32}P incorporated into the keratin fraction is high, however it decreases very rapidly during the chase period thus demonstrating that keratin phosphorylation undergoes a very high turnover. On the other hand, both K5 and K14 shared similar turnover, and only minor differences can be observed in the initial amount of ^{32}P incorporated in these polypeptides, K14 being slightly less phosphorylated than K5. To exclude the possibility that the observed turnover of phosphorylation was due to the turnover of keratin polypeptides, a similar experiment was performed by labelling the cells with [^{35}S]Met instead of ^{32}P. The corresponding fluorographs are shown in Fig. 1b. In this case the turnover of keratin polypeptides appeared to be very slow. To compare the turnover of the keratin polypeptides and their phosphorylation, densitometric analysis was performed. This analysis clearly confirms the differences between these two turnover processes as shown in Fig. 1c.

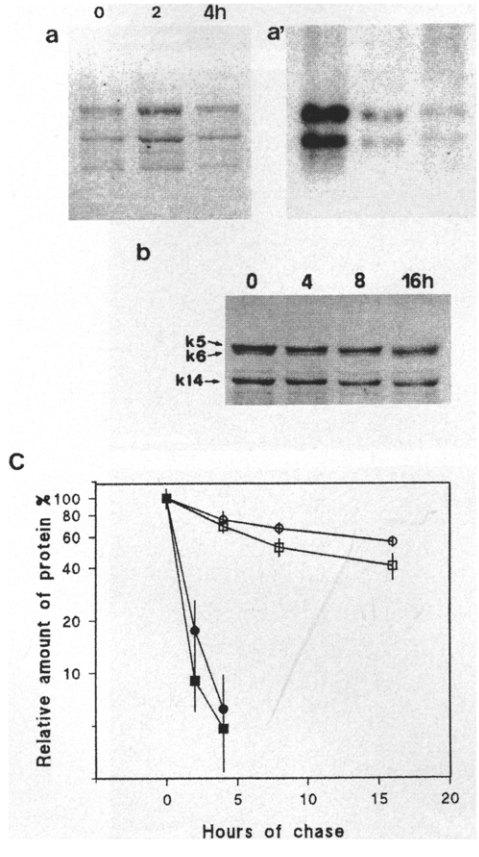


Fig. 1. Keratins undergo a high phosphate turnover as demonstrated by pulse-chase experiments. BMGE+H cells were labelled with ^{32}P for 1 h, at this time the cells were washed and incubated with non-radioactive medium for different periods (0, 2, and 4 h). Cytoskeletal preparations were obtained and analysed by SDS-PAGE and Coomassie staining (**a**) and autoradiography (**a′**). Note that without major alterations in the amount of keratin at each time point, there is a very rapid decrease in the ^{32}P content. **b)** Labelling with [^{35}S]Met demonstrates a very low turnover of keratin polypeptides. **c)** Corresponding densitograms for ^{32}P (*closed symbols*) and ^{35}S (*open symbols*) content of keratin K5 (*circles*) and K14 (*squares*).

Keratin dynamics in heterokaryons is affected by phosphorylation

We have recently reported the dynamic behaviour of keratins in cell heterokaryons [38]. To study the effects of protein phosphorylation in the dynamics of keratins the same experimental approach was used. Fusion experiments between BMGE+H and PtK2 cells were performed, but the reorganisation process was followed in the presence or absence of different chemicals. In this regard, we used 6DMAP and staurosporine as protein kinase inhibitors, okadaic acid as a protein phosphatase inhibitor, and TPA as an activator of protein kinase C. We followed the fate of K5 and K18 by double immunofluorescence using monospecific antibodies. Some examples of the results are shown in Fig. 2 and summarised in Fig. 3. As can be seen in control experiments in the absence of chemical treatment, the separate cytoskeletons of unfused parental cells (Fig. 2a) rapidly recombine in the fusion region (Fig. 2b). 2 h after the fusion, the parental nuclei are re-localised in the central region of the heterokaryon and there is only partial reorganisation of the two parental keratins in the heterokaryon cytoplasm (Fig. 2c). The process is, in most of the cases, finished after 6 h post fusion (Fig. 2d), being characterised by the complete reorganisation of the parental keratins in a hybrid cytoskeleton in which the parental polypeptides are colocalised in the same filaments. The presence of drugs causing the increase in protein phosphorylation, either by activating protein kinase C (Fig. 2e) or inhibiting protein phosphatases (Fig. 2g), increases the kinetics of such processes and only after 2 h post fusion there are a high number of heterokaryons showing the complete reorganisation of the keratin polypeptides. On the contrary, the inhibition of protein kinases, either by staurosporine (Fig. 2f) or 6DMAP (Fig. 2h), results in a considerable delay in the process of keratin reorganisation. In fact, after 6 h post fusion most of the heterokaryons displayed a partial re-distribution and a minor co-localisation of the parental keratins, in a pattern very similar to that displayed in control experiments after only 2 h post fusion (Fig. 2c). These effects are clearly shown by the quantitative analysis of the percentage of heterokaryons with a complete reorganisation of keratin K5 in the hybrid cytoskeleton at different times after fusion (Fig. 3). With respect to the architectural organisation of the keratin IF, cells treated with TPA (Fig. 2e) or with okadaic acid (Fig. 2g) displayed a somehow architecturally altered keratin cytoskeleton, characterised by short and twisted filaments and small keratin clumps, that formed a keratin network clearly different from that observed in untreated cells (Fig. 2d). This indicates that, besides increasing the dynamic reorganisation of keratins, these drugs provoked deep alterations in the overall architectural distribution of the keratin filaments in the cells.

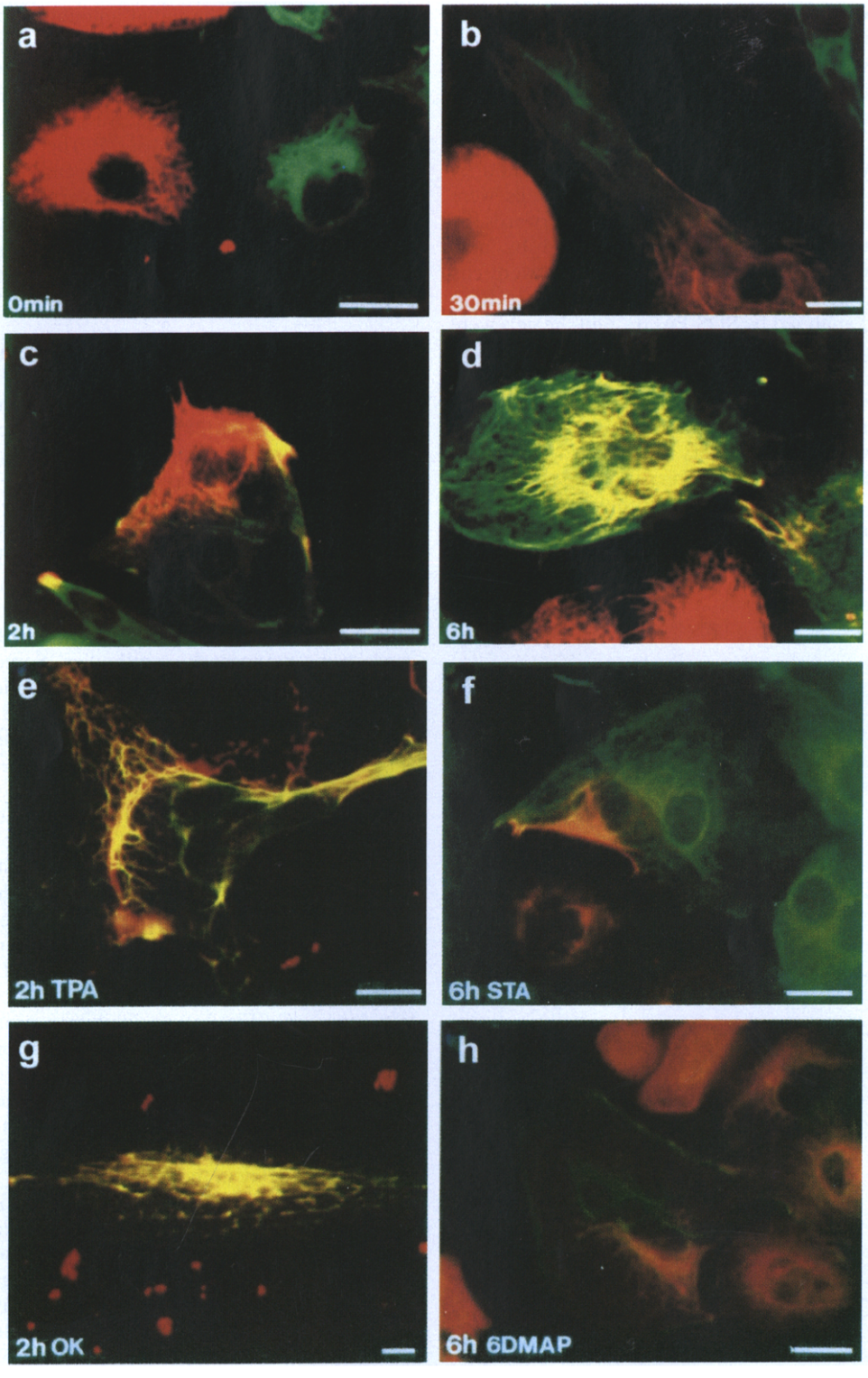


Fig. 2. Examples of keratin dynamics in PtK2/BMGE+H cell heterokaryons. At different times after fusion cells were stained with LE61 against keratin K18 (*FITC labelling*) and K5 using a rabbit polyclonal antiserum (*Texas Red labelling*). Note in unfused control cultures the two separate cytoskeletons (**a**) that 30 min after fusion begin to merge at the fusion area (**b**). After 2 h (**c**) the spreading and colocalisation (denoted by *yellow staining*) of the two parental keratins is limited to certain areas of the heterokaryon. The process is complete after 6 h (**d**). The presence of TPA (**e**) or okadaic acid (**g**) during the fusion accelerates this process and complete reorganisation of hybrid cytoskeleton is observed at 2 h post fusion. The inhibition of protein kinases either by staurosporine (**f**) or 6DMAP (**g**) treatment delayed the process, and the reorganisation observed after 6 h post fusion is similar to that displayed in control untreated cells after 2 h post fusion. Bars = 10 μm.

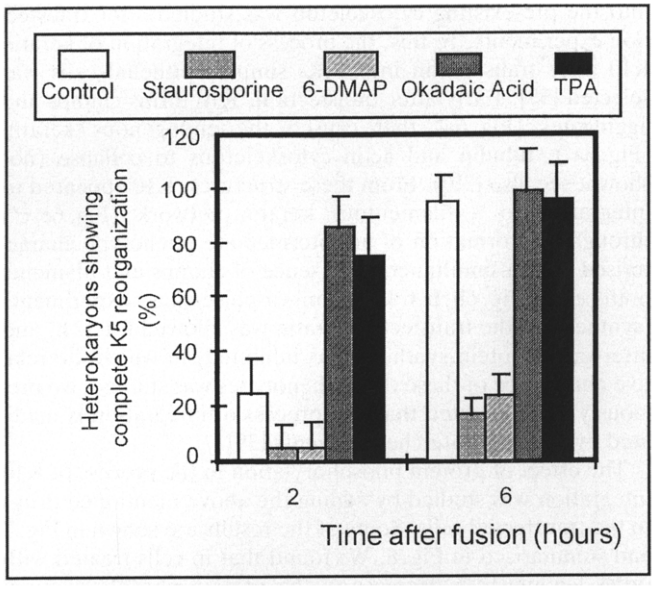


Fig. 3. Summary of keratin K5 dynamics in cell heterokaryons. At 2 or 6 hours after the fusion of BMGE+H and PtK2 cells the distribution of K5 and K18 was followed by immunofluorescence in the absence (control) or presence of different drugs as in Fig. 2. The number of cell hybrids in which K5 showed a complete spreading and co-localisation with K18 (see Fig. 2d, e or g) was determined for each time point. Results are the mean ± S.D. obtained from three to five independent experiments scoring at least 200 fusions for each experiment at each time.

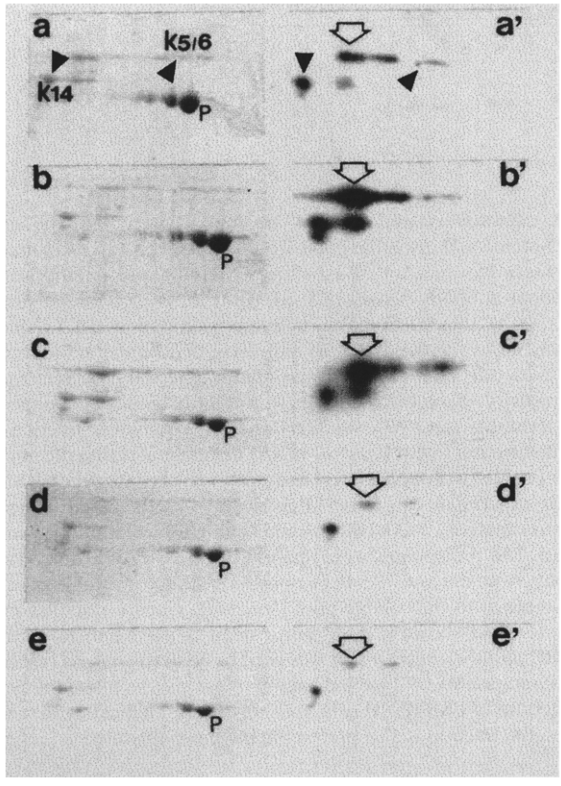


Fig. 4. The different drug treatments modify keratin phosphorylation. BMGE+H cells were labelled for 30 min with ^{32}P in the absence (**a, a'**) or presence of TPA (**b, b'**), okadaic acid (**c, c'**), staurosporine (**d, d'**), or 6DMAP (**e, e'**). Cytoskeletal extracts were obtained and processed by 2D gel electrophoresis (NEpHGE+SDS-PAGE). The corresponding gels were stained with Coomassie (**a–e**) and autoradiographed (**a'–e'**). Note the increase in phosphate content in samples after TPA or okadaic acid treatment and the decrease in those treated with staurosporine or 6DMAP. The corresponding position for each keratin is denoted by *arrowheads* in (**a, a'**). P represents in (**a–e**) the phosphoglycerate kinase added to the extracts as a marker. *Arrows* in (**a'–e'**) represent the presence of heterocomplexes which are not disrupted by electrophoresis in the first dimension (NEpHGE).

Different drugs affect keratin phosphorylation

The altered dynamic behaviour of the keratins observed after the in vitro treatment with the different drugs could be attributed to the alteration of essential mechanisms in the cells (an indirect effect) and/or to alterations of the keratin phosphorylation state (a direct effect). To confirm that the treatments directly altered keratin phosphorylation, we studied this in BMGE+H cells after incubation with the above mentioned different drugs. As expected, in untreated control cells there is a considerable incorporation of ^{32}P (Fig. 4a, a'), however, the treatment with TPA (Fig. 4b, b') and okadaic acid (Fig. 4c, c') promoted an increase in the incorporation of ^{32}P into keratins. On the contrary, treatment of the cells with protein kinase inhibitors staurosporine (Fig. 4d, d') and 6-DMAP (Fig. 4e, e') decreased the keratin phosphorylation. These results demonstrate that the changes in the dynamic behaviour observed can be correlated to changes in keratin phosphorylation, although the alteration of other cellular mechanisms independent of keratin phosphorylation but affecting the dynamics of these polypeptides can not be totally excluded.

Soluble keratins are predominantly phosphorylated

The above results strongly suggest that the increase in phosphorylation increases the dynamic behaviour of the keratin polypeptides. We have previously demonstrated that enhanced dynamics corresponded to the amount of keratin polypeptides in the soluble pool [38]. In this regard we have also shown that the different dynamics displayed by K5 and K14 in this system is not attributed to different phosphorylation of these two polypeptides [38]. However, we did not rule out the possibility that phosphorylation can increase or decrease the soluble amount for a certain keratin. To this, we have analysed the relative phosphorylation of K5 in the soluble and insoluble fractions. BMGE+H cells were cultured for 1 h in the presence of ^{32}P and the soluble and insoluble fractions were obtained after Triton X-100 and low salt extraction, and keratin K5 was immunoprecipitated using a rabbit antiserum against this polypeptide and analysed by SDS-PAGE and autoradiography. In advance the amount of K5 in these two fractions was analysed by Western blotting using the same antiserum, and was normalised in order to obtain similar amounts of the same polypeptide in the two fractions (Fig. 5a, b). This corresponded to approximately 50 times more K5 in the insoluble polymerised fraction (not shown). Afterwards, the same fractions were immunoprecipitated with the anti K5 antiserum and the ^{32}P content in this polypeptide was analysed. The results obtained (Fig. 5b') demonstrate a higher phosphate content in the soluble (Fig. 5b', lane sol) than in the insoluble (Fig. 5b', lane fil) pool of keratin K5. Since the immunoprecipitation procedure removes the vast majority of keratin K5 present in the soluble fraction (Fig. 5a, lane dep), and the treatment with alkaline phosphatase does

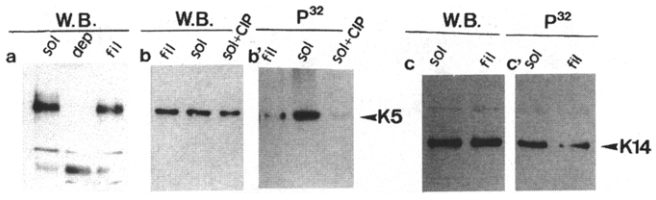


Fig. 5. Phosphorylated keratins are mostly present in the soluble pool. Soluble and insoluble pools from BMGE+H cells were analysed by Western blot or after ^{32}P-labelling by immunoprecipitation using a polyclonal antibody against K5. **a)** Western blot of soluble fraction (*lane sol*), soluble fraction after immunoprecipitation of K5 (*lane dep*) or filamentous fraction (*lane fil*) indicating that similar amounts of K5 were loaded in each lane and that immunoprecipitation removes the vast majority of soluble K5. **b)** Western blot of similar amounts of K5 from insoluble (*lane fil*), soluble (*lane sol*) or soluble fraction after phosphatase treatment (*lane sol+CIP*) demonstrates that the antibody reacts equally with phosphorylated and non-phosphorylated K5. **b'**) Similar experiments to those in (b) were performed, but instead of Western analysis, ^{32}P-labelling and immunoprecipitation was performed. The precipitates were analysed by SDS-PAGE and autoradiography. Note the low ^{32}P label associated with the insoluble pool (*lane fil*) compared to the soluble pool (*lane sol*). **c** and **c'**) Similar experiments to those shown in **b, b'** but instead of using anti-K5 antibody, RCK107 antibody against K14 was used in Western analysis (**c**). Immunoprecipitation of ^{32}P-labelled soluble (*lane sol*) or filamentous (*lane fil*) fractions of BMGE+H cells are shown in (**c'**) indicating that K14 in the soluble fraction is also preferentially phosphorylated.

not alter the ability of the antiserum to react with K5 (indicating that the antibody does not preferentially recognise the phosphorylated forms of this polypeptide Fig. 5b and b', lanes sol+CIP), these results clearly indicate that the soluble form of K5, which is the main form implicated in the dynamic exchange [38], is preferentially phosphorylated compared to K5 in its polymeric filamentous form.

K5 and K14 displayed a very different dynamics with respect to their abilities to redistribute and rebuild the heterokaryon cytoskeleton [38], K14 being delayed with respect to K5. Therefore, a similar approach was used to determine the levels of K14 phosphorylation in the soluble and insoluble fractions. From equivalent amounts of this protein in the soluble and filamentous fractions (Fig. 5c) the analysis of ^{32}P content by immunoprecipitation (Fig. 5c') again demonstrates the preferential phosphorylation of the soluble forms of K14. These results reinforce the above mentioned data suggesting that the soluble keratins display increased phosphorylation with respect to the polypeptides in the filamentous polymerised form. On the other hand, given the different dynamics displayed by K5 and K14 in cell heterokaryons [38], these results indicate that keratin phosphorylation does not seem to be the main mechanism responsible for such differential dynamics, in agreement with our previous observations [38].

Keratin K10 incorporation into PtK2 cytoskeleton is affected by different drugs

The preceding results indicate that the reorganisation of the keratin cytoskeleton is affected by phosphorylation, probably by increasing exchange of subunits between the soluble and filamentous forms of keratins. The existence of similar processes during the integration of a newly synthesised keratin into the pre-existing cytoskeleton was studied after transfection experiments. To this, the process of integration of keratin K10 after transfection into PtK_2 simple epithelial cells was selected [39]. Early after transfection, K10 forms clumps and aggregates (Fig. 6a) that caused the endogenous keratin (Fig. 6a'), tubulin and actin cytoskeletons to collapse (not shown, see also [39]). From these structures, K10 appeared to integrate into a filamentous keratin network (Fig. 6c, c') through the formation of an intermediate phenotype characterised by the simultaneous presence of clumps and filaments in the cells (Fig. 6b, b'). By means of pulse-chase experiments (synthesis of the transfected keratin was allowed for 12 h, and afterwards protein synthesis was inhibited) in which the relative abundance of these three phenotypes was studied, we previously demonstrated that this process of integration is mediated by dynamic interchange events [39].

The effect of protein phosphorylation in the process of K10 integration was studied by adding the above mentioned drugs to the transfected cells. Some of the results are shown in Fig. 7 and summarised in Fig. 8. We found that in cells treated with drugs leading to a decrease in the protein phosphorylation, K10 mostly appeared as aggregates (Fig. 7a, b and 8) which caused the endogenous keratin cytoskeleton to collapse (Fig. 7a', b') indicating a delayed integration process. On the contrary, those agents promoting an increase in the protein phosphorylation accelerated the integration of newly synthesised K10 into the pre-existing keratin cytoskeleton of the transfected cells, since the vast majority of the transfected cells treated with TPA or okadaic acid exhibited K10 integrated into filaments (Fig. 7c, c', d, d') in a higher proportion than transfected cells in the absence of chemical treatments (Fig. 8).

Keratin K10 is phosphorylated during its integration into the endogenous filamentous network

The above described results suggest that the increase in phosphorylation will lead to an accelerated integration of K10 into the endogenous filaments. To analyse this possibility, the changes in K10 phosphorylation during its integration into the endogenous keratin cytoskeleton were studied.

Western blot analysis of the expression of K10 at different times after transfection (pulse-chase experiments in the absence of protein synthesis [39]) revealed that, as the time increases, K10 is resolved into two discernible bands (arrowheads in Fig. 9a), suggestive of a posttranslational modification of K10. Interestingly, the presence of the upper band coincides with the kinetic evolution from clumps to filaments observed in the integration of K10 after transfection (Fig. 9b; see also [39]). To test whether this modification corresponded to changes in phosphorylation, immunoprecipitation experiments with mAb AE1 were performed using cell extracts from K10-transfected PtK2 cells under synthesis (pulse) and chase conditions, after 1 hour of labelling with ^{32}P. The results obtained (Fig. 9c) demonstrate that concomitantly with the integration of K10 into the endogenous keratin cytoskeleton of PtK2 cells there is an increase in the phosphorylation state of K10, thus confirming the role of this modification in the integration processes. Interestingly, the band corresponding to endogenous type I keratin (probably K19) also showed an increase in phosphorylation.

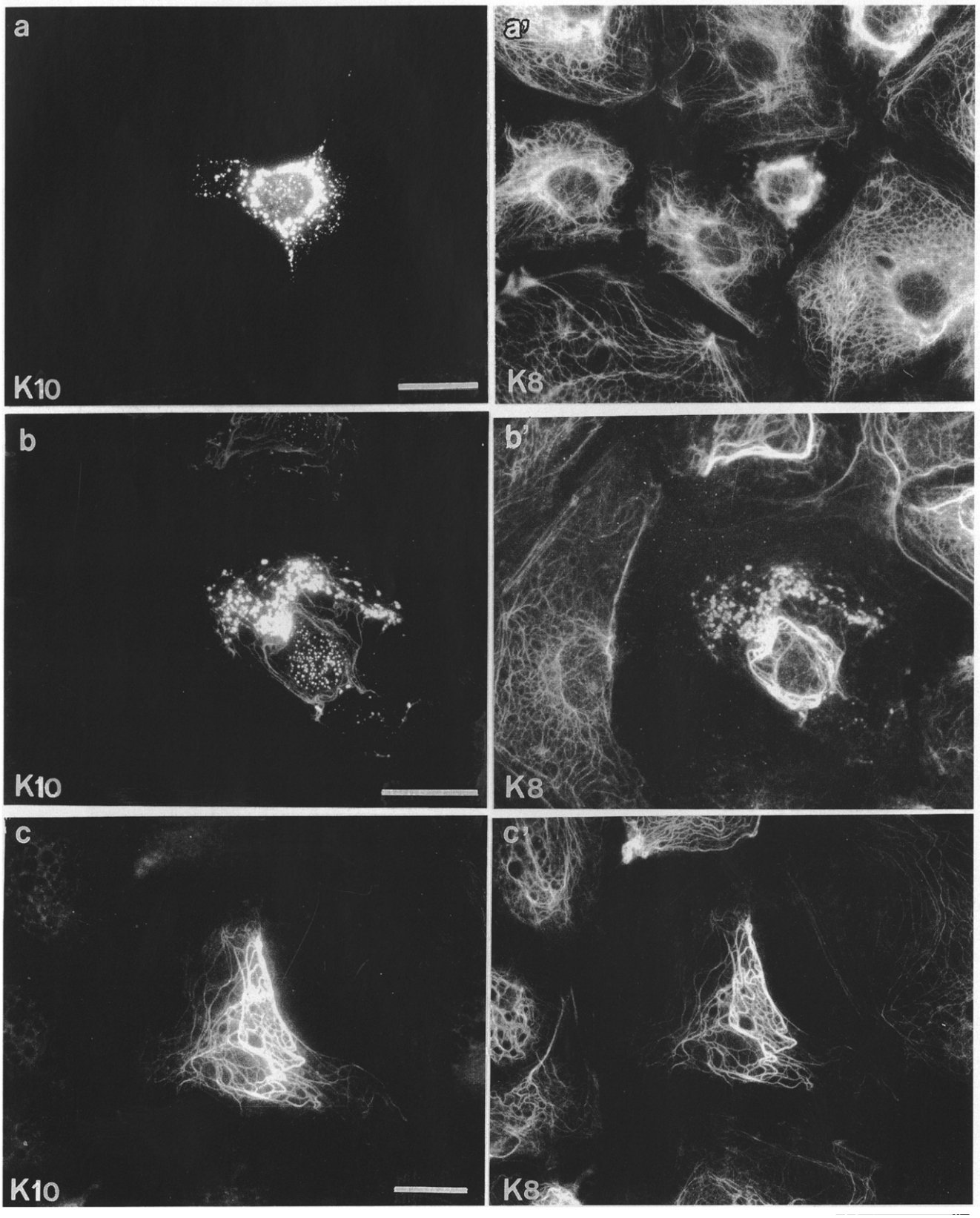
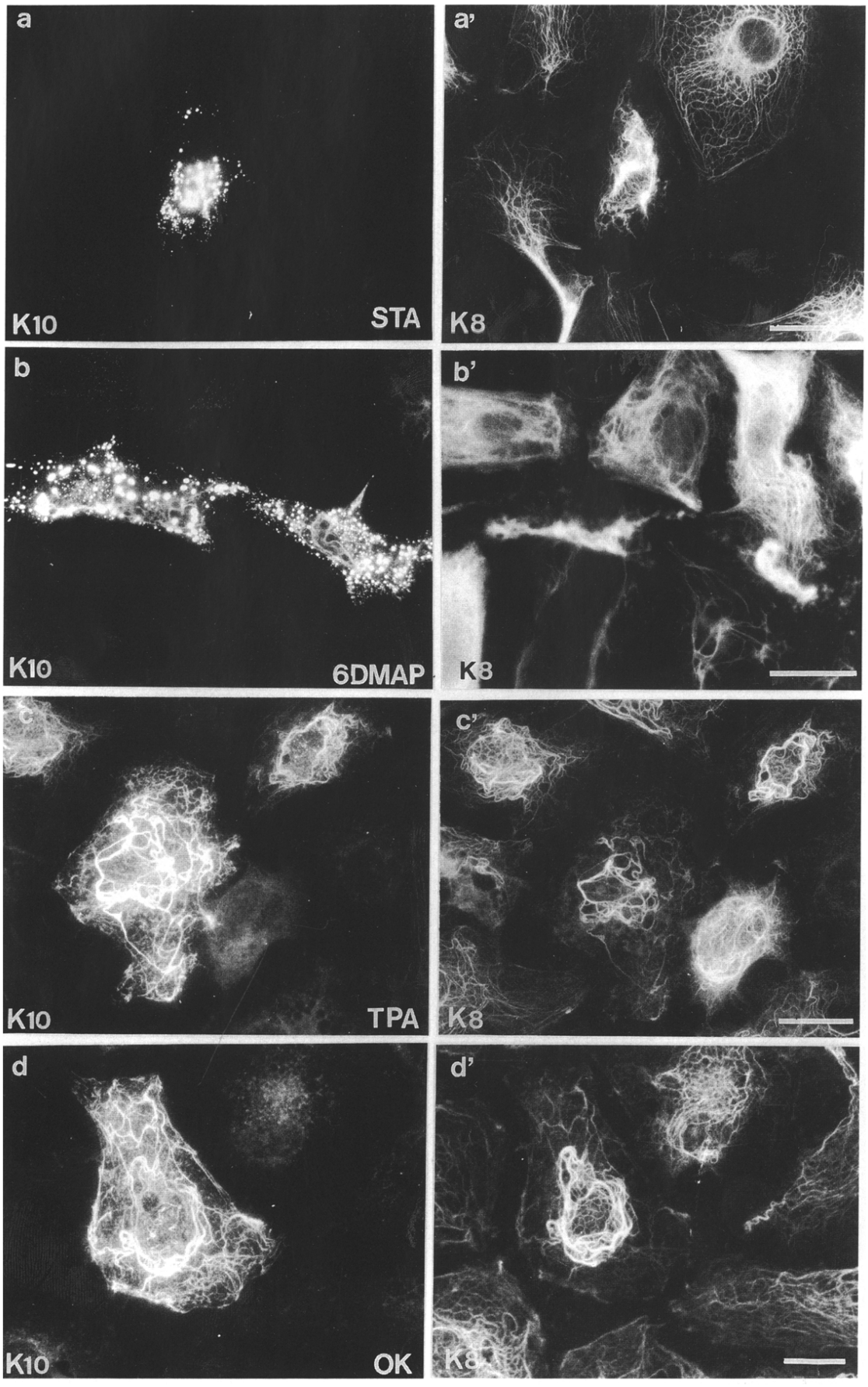


Fig. 6. Examples of the different phenotypes generated upon transfection of K10 into simple epithelial PtK_2 cells. Early after transfection, most of the transfected cells displayed K10 as aggregates (**a**) that caused the endogenous cytoskeletons to collapse (**a'**). At later stages these aggregates evolve to an intermediate phenotype characterised by the presence of small aggregates and filaments of the transfected K10 (**b**) and the endogenous keratins (**b'**). The final stage is the complete integration of K10 (**c**) into the endogenous cytoskeleton (**c'**). Double immunofluorescences using K8.60 (**a–d**) against K10 and Troma 1 against K8 (**a'–d'**) were performed as described [39]. Bars = 10 μm.


a
K10
STA
a'
K8
b
K10
6DMAP
b'
K8
c
K10
TPA
c'
K8
d
K10
OK
d'
K8

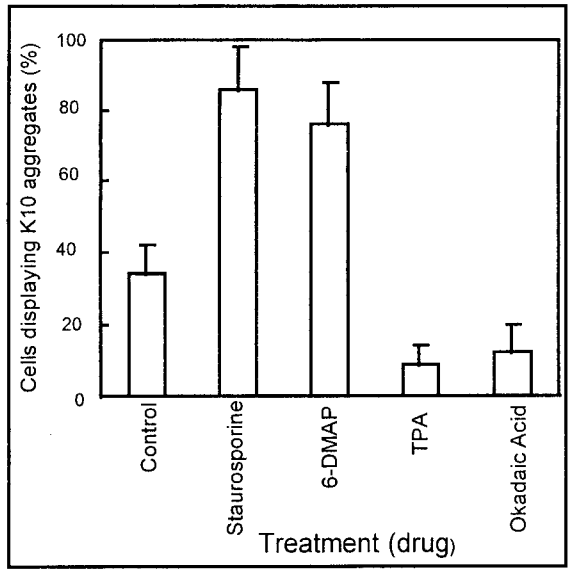


Fig. 8. Effect of different drugs on the assembly of transfected keratin K10 into the endogenous keratin cytoskeleton in PtK2 cells. At 48 hours after transfection the percentage of transfected cells showing K10 as perinuclear non-filamentous aggregates was determined. Note that the treatments causing the inhibition of protein phosphorylation almost inhibited the integration of transfected K10, while those increasing the phosphate content accelerated the process. Values are derived from three independent experiments scoring at least 100 transfected cell for each time point and represent mean ± S.D.

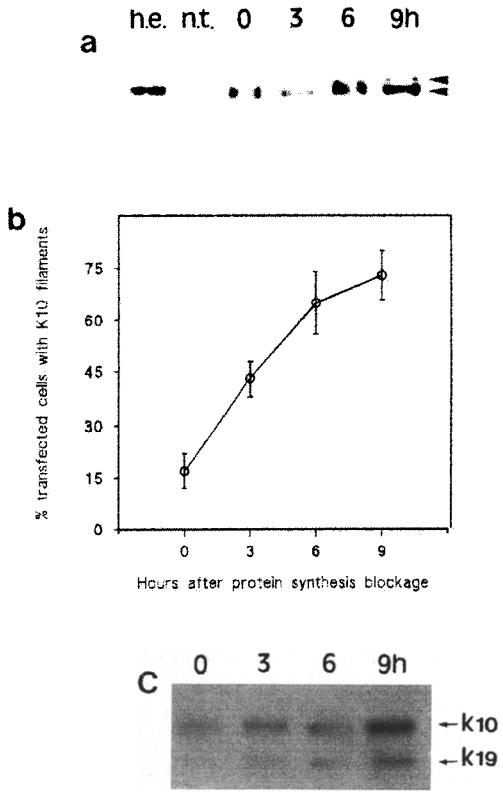


Fig. 9. Keratin K10 is phosphorylated concomitantly with its integration into the endogenous keratin cytoskeleton after transfection of PtK2 cells. Transfection and synthesis and chase experiments were performed as described [39]. **a)** Total protein extracts after transfection experiments at different times after protein synthesis blockage were analysed by Western blotting using K8.60 antibody along with protein extracts from human epidermis (*lane h.e*) and non-transfected PtK2 cells (*lane n.t.*). Note the presence of two discernible bands for K10 (denoted by *arrowheads*) at later times after protein synthesis blockage. These time points correspond to the widespread integration of K10 into the endogenous keratin cytoskeleton as shown in **b)**. In parallel experiments, cultures were labelled for 1 h with ^{32}P, and total extracts were immunoprecipitated using mAb AE1 which in PtK2 cells also reacts with the endogenous K19 (see [39]). Precipitates corresponding to the different time points of protein synthesis blockage were analysed by SDS-PAGE and autoradiography (**c**). Note the increase in ^{32}P content in K10 when this protein is integrated into endogenous filaments at 9 h.

Discussion

Our aim was to study the possible role of phosphorylation on the dynamics of the keratin polypeptides. We found that the activation or inhibition of cellular mechanisms responsible for protein phosphorylation led to alterations in the dynamic behaviour of keratin polypeptides, studied either in cell heterokaryons or after transfection.

Although keratins are considered to be relatively inert cytoskeletal elements due to their biochemical properties, different experimental approaches have demonstrated their dynamic nature. Moreover, the involvement of keratin mutations in the aetiology of different skin blistering diseases (see for reviews [10, 16, 17, 29, 31, 45]) has raised interest in knowing the molecular mechanism responsible for the assembly and dynamics of the keratins. In many cases, dynamic processes of IF polypeptides have been shown to be associated with phosphorylation/dephosphorylation events (see for reviews [42, 45]). Taking into account the highly dynamic nature and the slow turnover of these polypeptides in living cells, this association implies that keratin polypeptides must suffer a high phosphorylation turnover. In fact, this high turnover has been reported for vimentin, a type III IF protein [14], and a high phosphorylation rate occurs in mouse keratinocytes upon treatment with okadaic acid [27]. Pulse-chase experiments presented here (Fig. 1a, a′, c) clearly demonstrated that in untreated cultured cells the keratins undergo a very high phosphorylation turnover, which is in contrast to the turnover of the keratin polypeptides themselves (Fig. 1b, c) which appeared to be very slow. Similar slow polypeptide turnover has been described for rat liver keratins [13] and for vimentin [32].

Among the possible factors which can influence the soluble pool of IF proteins, phosphorylation appears to be an attractive regulator. In vitro phosphorylation of filamentous vimentin [8, 9, 24], rat liver keratins 8 and 18 [48], desmin [23], and neurofilaments [21] caused the disassembly of the filaments. On the other hand, the analysis of the phosphorylation state of the soluble fractions of vimentin [6, 25, 28, 43], keratins [7, 19] or neurofilaments [5, 36] suggested that phosphorylations are not involved in the generation of such soluble pools. Our previous results using cell heterokaryons suggested that the

◀ **Fig. 7.** Examples of double immunofluorescences using K8.60 (**a–d**) against K10 and Troma 1 against K8 (**a′–d′**) after transfection of K10 into PtK2 cells in the presence of staurosporine (**a, a′**), 6DMAP (**b, b′**), TPA (**c, c′**) or okadaic acid (**d, d′**). Bars = 10 μm.

dynamic nature of the keratins is related to the existence of such soluble pools [38]. The results presented here, using the same system of heterokaryon formation, demonstrate that the treatment with TPA or okadaic acid led to an increased dynamic behaviour (Fig. 2 and 3). We also demonstrated that these changes are associated with increased keratin phosphorylation (Fig. 4) and that phosphorylation affects preferentially the soluble form of the keratins (Fig. 5). A similar increase in the solubility of phosphorylated keratins has been described for mouse keratinocytes treated with okadaic acid [27]. On the other hand, the analysis of phosphorylation using G2/M-arrested cells in which there is an increased solubility of keratins showed no differences in the phosphorylation level between soluble and insoluble proteins [7]. Taking into account the high turnover of phosphorylation, the differences observed between the results presented here and those of Dr. Omary's group [7] could probably be attributed to the period of labelling. However, using relatively short labelling periods we have also observed an increase in phosphorylation of the polymerised keratins as a consequence of the okadaic acid and TPA treatment (Fig. 4), suggesting that the treatment of the cells also induced the phosphorylation of the filamentous forms of keratins. Another explanation for such apparent differences may be changes in the phosphorylation sites and/or the stoichiometry of phosphorylation. Alternatively, this increase in phosphorylation of the polymerised keratin fraction could be due to the integration of the previously phosphorylated soluble subunits. In this regard, we also observed an increase in phosphorylation during the integration of a newly synthesised keratin into the pre-existing filament scaffold (Fig. 9). In fact, this process is favoured by TPA or okadaic acid treatments upon transfection (Fig. 8), the transfected keratin K10 being phosphorylated during the process of incorporation (Fig. 9). These data reinforce the possibility that phosphorylation of soluble subunits increases interchange with filamentous polymerised keratins.

Collectively, the results presented here demonstrate that the dynamics of keratins in cells is highly dependent on processes of protein phosphorylation which affect keratin polypeptides, and suggest that the phosphorylation of soluble subunits favours their interchange with those in the filamentous polymerised state.

Acknowledgements. Special acknowledgement to Dr. J. L. Jorcano for his invaluable help during the course of these studies. Thanks to D. Roop and E. B. Lane for providing some of the antibodies used in this work and for helpful discussions. Also thanks for the technical support given by Montse Aldea and Elvira Cerezo and to Sole Moreno for her expertise work with the photographs. Thanks to Irwin McLean for giving his valuable help with the revision of the manuscript. This work has been supported partially from grant PB94-1230 from DGICYT (Spain).

References

[1] Achtstätter, T., M. Hatzfeld, R. A. Quinlan, D. C. Parmelee, W. W. Franke: Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. Methods Enzymol. **134,** 355–371 (1986).

[2] Albers, K., E. V. Fuchs: The expression of mutant epidermal keratin cDNAs transfected in simple epithelial and squamous cell carcinoma lines. J. Cell Biol. **105,** 791–806 (1987).

[3] Albers, K., E. V. Fuchs: Expression of mutant epidermal keratin cDNAs in epithelial cells reveals possible mechanisms for the initiation and assembly of intermediate filaments. J. Cell Biol. **108,** 1477–1493 (1989).

[4] Albers, K., E. V. Fuchs: The molecular biology of intermediate filament proteins. Int. Rev. Cytol. **134,** 243–279 (1992).

[5] Black, M. M., P. Keyser, E. Sobel: Interval between the synthesis and assembly of cytoskeletal proteins in cultured neurons. J. Neurosci. **6,** 1004–1012 (1986).

[6] Blikstad, I., E. Lazarides: Vimentin filaments are assembled from a soluble precursor in avian erythroid cells. J. Cell Biol. **96,** 1803–1808 (1983).

[7] Chou, C. F., C. L. Riopel, L. S. Rott, M. B. Omary: A significant soluble keratin fraction in "simple" epithelial cells. Lack of an apparent phosphorylation and glycosilation role in keratin solubility. J. Cell Sci. **105,** 433–444 (1993).

[8] Chou, Y. H., J. R. Bischoff, D. Beach, R. D. Goldman: Intermediate filament reorganization during mitosis is mediated by p34cdc2 phosphorylation of vimentin. Cell **62,** 1063–1071 (1990).

[9] Chou, Y. H., E. Rosevear, R. D. Goldman: Phosphorylation and disassembly of intermediate filaments in mitotic cells. Proc. Natl. Acad. Sci. USA **86,** 1885–1889 (1989).

[10] Compton, J. G.: Epidermal disease: faulty keratin filaments take their role. Nature Genet. **6,** 6–7 (1994).

[11] Coulombe, P.: The cellular and molecular biology of keratins: beginning of a new era. Curr. Opin. Cell Biol. **5:** 17–29 (1993).

[12] Coulombe, P., E. V. Fuchs: Elucidating the early stages of keratin filament assembly. J. Cell Biol. **111,** 153–169 (1990).

[13] Denk, H., E. Lackinger, K. Zatloukal, W. W. Franke: Turnover of cytokeratin polypeptides in mouse hepatocytes. Exp. Cell Res. **173,** 137–143 (1987).

[14] Eriksson, J. E., D. L. Brautigan, R. Vallee, J. Olmsted, H. Fujiki, R. D. Goldman: Cytoskeletal integrity in interphase cells requires protein phosphatase activity. Proc. Natl. Acad. Sci. USA **89,** 11093–11097 (1993).

[15] Franke, W. W., D. L. Schiller, C. Grund: Intermediate filament proteins in nonfilamentous structures: transient disintegration and inclusion of subunit proteins in granular aggregates. Cell **30,** 103–113 (1982).

[16] Fuchs, E. V.: Genetic skin disorders of keratin. J. Invest. Dermatol. **99,** 671–674 (1992).

[17] Fuchs, E. V., P. A. Coulombe: Of mice and men: Genetic skin diseases of keratin. Cell **69,** 899–902 (1992).

[18] Fuchs, E. V., K. Weber: Intermediate filaments: structure, dynamics, function and disease. Annu. Rev. Biochem. **63,** 345–382 (1994).

[19] Gall, L., E. Karsenti: Soluble cytokeratins in *Xenopus laevis* oocytes and eggs. Biol. Cell **61,** 33–38 (1987).

[20] Goldman, R. D., Y. H. Chou, G. Dessev, A. Goldman, J. E. Eriksson, R. Kohnken, S. Khuon, M. Lowy, K. Murphy, O. Skälli, P. Opal, R. K. Miller, K. Straube: Dynamic aspects of cytoskeletal and karyoskeletal intermediate filament (IF) systems during the cell cycle. Cold Spring Harbor Symp. Quant. Biol. **56,** 629–642 (1992).

[21] Gonda, Y., K. Nishizawa, S. Ando, S. Kitamura, Y. Minoura, Y. Nishi, M. Inagaki: Involvement of protein kinase C in the regulation of assembly-disassembly of neurofilaments in vitro. Biochem. Biophys. Res. Commun. **167,** 1316–1325 (1990).

[22] Hatzfeld, M., W. W. Franke: Pair formation and promiscuity of cytokeratins: formation in vitro of heterotypic complexes and intermediate-sized filaments by homologous and heterologous recombinations of purified polypeptides. J. Cell Biol. **101,** 1826–1841 (1985).

[23] Inagaki, M., Y. Gonda, M. Matsuyama, K. Nishizawa, Y. Nishi, C. Sato: Intermediate filament reconstitution in vitro. J. Biol. Chem. **263,** 5970–5978 (1988).

[24] Inagaki, M., Y. Nishi, K. Nishizawa, M. Matsuyama, C. Sato: Site-specific phosphorylation induces disassembly of vimentin filaments in vitro. Nature **328,** 649–652 (1987).

[25] Isaacs, W. B., R. K. Cook, J. C. Van Atta, C. M. Redmond, A. B. Fulton: Assembly of vimentin in cultured cells varies with cell type. J. Biol. Chem. **264,** 17953–17960 (1989).
[26] Kartasova, T., D. R. Roop, K. A. Holbrook, S. H. Yuspa: Mouse differentiation-specific keratins 1 and 10 require a preexisting keratin scaffold to form a filament network. J. Cell Biol. **120,** 1251–1261 (1993).
[27] Kasahara, K., T. Kartasova, X. Ren, T. Ikuta, K. Chida, T. Kuroki: Hyperphosphorylation of keratins by treatment with okadaic acid of BALB/MK-2 mouse keratinocytes. J. Biol. Chem. **268,** 23531–23537 (1993).
[28] Lamb, N. J. C., A. Fernandez, J. R. Feramisco, W. J. Welch: Modulation of vimentin-containing intermediate filament distribution and phosphorylation in living fibroblasts by the cAMP-dependent protein kinase. J. Cell Biol. **108,** 2409–2422 (1989).
[29] Lane, E. B.: Keratin diseases Curr. Opin. Genet. Dev. **4,** 412–418 (1994).
[30] Lane, E. B., S. L. Goodman, L. K. Trejdosiewicz: Disruption of the keratin filament network during epithelial cell division. EMBO J. **1,** 1365–1372 (1982).
[31] McLean, W. H. I., E. B. Lane: Intermediate filaments in disease. Curr. Opin. Cell Biol. **7,** 118–125 (1995).
[32] McTavish, C. F., W. J. Nelson, P. Traub: The turnover of vimentin in Ehrlich ascites tumour cells. FEBS Lett. **154,** 251–256 (1983).
[33] Miller, R. K., S. Khuon, R. D. Goldman: Dynamics of keratin assembly: exogenous type I keratin rapidly associates with type II in vivo. J. Cell Biol. **122,** 123–135 (1993).
[34] Miller, R. K., K. L. Vikstrom, R. D. Goldman: Keratin incorporation into intermediate filament networks is a rapid process. J. Cell Biol. **113,** 843–855 (1991).
[35] Moll, R., W. W. Franke, D. L. Schiller, B. Geiger, R. Krepler: The catalog of human cytokeratin polypeptides: patterns of expression of specific cytokeratins in normal epithelia, tumors and cultured cells. Cell **31,** 11–24 (1982).
[36] Nixon, R. A., S. E. Lewis, D. Dahl, C. A. Marotta, U. C. Drager: Early posttranslational modifications of the three neurofilament subunits in mouse retinal ganglion cells: neuronal sites and time course in relation to subunit polymerization and axonal transport. Mol. Brain Res. **5,** 93–108 (1989).
[37] Omary, B. M., G. T. Baxter, C. F. Chou, C. L. Riopel, W. Y. Lin, B. Strulovici: PKCε-related kinase associates with and phosphorylates cytokeratins 8 and 18. J. Cell Biol. **117,** 583–593 (1992).
[38] Paramio, J. M., M. L. Casanova, A. Alonso, J. L. Jorcano: Keratin intermediate filament dynamics in cell heterokaryons reveals diverse behaviour of different keratins. J. Cell Sci. **110,** 1099–1111 (1997).
[39] Paramio, J. M., J. L. Jorcano: Assembly dynamics of epidermal keratins K1 and K10 in transfected cells. Exp. Cell Res. **215,** 319–331 (1994).
[40] Peter, M., J. Nakagawa, M. Doreé, J. C. Labbe, E. A. Nigg: In vitro disassembly of the nuclear lamina and M phase-specific phosphorylation of lamins by cdc3 kinase. Cell **61,** 591–602 (1990).
[41] Quinlan, R., C. Hutchinson, E. B. Lane: Intermediate filament proteins. Protein Profile **1,** 779–911 (1994).
[42] Skalli, O., Y. H. Chou, R. D. Goldman: Intermediate filaments: not so tough after all. Trends Cell Biol. **2,** 308–312 (1992).
[43] Söellner, P., R. A. Quinlan, W. W. Franke: Identification of a distinct soluble subunit of an intermediate filament protein: tetrameric vimentin from living cells. Proc. Natl. Acad. Sci. USA **82,** 7929–7933 (1985).
[44] Steinert, P. M., R. K. Liem: Intermediate filament dynamics. Cell **60,** 521–523 (1990).
[45] Steinert, P. M.: Structure, function, and dynamics of keratins intermediate filaments. J. Invest. Dermatol. **100,** 729–733 (1993).
[46] Sun, T. T., R. Eichner, A. Schermer, D. Cooper, W. G. Nelson, R. A. Weiss: Classification, expression and possible mechanisms of evolution of mammalian epithelial keratins: a unifying model. In: A. Levine, G. F. Vande Woude, W. C. Toop, J. D. Watson (eds.): Cancer cells: The Transformed Phenotype. Vol. 1. pp 169–176. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY, USA 1984.
[47] Tölle, H. G., K. Weber, M. Osborn: Keratin filament disruption in interphase cells – How is it induced? Eur. J. Cell Biol. **43,** 35–47 (1987).
[48] Yano, T., T. Tokui, Y. Nishi, K. Nishizawa, M. Shibata, K. Kikuchi, S. Tsuiki, T. Yamauchi, M. Inagaki: Phosphorylation of keratin intermediate filaments by protein kinase C, by calmodulin-dependent protein kinase and by cAMP-dependent protein kinase. Eur. J. Biochem. **197,** 281–290 (1991).