Evidence for coordinated phosphorylation of keratins and vimentin during mitosis in transformed human amnion cells

Phosphate turnover of modified proteins

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Two-dimensional gel electrophoresis (IEF) analysis of short-term [^{32}P]orthophosphate-labelled intermediate-sized filament proteins (keratins and vimentin) from transformed mitotic amnion cells (AMA), have shown that these proteins are modified coordinately and that the half life of the phosphate is about 13 min for the keratins and 11 min for vimentin. These results support the notion that the preferential modification of intermediate-sized filament proteins during mitosis may play a role in modulating filament associations with organelles and other cellular structures.

Intermediate-sized filament protein *Modification* *Role in mitosis*
Two-dimensional gel electrophoresis

1. INTRODUCTION

We have previously shown that transformed amnion cells (AMA) contain 4 keratins (IEF 31, M_r = 50000; IEF 36, M_r = 48500; IEF 44, M_r = 44000 and IEF 46, M_r = 43500; HeLa protein catalogue number [1–3]) ([4–6], Celis et al., submitted) in addition to vimentin. In mitosis these intermediate filament proteins are phosphorylated above their interphase level and we have suggested that the modifications may be instrumental in assuring the process of cell division ([7–10], Celis et al., submitted).

Here, we present evidence indicating that in mitotic AMA cells all of the intermediate-sized filament proteins are coordinately modified and that the half-life of the phosphate is about 13 min for the keratins and 11 min for vimentin. These results are taken to support the notion that the preferential phosphorylation of intermediate-sized filaments during mitosis may play a role in modulating filament associations with organelles and other cell structures ([9–20], Celis et al., submitted).

Abbreviation: IEF, isoelectric focussing

2. MATERIALS AND METHODS

2.1. *Isolation of mitotic cells*

Mitotic cells were obtained by mechanical detachment as described for HeLa cells [21]. Three 250-ml flasks containing about 2 × 10^6 cells per flask were used. The distribution of phases in a mitotic population gave 16% prophase, 65% metaphase (early and late metaphase), 13% telophase and 8% interphase.

2.2. *Labelling of mitotic cells with [^{35}S]-methionine*

Mitotic cells harvested at 20°C were labelled at 37°C with [^{35}S]methionine for 10 min as in [21–23]. At the end of the labelling period the cells were centrifuged and resuspended immediately in lysis buffer [24].

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2.3. *Labelling of cells with [^{32}P]orthophosphate*

Mitotic cells harvested at 20°C were labelled at 37°C for various periods of time in 2 ml of phosphate-free medium containing 1 mCi of [^{32}P]orthophosphate. After labelling or chasing, the cells were centrifuged and resuspended immediately in lysis buffer.

The procedures for two-dimensional gel electrophoresis (IEF) [7,24], silver staining [10,25] and immunoprecipitation [5,9] have been previously described.

3. RESULTS AND DISCUSSION

Fig.1 shows an IEF gel of [^{35}S]methionine proteins from mitotic AMA cells obtained by mechanical detachment and labelled for 10 min at 37°C. The position of the 4 keratins present in these cells (IEF 31, M_r = 50000; IEF 36, M_r = 48500; IEF 44, M_r = 44000 and IEF 46, M_r = 43500; HeLa proteins catalogue number [1–31]) ([4–6], Celis et al., submitted), vimentin (v, IEF 26 in the HeLa protein catalogue), α- and β-tubulin and total actin are indicated. Some of the phosphorylated variants of the keratins and of vimentin are also indicated with their corresponding number in the HeLa protein catalogue. Similar gel electrophoretic analysis (IEF) of mitotic AMA proteins stained with silver nitrate are shown in fig.2 (only the relevant fraction of the gel is shown). Clearly, the modifications of keratins IEF 36 and 44 are quite substantial as compared to those of keratins IEF 31, 46 and vimentin.

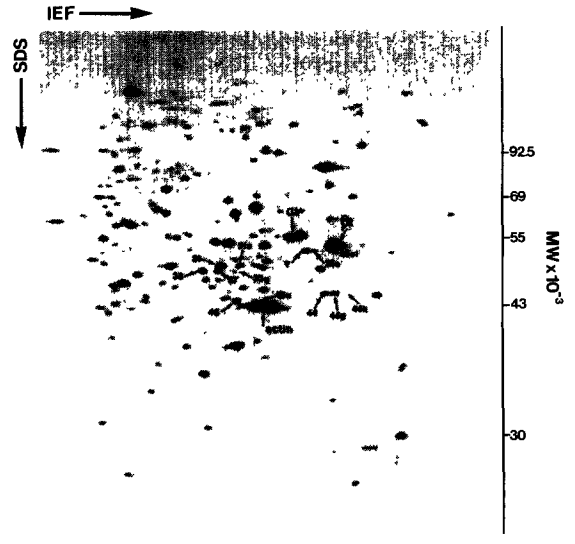

Fig.1. Two-dimensional gel electrophoresis (IEF) of [^{35}S]methionine-labelled proteins from mitotic AMA cells labelled for 10 min. IEF 31, 36, 44 and 46 correspond to keratins, v = vimentin; α-t = α-tubulin; βt = β-tubulin. Some modified variants of the keratins and of vimentin are also indicated.

Labelling of mitotic AMA cells with [^{32}P]orthophosphate (10 min) followed by analysis of the proteins by two-dimensional gel electrophoresis (IEF) revealed numerous phosphorylated variants of the 4 keratins and of vimentin (fig.3a; only the relevant fraction of the gel is shown; see also table 1). Since in all cases the intensity of the ^{32}P labelled spots is higher than that observed with [^{35}S]-methionine (fig.1) or by silver staining (fig.2), it seems reasonable to assume that these variants arise from increased phosphorylations. The spots which are preferentially observed during mitosis are underlined in table 1 (Celis et al., submitted). Nearly identical patterns to that presented in fig.3a have been obtained by labelling as short as 2 min. These results strongly suggest that the keratins as well as vimentin are coordinately modified. The authenticity of the modified keratins has been verified by immunoprecipitating [^{32}P]orthophosphate-labelled proteins from asynchronous AMA cells (fig.4a) with a broad specificity keratin antibody. The modified variants move to the acidic site of the keratins as judged from similar immunopre-

Fig.2. Two-dimensional gel electrophoresis (IEF) of proteins from mitotic AMA cells stained with silver nitrate. Only the relevant fraction of the gel is shown.

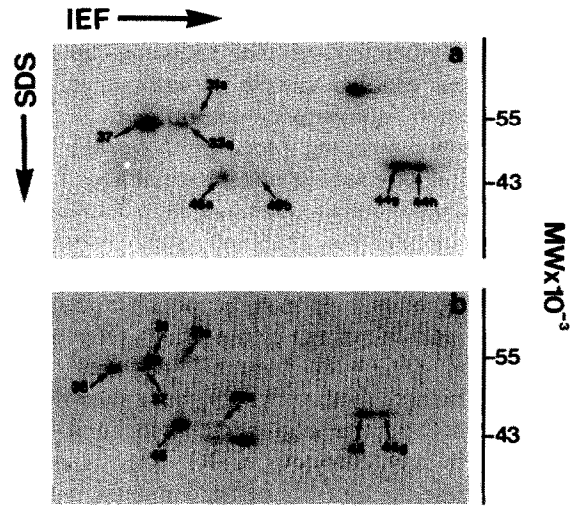

Fig.3

Fig.3. Immunoprecipitation of keratins using a broad specificity keratin antibody. (a) Immunoprecipitation of ^{32}P-labelled proteins from asynchronous AMA cells; (b) Immunoprecipitation of [^{35}S]methionine-labelled asynchronous AMA cells. Phosphovimentin (a) and actin (b) are usual contaminants in immunoprecipitates.

cipitation studies of [^{35}S]methionine-labelled AMA proteins (fig.4b).

To determine the half-life of the phosphate in the modified proteins we labelled mitotic AMA cells for 10 min with [^{32}P]orthophosphate (fig.3a) and then chased the radioactivity for various periods of time within the length of the AMA mitotic cycle. The relevant fraction of gels (IEF) from ^{32}P-labelled proteins chased for 5 min (fig.4b), 15 min (fig.4c) and 25 min (fig.4d) are

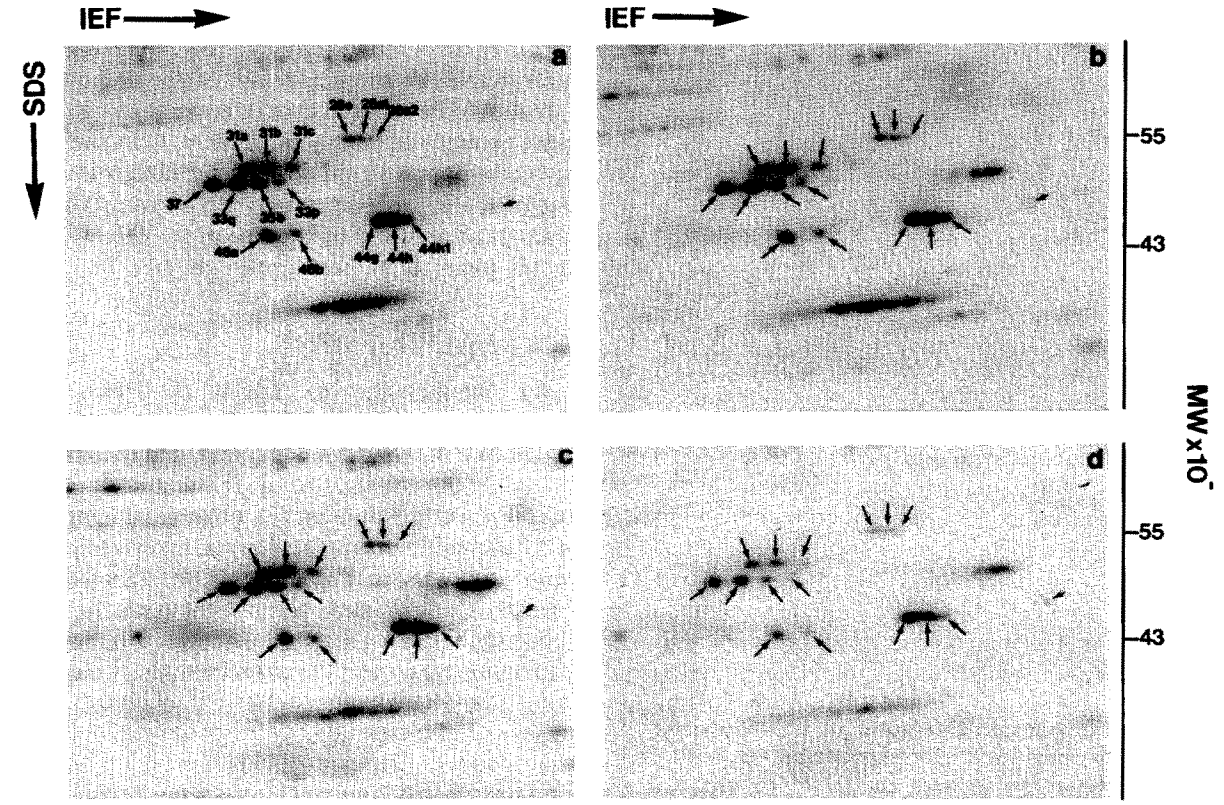

Fig.4. Phosphate turnover of phosphorylated keratins and vimentin. (a) Control cells labelled for 10 min with [^{32}P]orthophosphate; (b–d) As (a) but chased for 5 min (b), 15 min (c) and 25 min (d), respectively. The same total number of counts was applied to each gel. The short arrow indicates a spot whose radioactivity remains constant throughout the chase period.

Table 1

Phosphorylated products of keratins and vimentin

Intermediate-sized filament protein[a]	Phosphorylated spot[a,b]
Keratins	
IEF 31	31a, 31b, 31c
IEF 36	37, 33q, 35b, 33p
IEF 44	44g, 44h, $44h_1$
IEF 46	46a[c], 46b[c]
Vimentin	
Vimentin	26e, $26e_1$, $26e_2$

[a] Numbered according to the HeLa protein catalogue [1–3]

[b] The underlined numbers correspond to variants observed predominantly in mitosis (Celis et al., submitted)

[c] These numbers are new additions to the HeLa protein catalogue [1–3]

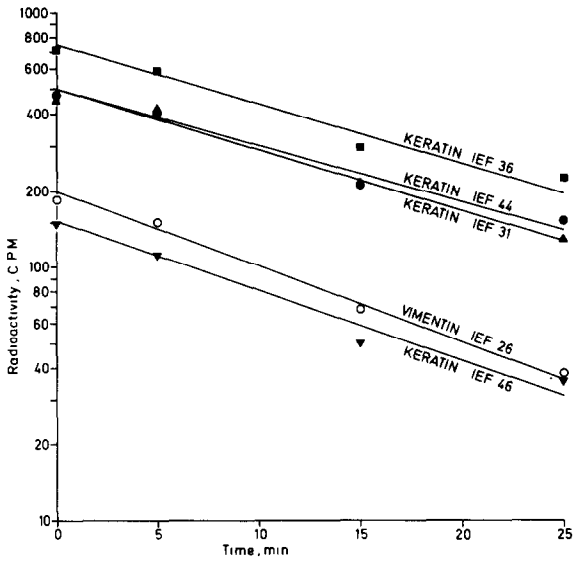

Fig.5. Rate of phosphate turnover of phosphorylated keratins and vimentin. Regions containing the labelled polypeptides (see fig.4) were cut from the gels and counted in scintillation fluid. The counts were corrected to the same total counts applied to the gel; and for radioactive decay. (▲—▲) keratin IEF 31; (■—■) keratin IEF 36; (●—●) keratin IEF 44; (▼—▼) keratin IEF 46; (○—○) vimentin (IEF 26).

shown in fig.4. To determine the half-life of the phosphate associated with the variants, the spots were cut out (pooled sample of variants in each case) and their radioactivity determined.

Fig.5 shows semi-log plots of these data. An estimated half-life for the phosphate of about 13 ± 2 min ($p < 0.05$) for keratins and 11 ± 3 min ($p < 0.05$) for vimentin was observed. These values are compatible with a regulatory role for phosphorylation during mitosis. It may be possible that the calculated half-life of the phosphate is affected by degradation [7,26–28] but this has not been analysed further. Authors in [29] have shown that the half life of the phosphate associated with vimentin in asynchronous CHO cells is about 60 min, while the half-life of the vimentin protein is considerably longer. Likewise, authors in [30] reported similar results in control and colcemide-treated CHO cells. At prsent we have not determine the half-life of the phosphorylated forms of the keratins and of vimentin during interphase (Celis et al., submitted), as this will require a careful analysis of each phase of the cell cycle.

It should be stressed that intermediate-sized filament proteins exhibit significantly different levels of modification in mitosis. Determination of the fate of the modified proteins will be a further step towards understanding the role of phosphorylation in this phase of the cell cycle.

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