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Transcriptional Modulation of Transin Gene Expression by Epidermal Growth Factor and Transforming Growth Factor Beta

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Transin is a transformation-associated gene which is expressed constitutively in rat fibroblasts transformed by a variety of oncogenes and in malignant mouse skin carcinomas but not benign papillomas or normal skin. It has been demonstrated that, in nontransformed Rat-1 cells, transin RNA expression is modulated positively by epidermal growth factor (EGF) and negatively by transforming growth factor beta (TGF-β); other peptide growth factors were found to have no effect on transin expression. Results presented here indicate that both protein synthesis and continuous occupancy of the EGF receptor by EGF were required for sustained induction of transin RNA. Treatment with TGF-β inhibited the ability of EGF to induce transin, whether assayed at the transcriptional level by nuclear run-on analysis or at the level of transin RNA accumulation by Northern (RNA) blot analysis of cellular RNA. TGF-β both blocked initial induction of transin transcription by EGF and halted established production of transin transcripts during prolonged treatment. These results suggest that TGF-β acts at the transcriptional level to antagonize EGF-mediated induction of transin gene expression.

Transin is a secreted protease of M_r 53,000 (9, 11) which shares significant amino acid homology with members of the metalloprotease family, such as human stromelysin (4, 15), human skin collagenase (5), and rabbit synovial cell activator (C. Brinckerhoff, personal communication). The transin gene was initially isolated by differential screening of a cDNA library from polyomavirus-transformed rat fibroblasts (9). Transin is constitutively expressed in significantly higher levels in rat cells transformed by polyomavirus, Rous sarcoma virus, and the oncogene h-*ras* than in the normal parental cell lines (9). Transin mRNA was detected in mouse skin squamous cell carcinomas but not in normal mouse skin or experimentally induced benign papillomas (7), suggesting a possible role in the tumorigenic capacities of transformed cells.

In nontransformed rat fibroblast cell lines, transin transcription is specifically induced by epidermal growth factor (EGF) but not by platelet-derived growth factor, fibroblast growth factor, insulin, or transforming growth factor beta (TGF-β); TGF-β has been found to inhibit the expression of transin when analyzed several hours after simultaneous addition of EGF and TGF-β (11). Neither the molecular mechanisms responsible for induction of transin by EGF nor the mechanisms responsible for the inhibitory effects of TGF-β are understood. To characterize the respective roles of EGF and TGF-β as positive and negative effectors of transin expression more fully, we conducted parallel studies on the cytoplasmic stability and nuclear transcription of transin RNA in response to these two effectors.

MATERIALS AND METHODS

Cell culture. Rat-1 fibroblasts (12) and their v-*src*-transformed derivative RM2 (supplied by Michael Weber) were cultured in Dulbecco modified Eagle medium (DMEM) containing 10% defined calf serum (Hyclone) and appropriate antibiotics at 37°C in a humidified 95% air–5% CO_2 atmosphere. Confluent cells were serum deprived with unsupplemented DMEM 16 to 24 h before stimulation with the various biological modifiers.

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Northern (RNA) blot analysis of RNA. Total cellular RNA was extracted and analyzed by Northern analysis as previously described (13). Cellular RNA was fractionated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) and prehybridized in the presence of a buffer containing 0.75 M NaCl, 75 mM sodium citrate, and 50% formamide at 42°C for 16 h as previously described (13). The prehybridization solution was replaced with hybridization solution containing the plasmid pTR1 labeled with ^{32}P by nick translation to a specific activity greater than 2×10^8 cpm/μg. pTR1 contains a 1.6-kilobase *Eco*RI fragment representing most of the full-length rat transin cDNA (11) (kindly supplied by L. Matrisian). After hybridization for 16 h at 42°C, the membranes were washed twice for 5 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS (sodium dodecyl sulfate) at room temperature, followed by two washes in 0.1× SSC–0.1% SDS at 50°C for 30 min per wash. The hybridized membranes were then exposed to Kodak X-Omat X-ray film with intensifying screens for 1 to 2 days at −70°C.

Transcriptional run-on analysis. Nuclei were prepared as described by Brown et al. (3), and all preparations and materials were kept at 4°C. The final nuclear pellet was suspended and stored at −80°C in 20 mM Tris hydrochloride (pH 7.9)–75 mM NaCl–0.5 mM EDTA–8.5 mM dithiothreitol–0.125 mM phenylmethylsulfonyl fluoride–50% glycerol. Initiated transcripts were elongated in the presence of 120 mM Tris hydrochloride (pH 7.8)–50 mM NaCl–0.35 M ammonium sulfate–4 mM manganese chloride–0.24 mM EDTA–1 mg of heparin per ml–1 mM each ATP, GTP, and UTP–20 to 25 μCi of [α-^{32}P]CTP (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.). Samples containing an identical amount of radioactivity (typically 10^7 cpm from an extract representing four 10-cm-diameter dishes) were then hybridized for 4 days at 42°C, as described by Brown et al. (3), against transin cDNA immobilized on diazobenzyloxymethyl-paper (DBM-paper) filters (Schleicher & Schuell). The filters were prepared by electrophoresis of *Eco*RI-digested pTR1 in a 0.8% agarose gel, followed by transfer onto

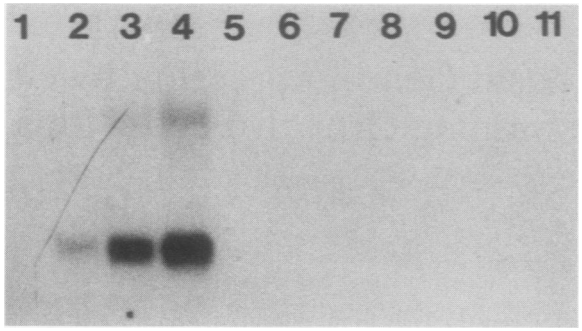

FIG. 1. Effects of EGF, TPA, and DOG on accumulation of transin RNA. Confluent Rat-1 cells (5×10^6 cells per 10-cm plate) were serum deprived for 16 h in DMEM and then exposed to EGF (10 ng/ml), TPA (100 ng/ml), DOG (100 μM), or the vehicular control dimethyl sulfoxide (0.1% vol/vol). Cells were then harvested, and total cellular RNA was prepared. Total cellular RNA (10 μg per lane) was size fractionated by electrophoresis in formaldehyde gels and transferred to nitrocellulose membranes. The transin cDNA pTR1 was labeled with ^{32}P to a specific activity of 5×10^7 to 10×10^7 dpm/μg and hybridized to the immobilized RNA in a 50% formamide–5× SSC hybridization buffer at 42°C for 16 h. Stringent washing for 1 h with 0.1× SSC–0.1% SDS at 50°C was followed by 1 to 2 days of exposure to Kodak X-Omat X-ray film with intensifying screens. Lane 1 shows the results with unstimulated control cells. Lanes 2, 3, and 4 show the increasing transin mRNA levels observed after exposure to EGF for 2, 4, and 6 h, respectively. Neither TPA for 2, 4, and 6 h (lanes 5 to 7) nor DOG for 2, 4, and 6 h (lanes 8 to 10) produced any detectable induction of transin RNA. Lane 11 represents the results obtained with cells exposed to the TPA and DOG solvent, dimethyl sulfoxide, for 6 h.

activated DBM-paper as described by Alwine et al. (1). After hybridization, the filters were washed three times in 2× SSC–0.1% SDS for 10 min at 42°C, treated with RNase A (50 μg/ml), and washed again in 2× SSC. Final washes were then performed in 0.1× SSC–0.1% SDS at 42°C. The filters were exposed overnight to Kodak X-Omat X-ray film at −70°C with intensifying screens.

Chemicals and reagents. EGF was prepared from mouse submaxillary glands essentially as described by Savage and Cohen (14) and further purified to homogeneity as previously described (10). TGF-β was prepared from human platelets as described by Assoian et al. (2) with the addition of a final purification step consisting of reversed-phase high-performance liquid chromatography on C3 columns and elution with an acetonitrile-trifluoroacetic acid gradient. 12-*O*-Tetradecanoyl phorbol-13-acetate (TPA) was purchased from Pharmacia and dissolved in dimethyl sulfoxide. L-*sn*-1,2-Dioctanoylglycerol (DOG) was purchased from Avanti Polar Lipids, Inc., Birmingham, Ala. Actinomycin D and anisomycin were both obtained from Sigma Chemical Co., St. Louis, Mo. Anti-EGF immunoglobulin G (IgG) was purified from rabbit antiserum as described previously (6).

RESULTS

Effects of EGF and protein kinase C activators on transin induction. To determine whether activation of protein kinase C might be involved in the induction of transin by EGF, we tested both EGF and activators of protein kinase C for their ability to induce transin RNA in Rat-1 cells. Confluent 10-cm plates were serum deprived for 24 h before the addition of the putative inducing agents. Cells were harvested at 2, 4, and 6 h after stimulation, and total cellular RNA was extracted for gel electrophoresis and Northern blot analysis. Stimulation of serum-deprived cells with EGF resulted in an elevation of transin RNA levels which gradually increased over the 6-h time course (Fig. 1). Transin was essentially undetectable in nonstimulated cells (Fig. 1, lane 1). In contrast to the stimulatory effects of EGF (lanes 2 to 4), neither TPA (lanes 5 to 7), DOG (lanes 8 to 10), nor the vehicular control dimethyl sulfoxide (lane 11) produced any induction of transin RNA over the 6-h period studied. Thus, under these conditions, induction of transin in nontransformed cells did not appear to be mediated by activation of protein kinase C.

EGF receptor occupancy and transin expression. To determine whether the continued presence of EGF was required for sustained maintenance of transin RNA expression, we first treated cells with EGF for 10 h to establish high levels of transin RNA expression. EGF was then removed by rinsing the cells in serum-free medium, and the cells were subsequently treated with anti-EGF IgG to prevent rebinding of any EGF released from cell surfaces. The optimal concentration of IgG was derived from the concentration of IgG which maximally inhibited binding of [^{125}I]EGF to cell surfaces (data not shown). Cells were harvested for RNA extraction and Northern analysis at different times after removal of EGF and addition of anti-EGF IgG. Transin levels gradually decreased over a 6-h period after removal of EGF (Fig. 2). Graphic analysis of the densitometric signal measured at 0, 2, 4, and 6 h after anti-EGF IgG treatment indicated that the half-life of transin RNA under these conditions was approximately 4 h. Experiments measuring the decrease in cellular transin RNA levels after treatment with the RNA synthesis inhibitor actinomycin D produced similar estimates for the half-life of transin RNA (data not shown). Thus, the sustained expression of transin for 10 to

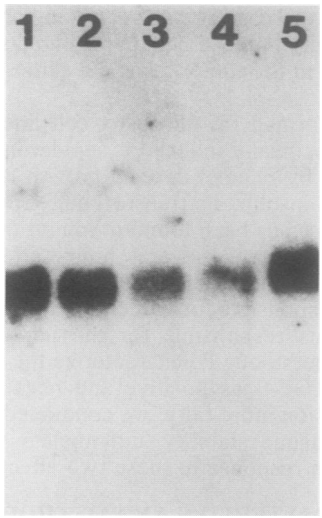

FIG. 2. Transin RNA expression after removal of EGF from extracellular medium. Confluent Rat-1 cells were serum deprived for 16 h in DMEM and then exposed to EGF for 10 h. The EGF-containing medium was removed and replaced with fresh DMEM containing levels of anti-EGF IgG sufficient to block EGF binding to the EGF receptor. Cells were incubated for an additional 2, 4, or 6 h and then harvested, and total cellular RNA was prepared as described in the text. Gel electrophoresis and Northern blot analysis were performed as described in the legend to Fig. 1, with ^{32}P-labeled pTR1 as the hybridization probe. Lanes 1 through 4, respectively, show the levels of transin RNA observed at 0, 2, 4, and 6 h after removal of EGF. Lane 5 represents a positive control.

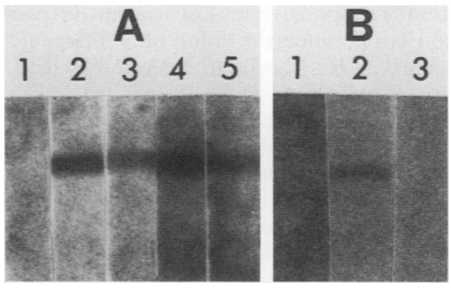

FIG. 3. Nuclear run-on analyses of transin transcription in Rat-1 cells. (A) Effect of EGF on transin transcription. Rat-1 cells were serum deprived and exposed to EGF for various times. Cells from four 10-cm dishes were then harvested, and nuclei were isolated for transcriptional nuclear run-on analysis as described in Materials and Methods. Lanes 1 to 5 show the hybridization signal produced by nuclear RNA extracted from cells exposed to EGF for 0, 0.5, 1, 6, and 10 h, respectively. (B) Effect of anisomycin on EGF-induced transin transcription. Rat-1 cells were exposed to EGF for 30 min in either the absence (lane 2) or the presence (lane 3) of 5 μg of anisomycin per ml added 15 min before EGF stimulation; untreated control Rat-1 cells were represented in lane 1. Transin sequences were released from the vector by *Eco*RI digestion, and the resulting fragments were size fractionated by gel electrophoresis before capillary transfer to DBM-paper. Nonspecific hybridization to vector sequences present on the DBM-paper strips was negligible under all experimental conditions. The autoradiograph shows the hybridization signal obtained from RNA transcripts obtained from nuclear extracts as described in Materials and Methods.

12 h observed in these and earlier experiments (9) appeared to result from continued binding of EGF to EGF receptors throughout the experimental exposure.

Transcriptional regulation of transin by EGF. To determine the temporal characteristics of transin transcription after EGF stimulation, transcription was monitored by nuclear run-on analysis. After serum deprivation for 24 h, nearly confluent Rat-1 cells were exposed to EGF for 0 to 10 h. Cells were harvested, and nuclei were isolated for nuclear transcriptional run-on analysis. Newly synthesized transin transcripts were evident as early as 30 min after EGF stimulation, and transcription continued for at least 10 h (Fig. 3A). Nuclei from cells which had not been exposed to EGF failed to produce detectable transcripts. The ability of EGF to induce transin transcription was inhibited by addition of the protein synthesis inhibitor anisomycin for 15 min before EGF stimulation (Fig. 3B). Hybridization to vector DNA sequences immobilized on the same DBM-paper strips was neglible under all experimental conditions.

Effects of TGF-β on induction of transin by EGF. To define more fully the interaction between EGF and TGF-β in modulating transin expression, we pretreated serum-deprived Rat-1 cells with EGF for various times before adding TGF-β. At 2 h after addition of TGF-β, the cells were harvested and total RNA was extracted for Northern analysis. A 2-h exposure to TGF-β was sufficient to decrease substantially the cellular levels of transin RNA, regardless of the length of pretreatment with EGF (Fig. 4). Transin RNA was not detected in the control cells containing the diluent for TGF-β additions.

Nuclear run-on analyses of transcription were performed on serum-deprived Rat-1 cells after cotreatment with EGF and TGF-β. Nuclei from cells exposed to EGF alone for 2 h displayed a high level of transin transcription (Fig. 5a, lane B), while nuclei from cells exposed to either TGF-β alone or TGF-β followed by EGF (lanes C and D) produced no discernible transin transcripts, although transcription of the ubiquitously expressed gene p1B15 (P. E. Danielson, S. Forss-Petter, M. A. Brow, L. Calavetta, J. Douglass, R. J. Milner, and J. G. Sutcliffe, DNA, in press) appeared to be unaffected by either EGF or TGF-β treatment (Fig. 5a). Treatment of Rat-1 cells with TGF-β completely blocked the ability of EGF to promote transin transcription, whether TGF-β and EGF were added simultaneously (Fig. 5b, lane 3) or whether TGF-β was added subsequent to establishment of transin induction by 2 h of pretreatment with EGF (Fig. 5b, lane 5). Thus, nuclear run-on analysis demonstrated that TGF-β could inhibit initiation of transin transcription whether or not the transin gene was being actively transcribed at the time of TGF-β addition.

DISCUSSION

The combination of transcriptional run-on analysis and Northern analysis of total transin RNA has provided specific information regarding the interactions between EGF and TGF-β which coordinately result in modulation of transin expression. Northern analyses of the decline in transin RNA levels after removal of EGF from the medium indicated that continuous occupancy of the EGF receptor is required for maintenance of transin transcription. Addition of TGF-β caused abrupt cessation of transin transcription, similar to that observed when EGF was removed, whether monitored at the level of transcription or that of cellular RNA accumulation. This observation strengthens the evidence indicating that continuous positive stimulation by EGF is necessary for active transin transcription. The minimal reduction in EGF binding observed in Rat-1 cells after TGF-β treatment (L. L. Muldoon, K. D. Rodland, and B. E. Magun, J. Biol. Chem.,

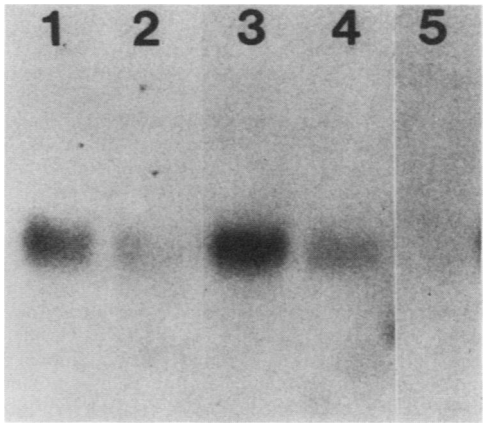

FIG. 4. Northern analysis of transin RNA expression in response to EGF and TGF-β. Confluent Rat-1 cells were serum deprived for 16 h in DMEM and then exposed to either EGF alone (10 ng/ml) or EGF plus TGF-β (10 ng/ml). Cells were harvested 2 h after addition of TGF-β or the TGF-β vehicle, and total cellular RNA was prepared as described in the text. Gel electrophoresis and Northern blot analysis were performed as described in the legend to Fig. 1, with ^{32}P-labeled pTR1 as the hybridization probe. Rat-1 cells were exposed to either EGF alone for 2 h (lane 1) or both EGF and TGF-β for 2 h (lane 2). In lanes 3 and 4, transin expression was induced by 2 h of exposure to EGF. At this point, either TGF-β (lane 4) or the TGF-β vehicle (lane 3) was added to the EGF-containing medium and the cells were incubated for an additional 2 h before harvesting. Lane 5 represents cells exposed to the TGF-β vehicle (20% acetonitrile, 0.1% trifluoroacetic acid) for 4 h.

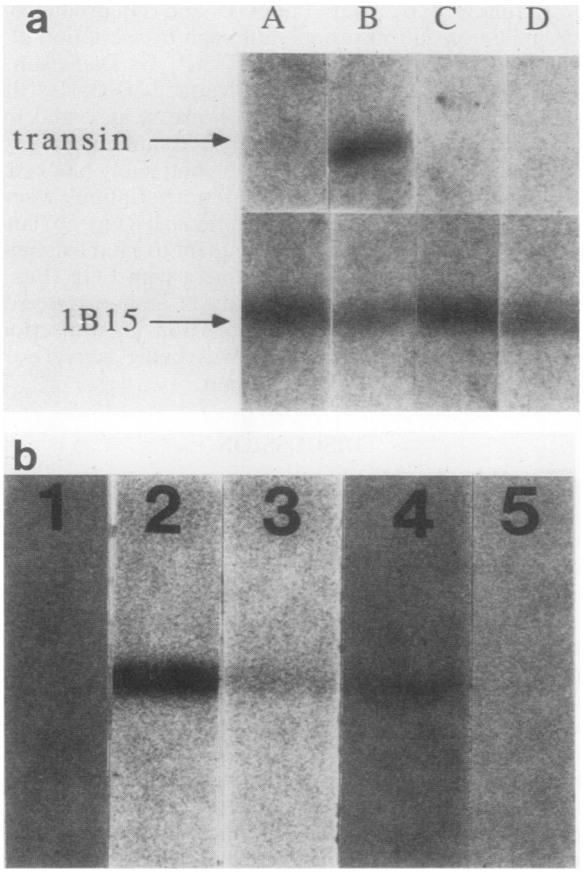

FIG. 5. Nuclear run-on assay of transin transcription in response to EGF and TGF-β. (a) Effect of TGF-β pretreatment on transin induction by EGF. Confluent 10-cm plates of Rat-1 cells were serum deprived for 16 h in DMEM and then exposed to either the TGF-β vehicle (lanes A and B) or TGF-β for 4 h (lanes C and D). EGF was added to lanes B and D during the final 2 h of incubation. Transcripts obtained from nuclear extracts as described in Materials and Methods were hybridized to DBM-paper strips containing either the 1.6-kilobase *Eco*RI transin insert (upper strips) or the ubiquitously expressed clone p1B15 (lower strips). (b) Effect of TGF-β on established induction of transin by EGF. Confluent 10-cm plates of Rat-1 cells were serum deprived for 16 h in DMEM and then exposed to either EGF alone (10 ng/ml) or EGF plus TGF-β (10 ng/ml) for various times as described below. Rat-1 cells exposed for 30 min to EGF alone or in the presence of TGF-β were represented in lanes 2 and 3, respectively. Cells represented in lanes 4 and 5 were pretreated with EGF for 2 h to establish induction of transin RNA. At that point, either TGF-β (lane 5) or the TGF-β vehicle (lane 4) was added and the cells were incubated for an additional 2 h before harvesting. Untreated control cells are represented in lane 1. Transcripts prepared from these nuclear extracts were hybridized to the 1.6-kilobase *Eco*RI fragment of pTR1 immobilized on DBM-paper.

in press) was not sufficient to account for the extent of the inhibition observed. The dependence of transin RNA expression on protein synthesis demonstrated in the anisomycin experiments suggests, but does not prove, that TGF-β modulates transin expression by inducing the synthesis or transcription of a separate gene product required for induction of transin transcription.

Although both EGF and protein kinase C are independently capable of inducing expression of the VL30 gene in serum-deprived Rat-1 cells (13), neither an exogenous nor an endogenous activator of protein kinase C (TPA and DOG, respectively) was capable of inducing transin expression. This differential responsiveness of transin RNA expression to TPA and EGF parallels the differential mitogenic response of serum-deprived Rat-1 cells to TPA and EGF; i.e., EGF, but not TPA, is mitogenic in Rat-1 cells (8).

In nontransformed Rat-1 cells, transin expression appeared to be tightly regulated by the coordinated actions of EGF and TGF-β, suggesting that growth factor modulation of this gene is important in vivo. Whereas the physiological role of transin in normal cells is unknown, evidence derived from sequence homology at both the nucleotide and amino acid levels suggests that transin is a member of a broadly defined metalloprotease family (4, 5, 15), and transin appears to be closely related to human stromelysin (15). Constitutive expression of transin appears to be a corollary of the transformed state, since transin expression is observed in a variety of oncogene-transformed cell lines (9) and in malignant mouse skin carcinomas but not benign papillomas or normal mouse skin (7). Whereas the molecular mechanisms involved in this constitutive expression are unknown, enhanced understanding of the normal interactions between EGF induction and TGF-β inhibition may lead to identification of the specific defect(s) responsible for the constitutive expression of transin in transformed cells. The ability to sort out the relative importance of positive and negative effectors in transformation-induced changes in gene regulation may contribute to a better understanding of the role of growth factors in modulating the expression of specific genes in neoplastic cells.

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