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STIMULATED PHOSPHORYLATION OF NON-HISTONE PHOSPHOPROTEINS IN SV-40 TRANSFORMED WI-38 HUMAN DIPLOID FIBROBLASTS

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Summary

Because the phosphorylation of non-histone proteins has been suggested to play a role in the regulation of eukaryotic gene transcription, we have compared the phosphorylation of these proteins in normal and SV-40 transformed WI-38 human diploid fibroblasts. The rate of phosphorylation was found to be roughly ten-fold higher in the transformed cells, and this striking difference could not be accounted for by either an increased rate of phosphate transport or by the synthesis of new species of non-histone proteins which subsequently become phosphorylated. To our knowledge this is the most dramatic alteration in non-histone protein phosphorylation ever described, and therefore may have important implications for our understanding of malignant transformation.

In recent years evidence accumulated from a variety of experimental approaches had led to the view that the non-histone proteins of eukaryotic cells play an important role in the regulation of gene activity (reviewed in ref. 1). Although the mechanism by which this control is exerted is not understood at the molecular level, one possibility which has been suggested is that the interaction of non-histone proteins with the genome is modulated via phosphorylation and dephosphorylation of specific sites in the non-histone protein molecules (reviewed in refs 2 and 3). Support for this hypothesis has come from the observations that phosphorylated non-histone proteins, (i) exhibit changes in phosphorylation which correlate with alterations in gene activity [3], (ii) bind specifically to DNA [3—5], (iii) are heterogeneous and tissue specific [3, 5—7], and (iv) stimulate RNA synthesis when added to cell-free systems [3, 5, 8—10]. In addition, the phosphorylation of individual non-histone proteins has been shown to be under the control of a family of protein kinases whose activities are controlled to varying extents by cyclic AMP [11].

Because the transformation of mammalian cells by DNA viruses is accompanied by changes in gene expression, it is not surprising that several laboratories have observed alterations in the composition and metabolism of

non-histone proteins in transformed cells [12—17]. In one of these studies [16] a modest increase in the incorporation of ^{32}P into the total non-histone protein fraction was observed in SV-40 transformed fibroblasts. In order to attempt to identify the nature of the proteins whose phosphorylation is enhanced, we have now undertaken a comparison of the phosphorylation of nuclear phosphoproteins in normal and SV-transformed WI-38 human diploid fibroblasts.

Human diploid fibroblasts and SV-40 transformed WI-38 fibroblasts were grown in monolayer culture in Eagle's Basal Medium containing 10% fetal calf serum. All experiments were carried out utilizing exponentially growing cells. The normal fibroblasts employed in these studies ranged from passage 28 to 32. Cells were labeled by incubation for one hour in phosphate-free Eagle's Basal Medium containing 100 mCi/ml $^{32}P_i$, after which they were washed three times with cold Earle's balanced salt solution and then lysed in 80 mM NaCl/

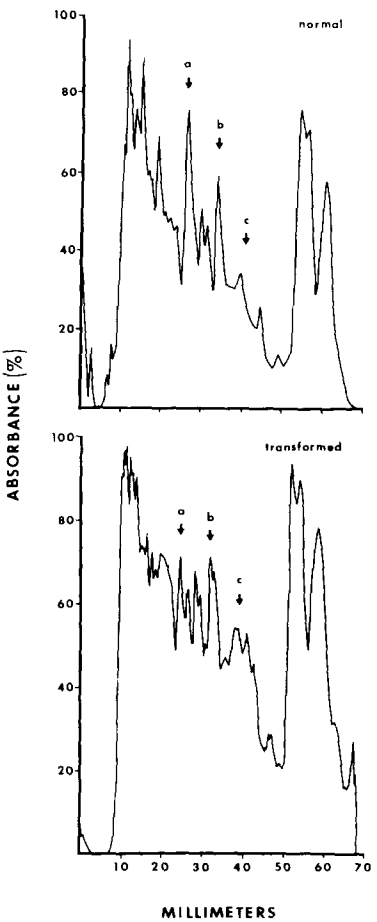

Fig. 1. Comparison of densitometer scans of non-histone phosphoproteins prepared from normal and SV-40 transformed human diploid fibroblasts. Proteins were fractionated on sodium dodecyl sulfate-polyacrylamide gels and stained with Coomassie Blue as described elsewhere [6]. Although the overall profiles are quite similar, differences in the relative amounts of protein contained in regions "a", "b", and "c" are evident.

20 mM EDTA/1% Triton-X-100 (pH 7.2). Nuclei were isolated in the presence of the protease inhibitor 1-1-tosylamide-2-phenyl-ethylchloromethyl-ketone (50 mg/ml) as described elsewhere [16].

Nuclear pellets were suspended in 10 volumes of 0.14 followed by an equal volume of 2.0 M NaCl/0.03 M Tris, pH 7.5. After a 20 s treatment with a Polytron homogenizer at 72 V, 1.5 volumes of 0.02 M Tris, pH 7.5 was added slowly with continuous stirring. After formation of the nucleoprotein gel the samples were centrifuged at 95 000 x *g* for 60 min. The supernatant, which is enriched in phosphorylated non-histone proteins, was electrophoresed in acrylamide gels employing the sodium dodecyl sulfate/phosphate system of Weber and Osborn [18]. Gels were stained for protein with Coomassie Blue or sliced for radioactivity measurements as described elsewhere [6].

Analysis of sodium dodecyl sulfate acrylamide gels stained for protein indicates that the non-histone phosphoprotein fraction prepared from diploid fibroblasts is highly heterogeneous, with up to 27 bands being routinely resolvable. Comparison of densitometer scans of gels containing proteins prepared from either normal and SV-40 transformed fibroblasts shows that the composition of this fraction is similar in the two cases (Fig. 1). There is, however, an indication of small quantitative differences in the relative amounts of several protein species.

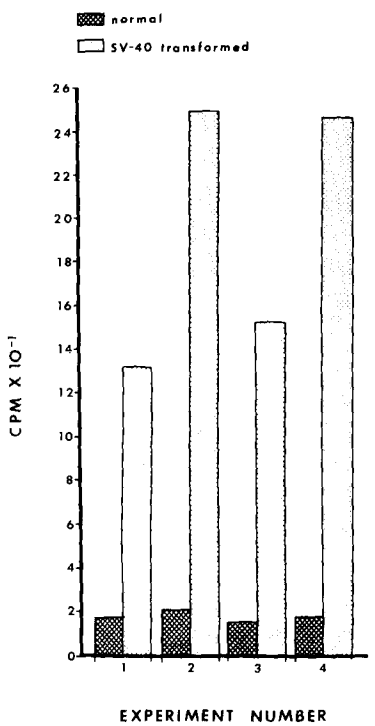

Fig. 2. Comparison of the rates of phosphorylation of non-histone phosphoproteins from normal and SV-40 transformed human diploid fibroblasts. The incorporation of ^{32}P into protein during a one hour pulse is given for four separate experiments. In experiment number four, cycloheximide (5 μg/ml) was present. Note the dramatic elevation of phosphorylation in the SV-40 transformed cells.

In contrast to the small differences observed between normal and SV-40 transformed fibroblasts in terms of the non-histone phosphoprotein species present, there are marked quantitative and qualitative differences in protein phosphorylation. In terms of the rate of phosphorylation of the total phosphoprotein fraction, stimulations of between 7- and 13-fold have been repeatedly observed in log phase cells pulsed for one hour with ^{32}P (Fig. 2). In order to determine whether this dramatic increase in ^{32}P-labeling might be accounted for by an increased transport of the radioisotope into the cell, measurements were made of the radioactivity in the intracellular pool. After 1 h incubation with ^{32}P, the specific activity of the acid-soluble phosphate pool was found to be indistinguishable between normal and transformed fibroblasts, indicating that change in the specific activities of the phosphate pool could not account for the enhanced incorporation of ^{32}P into non-histone proteins.

Experiments were also performed in the presence of cycloheximide (5 μg/ml) to determine whether the enhanced labeling of non-histone phospho-

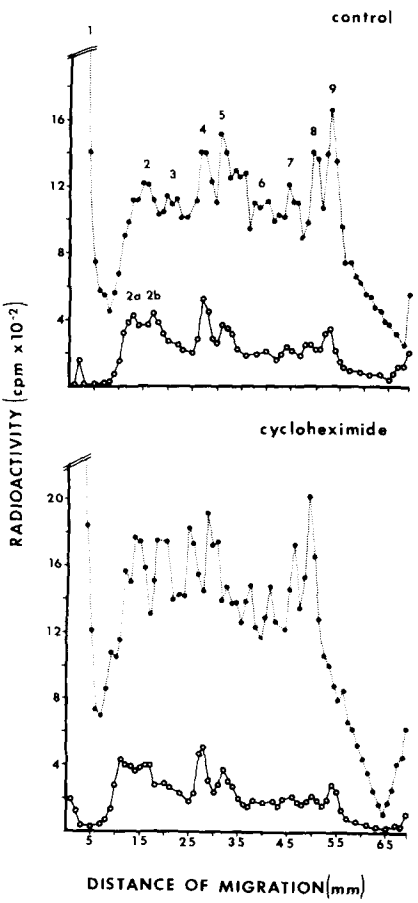

Fig. 3. Comparison of phosphorylation patterns of non-histone phosphoproteins isolated from normal (○—○) and SV-40 transformed (●—●) human diploid fibroblasts. A 1 h pulse of ^{32}P was employed either in the absence (upper graph) or presence (lower graph) of 5 μg/ml cycloheximide. The results show that a striking elevation in phosphorylation of pre-existing non-histone proteins, especially those in peaks 3, 5, 6, 7, and 8, occurs in the transformed cells.

proteins with ^{32}P could be accounted for by the synthesis of new protein species which were subsequently becoming phosphorylated. The data indicated that blocking protein synthesis with cycloheximide had no effect on the total incorporation of ^{32}P into the non-histone proteins of either normal or transformed fibroblasts during a one hour pulse, indicating that this phosphorylation is occurring on pre-existing protein species.

In order to determine whether the difference in non-histone protein phosphorylation between normal and transformed fibroblasts was exerting any selective effects on individual protein species, ^{32}P-labeled proteins were fractionated on sodium dodecyl sulfate acrylamide gels. As is shown in Fig. 3, a large number of different protein species are being phosphorylated in this system. Not only is there a general increase in the phosphorylation of all the resolved protein species in transformed cells, but there are also indications of several selective effects. Thus peaks 3, 5, 6, 7, and 8 have higher relative rates of phosphorylation in the SV-40 transformed cells. Treatment with cycloheximide has no effect on the phosphorylation pattern, indicating that these selective alterations in ^{32}P-labeling of individual peaks can be accounted for solely by changes in the state of phosphorylation of preexisting proteins.

To our knowledge this is the most dramatic alteration in non-histone protein phosphorylation which has been described to date in any system. In most situations where alterations in non-histone protein phosphorylation have been observed the level of phosphorylation can be roughly correlated with the RNA synthetic capacity of the genome. In the present situation, however, we are dealing with a difference in phosphorylation of roughly ten-fold between normal and SV-40 transformed WI-38 fibroblasts, which is certainly far beyond any difference in overall synthetic capacity of their genomes. Although we have already eliminated the possibility that the enhanced ^{32}P incorporation is due to a pool effect or an increased rate of synthesis of new proteins to be phosphorylated, several possible interpretations still remain. Since exchanges of non-histone chromosomal proteins between the genome and other cellular compartments have been reported [19], the increased ^{32}P-labeling of the non-histone proteins of SV-40 transformed cells may be accounted for by an increased rate at which nucleoplasmic or cytoplasmic proteins become associated with the genome, as well as by an increased rate of in situ phosphorylation of pre-existing chromosomal proteins. Whatever the relative contributions of these two phenomena, the present findings make it clear that the rate at which phosphorylated proteins become associated with the genome undergoes a dramatic alteration in transformed cells. This conclusion is consistent with other reports of alterations in non-histone protein phosphorylation in malignant cells [20-23], although the present results are much more dramatic and suggest that SV-40 transformed fibroblasts may be an ideal system in which to explore the functional implications of chromosomal protein phosphorylation.

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