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TGF-β_1 induces phosphorylation of the cyclic AMP responsive element binding protein in ML-CCl64 cells

IJsbrand M.Kramer[1], Irma Koornneef, Siegfried W.de Laat and Adriana J.M.van den Eijnden-van Raaij

Hubrecht Laboratory, Netherlands Intitute for Developmental Biology, Uppsalaan 8, 3584 CT Utrecht, The Netherlands

[1]Present address: Department of Biochemistry SJ-70, University of Washington, Seattle, WA 98195,USA

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Type β transforming growth factors represent a family of polypeptides that modulate growth and differentiation. TGF-β exerts its effects on target cells through interaction with specific cell surface receptors, but the signal transduction pathways are largely unresolved as yet. In this study we report that TGF-β_1 induces a rapid phosphorylation of the cyclic AMP responsive element binding protein (CREB) in mink lung CCl64 cells. Phosphorylation induced by TGF-β_1 is not mediated by the cAMP-dependent protein kinase. Parallel to the increase in phosphorylation of CREB, an increase in binding to the collagenase TPA responsive element was observed. CREB participates in the binding to this element, probably as a heterodimer with another as yet unknown protein. The modification imposed on CREB and its involvement in an enhanced TRE-binding could be a mechanism by which TGF-β_1 induces the TRE-mediated transcriptional activation.

Key words: CRE binding/*jun B*/ML-CCl64 cells/nuclear protein phosphorylation/TGF-β_1

Introduction

Type β transforming growth factors (TGF-β) represent a family of polypeptides that modulate growth and differentiation of a wide variety of cell types (Massagué, 1987; Sporn *et al.*, 1986). In initial studies TGF-β was recognized by its ability to induce phenotypic transformation of normal rat kidney cells in the presence of epidermal growth factor (EGF) or transforming growth factor α (De Larco and Todaro, 1978). Subsequently, it has been demonstrated that TGF-β stimulates growth for instance of AKR-2B cells (Leof *et al.*, 1986) and of osteoblast cells (Robey *et al.*, 1987) and that it induces mesoderm differentiation in early amphibian development (Smith, 1989). More recently TGF-β has been recognized to be a member of a large family of growth inhibiting ligands (Massagué, 1987; Roberts *et al.*, 1985). Three types of TGF-β receptor have been characterized, only one of which seems to transduce the TGF-β signal effectively (Cheifetz *et al.*, 1986). Despite the wide interest in the mode of action of TGF-β, very little is known about the receptor-mediated signal transduction mechanism. Although TGF-β counteracts the stimulatory action of FGF, PDGF, EGF or insulin/insulin-like growth factor, it does not do so by blocking early events in signal transduction (Fanger *et al.*, 1986; Libby *et al.*, 1986; Chambard and Pouysségur, 1988). At the nuclear level it was shown that transcriptional activation by TGF-β_1 is mediated by the TPA responsive element (TRE) (de Groot and Kruijer, 1990; Kim *et al.*, 1990; Scotto *et al.*, 1990), and it is suggested that phorbol ester and TGF-β_1 share a common mechanism of transcriptional activation. Recently we observed that TGF-β_1 causes a rapid phosphorylation of a number of nuclear proteins in mink lung CCl64 cells (Kramer *et al.*, 1991), in particular the phosphorylation of a 43 kd, pI 5.7–6.0 protein. Parallel to the induction of phosphorylation we demonstrated the induction of expression of the immediate early gene *jun B*. Augmentation or inhibition of the induced phosphorylation, in particular of the 43 kd, pI 5.7–6.0, coincides with a similar change in gene expression. We concluded therefore that protein phosphorylation may be important for the observed induction of immediate early gene expression. The characteristics of the 43 kd, pI 5.7–6.0 protein resembles those of the cyclic AMP responsive element binding protein (CREB) (Montminy and Bilezikjian, 1987; Zhu *et al.*, 1989) and we investigated therefore the role of CREB in the action of TGF-β_1 using CREB-specific antibodies (Yamamoto *et al.*, 1990). We now report that TGF-β_1 induces rapid phosphorylation of CREB but this phosphorylation does not involve the cAMP signal transduction pathway. Furthermore we show that TGF-β_1 induces an increased binding of nuclear extracts to the consensus sequence of the collagenase TRE. CREB participates in the binding to this element probably as a heterodimer. It may be this mechanism of action that is responsible for the TRE-mediated changes in gene transcription.

Results

TGF-β_1 and forskolin induced phosphorylation of ML-CCl64 nuclear protein

Because we had established that induction of nuclear protein phosphorylation is an early event in TGF-β_1 action (Kramer *et al.*, 1991), next we set out a series of experiments to establish the identity of one of the target proteins. ML-CCl64 cells were labelled overnight with [^{32}P]orthophosphate and challenged with either TGF-β_1 or forskolin for 15 or 25 min. Nuclear extracts were prepared and analysed on a two-dimensional gel electrophoresis system. As shown in Figure 1 (first row) addition of forskolin or TGF-β_1 causes an increase in phosphorylation of a number of proteins. Phosphorylation induced by TGF-β_1 or forskolin was in part comparable, most notably the phosphorylation of a 43 kd protein with an apparent isoelectric point of 5.7–6.0 (depending on the extent of phosphorylation). The molecular characteristics and the observation that the protein is phosphorylated upon a rise in cAMP, closely resemble the characteristics of the transcription factor CREB (M.R.

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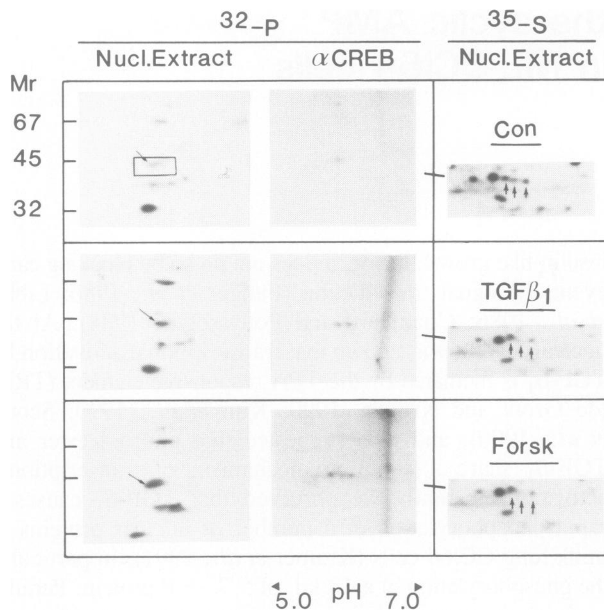

Fig. 1. Effects of TGF-β_1 or forskolin on nuclear protein phosphorylation and protein synthesis in ML-CCl64 cells. Cells were labelled in 4 ml of medium supplemented with 200 μCi of [^{32}P]orthophosphate or 100 μCi of [^{35}S]methionine for 12 h. Cells were next challenged with TGF-β_1 or forskolin for 15 or 25 min. After the challenge, they were washed with PBS–EDTA and frozen in liquid nitrogen. While thawing, 1 ml of sucrose, NaF, EDTA, DTT and PMSF was added and nuclei were isolated. Protein was extracted from the nuclear fraction with 400 mM salt (KCl plus NaF), and after reduction of the salt concentration, the samples were mixed with sample buffer (1:1 volume). Nuclear protein was immunoprecipitated as described in Materials and methods. All preparations were run on a two-dimensional gel system. After drying of the gel, films were exposed for 2 to 3 days. This experiment represents 1 out of 5. Control cells were quenched at 20 min. The arrows indicate the 43 kd, pI 5.7–6.0 protein. αCREB, CREB specific antibody, αCREB244. The ^{35}S-labelled protein spots are a two-fold magnification of the area as indicated on the two-dimensional gel of the total ^{32}P nuclear extract.

Montminy, personal communication). We studied therefore protein phosphorylation with the use of a CREB-specific antibody, αCREB244 (Yamamoto *et al.*, 1990), and found that this antibody specifically immunoprecipitates a 43–45 kd protein, of which the phosphorylation is increased upon treatment of cells with both TGF-β_1 and forskolin (Figure 2). The protein appears as a doublet on a 10% SDS–polyacrylamide gel (see also Yamamoto *et al.*, 1990). Two-dimensional gel electrophoretic analysis of the same SDS-treated preparation revealed that the immunoprecipitated 43–45 kd protein indeed resembles the 43 kd, pI 5.7–6.0 phosphoprotein as present in the gels of nuclear extracts (Figure 1, second vertical row). However, probably because of the SDS treatment and a slightly flatter pH gradient, the protein now appears as three individual spots, probably representing phosphorylated isotypes of the 43 kd protein (whole nuclear extracts could not be treated with SDS because focusing was disturbed. With the immunoprecipitated protein this was not a problem).

The observed increase in phosphorylation of the 43 kd protein could be due either to an increased phosphorylation or to an increased amount of protein. To distinguish between the two possibilities we performed [^{35}S]methionine labelling of the cells and analysed ^{35}S-labelled protein of cells treated

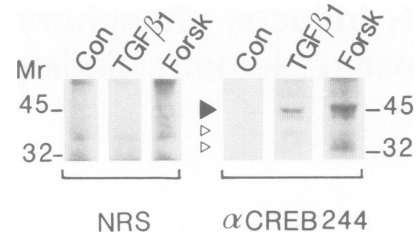

Fig. 2. Immunoprecipitation of ML-CCl64 nuclear protein by αCREB244. Cells were labelled with [^{32}P]orthophosphate for 12 h followed by the addition of TGF-β_1 or forskolin. After quenching of the phosphorylation, nuclear extracts were prepared as described in Materials and methods. Nuclear protein was immunoprecipitated with αCREB and the immuno-pellet was analysed on a 10% polyacrylamide gel. NRS; normal rabbit serum. αCREB; CREB specific antibody αCREB244.

with TGF-β_1 or forskolin. Within a time period of 25 min, the two ligands had no effect on the amount of ^{35}S-label in protein as analysed by two-dimensional gel electrophoresis (Figure 1, third vertical row). The ^{35}S-spots that correspond with the ^{32}P-spots are indicated with arrows. Thus within 25 min both TGF-β_1 and forskolin increased the phosphorylation state of the 43 kd protein.

CRE binding of ML-CCl64 nuclear extracts

Next we tested, in a bandshift experiment, whether αCREB244 indeed recognizes a CRE-binding protein in nuclear extracts of ML-CCl64 cells. As shown in Figure 3, these cells contain specific CRE-binding activity, that is, binding can be competed by a 20-fold excess of unlabelled CRE and is not sensitive to the addition of pUC DNA. Preincubation of the nuclear extracts with αCREB244 resulted in a complete (super)shift of the retarded protein–DNA complexes (Figure 3, last lane). Normal rabbit serum had no effect on the mobility of the retarded complexes (Figure 4, lanes 4–6). The antisera alone did not cause retardation of the oligonucleotide (Figure 4, last two lanes). Thus the αCREB244 antibody recognizes a CRE-binding protein. In combination with the previous data we conclude that the phosphorylation of the 43 kd protein is induced by TGF-β_1, which matches the characteristics of CREB as identified by Montminy and Bilezikjian (1987) (see also Yamamoto *et al.*, 1990).

Effect of TGF-β_1 on CRE and TRE binding of ML-CCl64 nuclear extracts

Although TGF-β_1 induces phosphorylation of a CRE-binding protein, most actions of TGF-β_1 on gene transcription seem to be mediated via the TRE (Kim *et al.*, 1990; Scotto *et al.*, 1990). In ML-CCl64 cells in particular it was shown, in transient transfection experiments with a CAT construct, that TGF-β_1 has no effect on constructs containing a CRE but has an effect on constructs containing the TRE (de Groot and Kruijer, 1990). We therefore studied the effect of TGF-β_1 on CRE- and TRE-binding. Nuclear extracts obtained from control and TGF-β_1 treated cells displayed a similar binding to the CRE as determined in a bandshift assay (Figure 4, lanes 1–3). The presence of αCREB244 caused an almost total supershift of the retarded band for both control and TGF-β_1 treated cells (Figure 4, lanes 7–9). This indicates that neither total binding activity nor the relative contribution of CREB to the CRE-binding

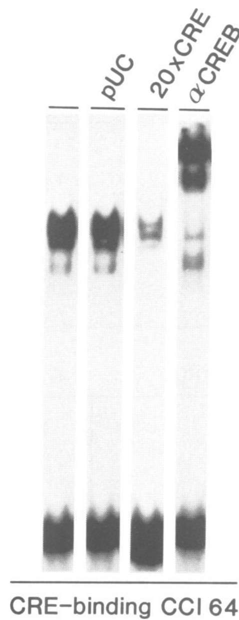

Fig. 3. CRE-binding of ML-CCl64 nuclear extracts. Nuclear extracts were prepared as described for phosphorylation except that the salt content was reduced by dialysis in the presence of 25% (w/v) of glycerol. Nuclear extracts were incubated with a fibronectin [^{32}P]CRE and the protein–DNA complex was analysed on a 5% polyacrylamide gel and visualized by autoradiography. To assay specificity of binding, [^{32}P]CRE binding was challenged with the simultaneous addition of 20-fold CRE, or a similar amount (ng) of pUC DNA. αCREB antibodies were added to the nuclear extract, 5 min prior to the addition of the labelled oligonucleotide. αCREB; CREB specific antibody αCREB244.

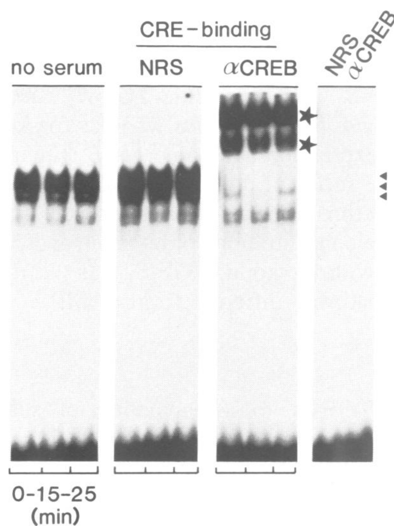

Fig. 4. Effect of TGF-β_1 on CRE-binding in ML-CCl64 cells. Cells were treated with TGF-β_1 for 15 or 25 min. The incubation was stopped by washing the cells with PBS–EDTA followed by rapid freezing in liquid nitrogen. Nuclear extracts were prepared as described for phosphorylation except that reduction of the salt content was obtained by dialysis. Antibodies were added 5–10 min prior to the addition of the labelled oligonucleotides. To test nonspecific effects of the antibodies on DNA binding, the [^{32}P]CRE was incubated in the absence of nuclear extract (last column). The stars indicate the supershifted protein–DNA bands; the triangles indicate the retarded bands that are diminished in the presence of a 20-fold excess of unlabelled CRE. NRS; normal rabbit serum; αCREB; CREB specific antibody αCREB244.

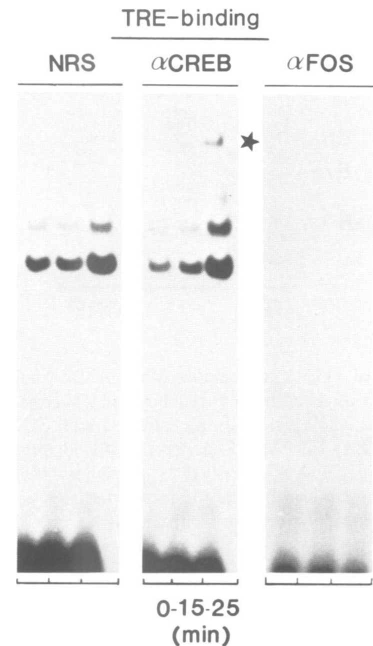

Fig. 5. Effect of TGF-β_1 on TRE-binding in ML-CCl64 cells. Cells were treated with TGF-β_1 for 15 or 25 min. The incubation was stopped by washing the cells with PBS–EDTA followed by rapid freezing in liquid nitrogen. Nuclear extracts were prepared as described for phosphorylation except that reduction of the salt content was obtained by dialysis in the presence of 25% (w/v) of glycerol. Antibodies were added 5–10 min prior to the addition of the labelled oligonucleotides. The star indicates the supershifted protein–DNA band; the triangles indicate the retarded bands that are diminished in the presence of the αFOS antiserum. αCREB; CREB specific antibody αCREB244. αFOS; antibody αFOS M2 that recognizes FOS and FOS-related antigens.

complex changes [similar experiments have been performed with forskolin treated cells but no effect was found either (Montminy and Bilezikjian, 1987)]. Next we assayed TRE binding. ML-CCl64 nuclear extracts only showed a moderate binding to the TRE (20% binding activity as compared with the CRE), however, this binding increased 4- to 5-fold in nuclear extracts of TGF-β_1 treated cells (scintillation counting of retarded protein–DNA bands) (Figure 5). This increase becomes apparent after 15–25 min of TGF-β_1 treatment. The retarded bands appear as a doublet (Figure 5, at the positions of the triangles) in the gel at a different level compared with any of the bands in the CRE binding assay. The relative contribution of these two bands varied within the experiments for a, thus far, unknown reason. Interestingly, addition of αCREB244 to the nuclear extract revealed that CREB is a component of the TRE-binding complex after treatment of the cells with TGF-β_1 (Figure 5, lanes 4–6). As estimated by the amount of label that is supershifted, its contribution to the TRE-binding seems much less compared with that of the binding to the CRE. The antibody supershifts ~20% of total label present in the retarded protein–DNA complex as calculated by scintillation counting of the excised bands. Because it was shown by Ransone *et al.* (1990) that CREB homodimers cannot bind to the collagenase TRE, we analysed further the configuration in which CREB participates in the TRE binding. We added an αFOS antibody and found a complete disappearance of the retarded complexes (Figure 5, last three lanes). This result indicates that a FOS-related antigen is

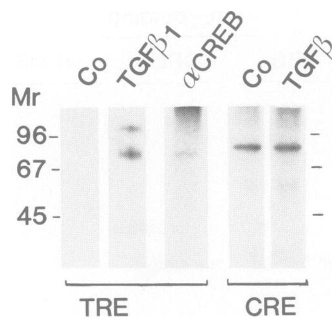

Fig. 6. Effect of TGF-β_1 on binding of ML-CCl64 nuclear extracts to a TRE or CRE as determined by analysis of UV-crosslinked protein–DNA complexes. Cells were treated with TGF-β_1 for 25 min, washed with PBS–EDTA and quenched in liquid nitrogen. Nuclear extracts were prepared as described for phosphorylation except that the salt content was reduced by dialysis in the presence of 25% (w/v) of glycerol. Nuclear extracts were incubated with [^{32}P]CRE or [^{32}P]TRE for 5 min followed by an exposure to short-wave UV light for 15 min. The incubation was terminated by the addition of Laemmli sample buffer (Laemmli, 1970) and protein–DNA complexes were analysed on a 10% SDS–polyacrylamide gel. To analyse the presence of CREB in the labelled protein bands, the TRE–protein preparation was diluted after UV treatment and protein was immunoprecipitated with αCREB244 as described in Materials and methods (middle lane). αCREB; CREB specific antibody αCREB244.

involved in the TRE-binding but it also indicates that CREB is likely to be involved in this binding as a heterodimer, because in a parallel experiment with a CRE oligonucleotide, αFOS left the (homodimer) CRE-binding unaffected (data not shown). The finding that αFOS prevents TRE-binding is in agreement with the findings of Sassone-Corsi *et al.* (1990) (see also Curran *et al.*, 1985) using similar antibodies.

Crosslinking of protein–DNA complexes

To substantiate further the identity of the proteins involved in the TRE-binding we performed crosslink experiments and analysed the presence of CREB with the αCREB244 antiserum. Treatment of nuclear extracts plus [^{32}P]TRE with UV for 15 min resulted in the formation of two ^{32}P-labelled protein bands in nuclear extracts from TGF-β_1 treated cells (Figure 6). These radiolabelled bands do not appear on the gel in the absence of UV treatment or with addition of a 40-fold excess of unlabelled TRE (data not shown). The identity of the bands cannot be deduced from their relative mobility because the proteins may appear as monomers or dimers and the contribution of the probe to relative mobility cannot be estimated. However, immunoprecipitation of the crosslinked complexes revealed that αCREB244 recognizes a 78–80 kd protein (Figure 6, middle lane). In previous studies with ^{32}P-labelled nuclear extracts we showed that αCREB recognizes a 43 kd protein thus, according to the molecular weight of the UV-crosslinked TRE protein complex (78–80 kd), CREB must share the TRE-binding with another protein (or proteins). When the same experiment is performed with a CRE, only one band of ~88 kd is visible (Figure 5). This indicates that the proteins appear in the gel as crosslinked dimers.

TGF-β_1 and the cAMP pathway

TGF-β_1 and cAMP seem to mimic each other with respect to induction of substrate phosphorylation, so we tested the effect of TGF-β_1 on the formation of intracellular cAMP. TGF-β_1 did not alter the levels of cAMP (Figure 7A), either in a positive or in a negative way (forskolin plus TGF-β_1, data not shown). As a positive control forskolin treatment caused a substantial elevation of cAMP levels. In addition we compared immediate early gene expression between TGF-β_1 and forskolin. TGF-β_1 caused mainly expression of *jun B* and no c-*fos* whereas forskolin caused mainly c-*fos* expression and some *jun B* (Figure 7B). We tested a whole series of concentrations of the two ligands (of which only three are presented) to rule out a dose effect. At none of the concentrations of either ligand can TGF-β_1 be compared with forskolin. TGF-β_1 acts through a signal transduction pathway different from cAMP.

A

	cAMP (pmol/10^{6}cells)	
	TGFβ_1	Forskolin
time 0	1.2	1.8
5	1.4	14.2
10	0.7	16.8
20	1.1	16.2
30 (min)	1.3	15.4

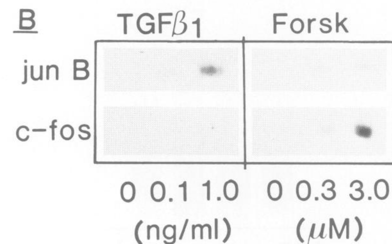

Fig. 7. A. Measurement of intracellular cAMP after treatment of ML-CCl64 cells with TGF-β_1 or forskolin. Cells were treated with TGF-β_1 or forskolin for the time periods indicated. Quenching was assured by removal of the medium followed by the addition of ice-cold 90% (v/v) propanol. The samples were left overnight at 4°C, the propanol was evaporated and cAMP was measured using the Amersham radioimmunoassay kit. **B.** Immediate early gene expression as induced by TGF-β_1 or forskolin in ML-CCl64 cells. Cells were treated with various concentrations of TGF-β_1 or forskolin for 45 min. RNA was isolated, size fractionated and analysed by Northern blotting. Hybridization was performed with *jun B* and c-*fos* DNA.

Discussion

Addition of TGF-β_1 to a population of sub-confluent ML-CCl64 cells induces rapid phosphorylation of a 43 kd, pI 5.7–6.0 nuclear protein that subsequently has been identified as the CREB (Montminy and Bilezikjian, 1987). Identification of this protein has been based on the criteria that: (i) the protein is recognized by the CREB specific antibody αCREB244; (ii) the protein has an identical molecular weight and isoelectric point (M.R.Montminy, personal communication; Yamamoto *et al.*, 1990); (iii) the antibody does detect a CRE-binding protein in nuclear extracts of ML-CCl64 cells as determined in a gel mobility shift assay; (iv) the protein can be phosphorylated in a cAMP dependent manner; and (iv) the protein elutes from the nucleus at a minimal salt concentration of 300 mM (NaF + KCl). The immunoprecipitated protein appears as a number of spots on a two-dimensional gel, whereas in total nuclear extracts, this resolution cannot be obtained and the

protein appears as a streak. With increasing phosphorylation the spot stretches to the acidic side of the gel which in the immunoprecipitated protein preparation is observed as a subsequent phosphorylation of additional (more acidic) spots. The increase in phosphorylation is not due to an increased expression of protein because within a similar time span we did not observe any changes in ^{35}S-labelled protein (of selected spots that migrate at a similar position to ^{32}P-labelled CREB) after addition of TGF-β_1 or forskolin. In addition, these data also reveal that in a non-stimulated cell, a similar set of ^{35}S-proteins is present to that in the stimulated one and that the subsequent appearance of additional phosphoprotein spots is not likely to be the consequence of an increased acidification of one protein but rather an additional phosphorylation of a more acidic protein spot. The relative molecular weight of the protein spots does increase slightly after addition of TGF-β_1 or forskolin, which might be a consequence of phosphorylation. This implies that CREB is present in at least three forms (isotypes) (see also Yamamoto *et al.*, 1990; Berkowitz and Gilman, 1990).

Although phosphorylation of CREB would predict an involvement of the CRE in changes in gene transcription, we were unable to detect any effect of TGF-β_1 on CRE-binding, also TGF-β_1 did not induce CAT expression in a CRE−CAT construct, as assayed in a transient transfection assay in ML-CCl64 cells (de Groot and Kruijer, 1990). It became apparent from a number of studies that TGF-β_1 acts through a TRE (Kim *et al.*, 1990; Scotto *et al.*, 1990) and it was these observations that made us consider the role of CREB in TRE-binding. Treatment with TGF-β_1 causes a rapid increase in TRE-binding and from data obtained with αCREB244 antiserum it became apparent that CREB is a component of the protein complex that binds to the TRE. The observation that the CREB is involved in binding to a consensus TRE of the collagenase gene appears unprecedented. Although CREB homodimers can bind the TRE of the *jun* gene it has not been shown to be able to bind to the collagenase TRE (Ransone *et al.*, 1990). Thus CREB must be present in the protein−DNA binding complex in a configuration different to that of a homodimer. The binding to the TRE can be prevented by the addition of an antibody that recognizes FOS or a FOS-related antigen (Curran *et al.*, 1985; Sassone-Corsi *et al.*, 1990; Franza *et al.*, 1990) which indicates that FOS or a FOS-related antigen is also involved in binding to TRE. The observation that both αFOS and αCREB244 affect the DNA-binding complex suggests that CREB may bind to the TRE as a heterodimer together with FOS or a FOS-related antigen, however, no clear proof is yet given. This protein is unlikely to be the product of the c-*fos* gene itself, because expression of this gene is not detectable in ML-CCl64 cells and the half-life of the protein is very short (Ryder *et al.*, 1988). In an attempt to analyse further the protein complex that binds to the TRE (using a simple UV-crosslink protocol) we confirmed that the protein complex consists of more than one type of protein and that αCREB244 does indeed recognize one of the protein bands. The band that is recognized has a mol. wt of ~78 kd, which is not the same size as the mol. wt of the protein band that is detected in the [^{32}P]CRE binding assay. In the CRE-binding assay, a 88 kd protein is detected. This latter observation is in agreement with the findings of Yamamoto *et al.* (1988) who observed two types of UV-crosslinked protein−DNA complexes, one of 45 and one of 88 kd.

The extent of dimerization (88 kd protein) was dependent on the phosphorylation state of purified CREB. In ML-CCl64 cells, the 43 kd protein is always present in a phosphorylated state (although only in small amounts); this might explain why we observe, both in nuclear extracts of stimulated and unstimulated cells, mainly a 88 kd protein band. Assuming that the 88 kd protein band consists of a CREB homodimer, it is again evident that such a complex is not present in the TRE−protein complex. The precise identity of the protein complex and the explanation of why CREB can bind to the TRE in the configuration of a heterodimer awaits further investigation.

The role of phosphorylation of CREB in relation to the increased TRE-binding and TRE−CAT transcription remains to be established. In a previous study we presented data indicating that protein phosphorylation induced by TGF-β_1 is relevant for the expression of the immediate early gene *jun B* (Kramer *et al.*, 1991). From those data it appeared that interference of TGF-β_1-induced phosphorylation of nuclear protein by a protein kinase inhibitor or a protein phosphatase inhibitor, is matched by an interference of the induction of *jun B* expression. This correlation was particularly clear for the phosphorylation of the 43 kd, pI 5.7−6.0 protein. Moreover in a previous study we showed that increased expression of *jun B* is a consequence of enhancing its expression via modification of existing transcription factors. It seems likely therefore that CREB phosphorylation is involved in the changes in gene expression. So far, *in vitro* phosphorylation of CREB and the effect on DNA binding and gene transcription has been studied with the use of protein kinase A or C (Yamamoto *et al.*, 1988). Our studies suggest that these protein kinases are unlikely candidates in phosphorylation of CREB in ML-CCl64 cells. Concerning the unusual behaviour of CREB to bind to the collagenase TRE, this may be explained by the possibility that phosphorylation of CREB as induced by TGF-β_1 may impose new properties on the CREB protein, in that it directs its affinity towards different transcription factors or DNA binding sites. Conclusive studies could only be performed in a cell-free system with the presence of exogenous CREB and CREB mutants lacking phosphorylation sites (Gonzalez and Montminy, 1989; Gonzalez *et al.*, 1989). The signal transduction data presented in this report show that although the cAMP-mediated phosphorylation can mimic the phosphorylation induced by TGF-β_1, no increase in intracellular cAMP was found and in addition immediate early gene expression differed between TGF-β_1 and forskolin. Thus TGF-β_1 does not activate the cAMP-dependent pathway (see also Kerr *et al.*, 1988). In addition it should be noted that the PKC pathway and very likely the Ca^{2+}/calmodulin pathway are not used either (Kramer *et al.*, 1991). For example TPA does not induce phosphorylation of the 43 kd, pI 5.7−6.0 protein in ML-CCl64 cells (unpublished observation). However, the identification of one substrate should facilitate the search for a protein kinase that is involved in the cascade of events following addition of TGF-β_1.

In conclusion, TGF-β_1 induces the phosphorylation of CREB, which in time relates to the immediate early gene expression measured in parallel. The phosphorylation of CREB coincides with an increased binding of nuclear extracts to the collagenase TRE. CREB participates in this binding

together with at least one other component, amongst which a FOS-related antigen is a likely candidate. Thus modification of CREB may result in an increase in TRE binding and this mechanism of action could explain the effects of TGF-β_1 on expression of (immediate early) genes containing a TRE in the promoter region.

Materials and methods

Materials

Pharmalyte ampholines (3–10 and 6–8) and IBMX were obtained from Sigma Chemical Company (St Louis, MO). Urea and agarose were from BRL (Bethesda, DC). Tissue culture media were from Flow Laboratories (Zwanenburg, The Netherlands). [α-^{32}P]-dCTP, [α-^{32}P]-dATP, [^{32}P]-orthophosphate, [^{35}S]-methionine, multiprime DNA labelling kit, cAMP radioimmunoassay kit, Amplify and Hybond-N were purchased from Amersham Co. (UK). αCREB244 was obtained from M.Montminy (Salk Institute, San Diego, CA, USA) and αFOS (M2 peptide) was obtained from T.Curran (Roche Institute of Molecular Biology, NJ, USA).

Cell culture

Mink Lung CCl64 cells were grown in Dulbecco's Modified Essential Medium (DMEM, Flow Laboratories) supplemented with 7.5% FCS and buffered with bicarbonate (44 mM). Cells were grown to near confluency in 60 cm^2 Petri dishes before challenging with TGF-β_1.

TGF-β_1

TGF-β_1 was purified from human platelets according to the method described by van Eijnden-van Raaij *et al.* (1988). In a standard cell growth assay, an 80% growth inhibition within 36 h of incubation could be obtained at 1 ng/ml of TGF-β_1 as determined by [^{3}H]thymidine incorporation.

Nuclear protein phosphorylation and [^{35}S]methionine incorporation

Cells were preincubated for 12 h with 200 μCi of [^{32}P]orthophosphate or [^{35}S]methionine in 4 ml of DMEM plus 7.5% serum. After addition of TGF-β_1 (3 ng/ml) or forskolin (3 μM), cells were left for further incubation at 37°C for times varying from 15 to 25 min. At the appropriate time, medium was removed, cells were washed once with 5 ml of PBS/5 mM EDTA (37°C) and rapidly frozen in liquid nitrogen. While thawing, 1 ml of Tris–HCl pH 8.2 (10 mM), sucrose (0.34 M), NaF (50 mM), and DTT (1 mM) plus PMSF (1 mM) was added. Complete breakage of cells was ensured by homogenization in a Dounce type B homogenizer. Nuclei were spun down at 10 000 g for 20 s and the supernatant was removed. To extract protein from the nuclear pellet, 100 μl of Tris–HCl pH 8.0 (30 mM), NP-40 (0.1% v/v, KCl (370 mM), NaF (50 mM), $MgCl_2$ (2.5 mM) plus DTT (1 mM) and PMSF (1 mM) was added and left at 4°C for 30 min while being vortexed every 5 min. At the end of the incubation 300 μl of NP-40 (0.1% v/v) was added and non-extracted material was spun down at 10 000 g for 3 min. This preparation was either used for two-dimensional gel electrophoresis or for immunoprecipitation.

Two-dimensional gel electrophoresis

Nuclear extracts were mixed with one volume of urea (8 M), ampholines (2.0%, pH range 3–10. 0.2%, pH range 6–8), β-mercaptoethanol (2% v/v) and NP-40 (2% v/v). Radioactivity in each sample was roughly estimated to be equivalent to the amount of sample added onto the gel. Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell *et al.* (1975). Gels were stained with Coomassie blue, dried and left with film for 1 or 2 days in the presence of an enhancer screen. In the case of [^{35}S]methionine, gels were soaked in Amplify for 15 min before drying.

Immunoprecipitation

Protein A–Sepharose 6MB was washed three times in 1 ml of 10 mM Tris (pH 8.0), 0.1 mM EGTA, 1.0 mm $MgCl_2$, 0.1% (v/v) NP-40, 10 mM NaF, 100 mM KCl, 1 mM DTT and 1 mM PMSF. Nuclear extracts were preincubated with protein A–Sepharose (~7 mg) for 60 min at 4°C with gentle shaking. The supernatant was added to 8 μl of the αCREB244 antiserum (1:50 dilution) and the incubation was continued for another 4 h at 4°C with gentle shaking. The nuclear extract was then centrifuged for 3 min in an Eppendorf centrifuge at 10 000 g to remove all particulate components. The supernatant was added to a pellet of 5 mg of protein A–Sepharose. After mixing, the extract was left for a further 1 h at 4°C, with gentle shaking. The protein A–Sepharose was pelleted and washed six times with 1 ml of the buffer that was also used to wash the protein A–Sepharose. Before the final wash the tubes were changed to eliminate non-specific binding to the tube.

Gel mobility shift assay and UV crosslinking

Oligonucleotides containing the collagenase TRE (Angel *et al.*, 1987) (5'-AGCTTGGTGACTCATCCG-3' and 3'-ACCACTGAGTAGGCC-TAG-5') were synthesized in a nucleotide synthesizer. The fibronectin CRE (Dean *et al.*, 1987) 5'-TGACGTCACCCG-3' and 3'-GGGCACTGCA GTGGGCTCGA-5') was cloned into a pGEM plasmid (Promega). The oligonucleotide was excised from the plasmid by *Xho*I and *Pst*I and isolated via a 10% (w/v) polyacrylamide gel. The cohesive ends of the fragments were labelled with [α-^{32}P]dATP and [α-^{32}P]dCTP (5000 Ci/mol) using large fragment Klenow DNA polymerase I. Labelled oligonucleotides were separated from the reaction mixture constituents by gel filtration using Sephadex G-50 spin columns equilibrated in 10 mM Tris–HCl, (pH 8.0), 1 mM EDTA and 150 mM NaCl. Isolation of nuclear extracts was performed as described in this paper, except that non-labelled cells were used and the samples were not diluted but dialysed against 500 ml of 20 mM HEPES, (pH 8.2), 100 mM KCl, 25% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT and 1 mM PMSF, for 2 h at 4°C in an Eppendorf tube sealed with a dialysis membrane. Protein concentration was determined by the BioRad protein assay according to the manufacturer's protocol. Approximately 10 μg of protein was used per gel mobility shift sample.

The electrophoretic mobility shift assay was performed as described by Fried and Crothers (1981) and de Groot and Kruijer (1990), except that electrophoresis was carried out overnight at 20 V. When antibodies were applied, these were added to the nuclear extracts (1:50 dilution) 5–10 min prior to the addition of the labelled nucleotides. For UV-crosslink experiments, the nuclear extract combined with the labelled oligonucleotide was placed on SARAN wrap and exposed for 15 min to short-wave UV light, at a distance of 5 cm, in a humidity box. Samples were removed from the SARAN after the addition of Laemmli sample buffer (Laemmli, 1970), heated in boiling water for 5 min and analysed on a 10% SDS–polyacrylamide gel.

RNA preparation and Northern hybridization

For gene expression experiments, cells were challenged with TGF-β_1 or forskolin for 45 min. After removal of medium, cells were lysed *in situ* by sarkosyl–guanidium thiocyanate–β-mercaptoethanol. RNA was isolated by centrifugation through a caesium chloride cushion followed by ethanol precipitation. Total RNA was denatured for 15 min at 68°C in 50% formamide, 2.2 M formaldehyde, 2 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA and then size-fractionated by electrophoresis through a 0.8% agarose gel containing 2.2 M formaldehyde. Hybridization was performed with a 1.5 kb *Eco*RI cDNA fragment of *jun B* (Curran *et al.*, 1982) and a 1.0 kb fragment of c-*fos* (Ryder *et al.*, 1988). A 1.4 kb cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to quantify the RNA that was transferred to the blot (data not shown). Probes were labelled by random priming with [α-^{32}P]dCTP.

Measurement of cAMP

Cells (1 × 10^6/well) were preincubated with 0.5 mM of IBMX for 10 min prior to the addition of TGF-β_1 (3 ng/ml), forskolin (3 μM) or a combination of the two. The cells were left in the presence of the ligand for various time intervals. Quenching was assured by removal of the medium followed by addition of ice-cold 90% (v/v) propanol. The samples were left overnight at 4°C, the propanol was evaporated and cAMP was measured using the Amersham radioimmunoassay kit.

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