High Activity of Serum Response Factor in the Mesenchymal Transition of Epithelial Tumor Cells Is Regulated by RhoA Signaling*

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The serum response factor (SRF) regulates the transcription of target genes by binding to serum response elements in dimeric form and by interacting with ternary complex factors. In this study, we have analyzed the role of the serum response factor and mechanisms that regulate its activity in tumor progression utilizing a multistage model of mouse skin carcinogenesis. We demonstrate elevated SRF DNA binding activity only in the cell lines that have undergone an epithelial to mesenchymal transition and have increased actin stress fiber formation. Transient transfection experiments of activated or dominant negative forms of RhoA showed that the high activity of SRF and the induced formation of actin stress fibers in cells with spindle morphology were mediated by RhoA signaling. A dominant negative form of SRF inhibited RhoA-induced actin polymerization and stress fiber formation. The DNA binding activity of SRF in mesenchymal tumor cells was also correlated with elevated expression of SRF target genes, similar to SRF itself, actin, and vinculin. These observations suggest for the first time that SRF may play an important role in tumor progression, specifically at the transition to an invasive metastatic stage of carcinogenesis.

Serum response factor (SRF) is an important regulator of many genes through binding to the sequence CC(A/T)G/G terming the CARG box (1). SRF has been shown to be implicated in cell processes like immediate early and tissue-specific gene expression, cell proliferation, differentiation, and apoptotic pathways (2–6). One of the SRF targets is c-fos, an immediate early gene that can be activated by a variety of growth factors and mitogens through several different signaling pathways (7). The activation of c-fos by most of these pathways is mediated through the serum response element (SRE) where a protein complex consisting of SRF and ternary complex factors (TCFs) can be formed (8). The TCF family of transcription factors is composed of three members, the Elk-1, Sap1a, and Sap-2-ErpNet, that bind DNA through their Ets domain and are recruited to the SRE by direct protein-protein interaction with the SRF (9). Signaling pathways have been reported to induce transcription through the SRE, being dependent either on TCFs or SRF transcription factors. TCFs are activated after phosphorylation by ERK, c-Jun NH2-terminal kinase, and p38 pathways (10–12). SRF activation involves the pathway of the Rho family of GTPases (13). Rho family members regulate diverse processes including cytoskeletal rearrangements, gene transcription, cell-cycle progression, cell transformation, and cytokinesis (14). In mammalian cells, Rho proteins control the cytoskeletal organization by regulating the formation of actin stress fibers and focal adhesions (15). The formation of the focal adhesions is mediated by the association of integrins with molecules like vinculin (16). The transformation by oncogenic Ras is accompanied by dramatic alterations in actin cytoskeleton, and it is known that GTPases of the Ras subfamily can activate cascades of the Rho family members (17). Functional RhoA is also required for serum- and lysophosphatidic acid-induced activation of SRF (18). It has also been shown that changes in actin dynamics regulated by RhoA and LIM kinase-1 are critical to SRE activation (19, 20).

In this study, we investigated the role of SRF in chemically induced multistage carcinogenesis. One of the best characterized animal models for studying the genetic and biological alterations in tumor initiation, promotion, and progression is the mouse skin system. Treatment with the chemical carcinogen 7,12-dimethylbenz[a]anthracene causes H-ras gene mutations during tumor initiation. Additional changes have been detected at the H-ras locus in tumor promotion induced by further treatment of the initiated lesions with 12-O-tetradecanoylphorbol-13-acetate (21). A series of cell lines representative of different stages of mouse skin carcinogenesis has been used for our studies.

It has been previously shown that the product of the c-fos gene is overexpressed in malignant tumors in the multistep mouse skin carcinogenesis model (22). Furthermore, c-fos-deficient mice carrying a H-ras transgene fail to undergo malignant progression (23). Therefore, we hypothesized that the main factors regulating c-fos transcription like SRF and TCFs could be important in tumor progression.

In this study, we show that SRF is overexpressed and highly active only in tumor cells that have undergone the epithelial-mesenchymal transition and acquired a spindle phenotype. In addition, SRF target genes that encode for different cytoskeletal factors like vinculin and actin are overexpressed in the spindle tumor cells. The activation of SRF and induction of

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The abbreviations used are: SRF, serum response factor; SRE, serum response element; TCF, ternary complex factor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; TGF-β, transforming growth factor β; RBD, Rho binding domain.

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RESULTS

SRF DNA Binding Activity on SRE Is Enhanced in Spindle Cell Carcinomas—Because c-fos has been shown to be involved in mouse skin tumor progression (22), we tested the hypothesis that serum response factor, an important transcriptional regulator of c-fos, could play a role at a specific stage of progression in this system. For this purpose, we made use of a series of epidermal cell lines representing all of the important stages of skin tumor development. C50 is a nontumorigenic immortalized cell line. P1 is a papilloma cell line, B9 is a squamous cell carcinoma from a multiple 7,12-dimethylbenz[a]anthracene-treated mouse, and A5 is a spindle cell carcinoma isolated from the same tumor (30). CarB is a highly aggressive spindle cell carcinoma. C50, P1, and B9 have a typical epithelioid morphology. A5 and CarB have a mesenchymal or fibroblastoid morphology and give rise to spindle cell carcinomas upon injection into nude mice (31). Nuclear protein extracts were prepared from exponentially growing cells.

To test the DNA binding activity on SRE in these cell lines, electrophoretic mobility shift assay was performed (Fig. 1A). The same nuclear extracts used for the Western blot of C50, P1, B9, A5, and CarB cell lines were incubated with 32P-labeled SRE oligonucleotide containing Ets and SRF binding sites. As a control, the samples showing DNA binding of in vitro translated SRF and Elk-1 proteins were included. Protein-DNA complexes were formed with gradually increasing binding activity from the nontumorigenic to the fully malignant cell lines. The highest activity was observed at the late stages of carcinogenesis, where the SRF DNA binding activity was elevated by ~80-fold in the A5 and 60-fold in the CarB cells compared with the C50 cells. Also, in B9 cells, the SRF DNA binding activity was increased by 10-fold compared with C50 cells. In addition, the SRF-TCF ternary complex was formed only in A5 and CarB cell lines.

The contribution of SRF and Ets binding sites on the protein-DNA complex formation was further examined (Fig. 1B). For that purpose, we used an oligonucleotide with mutated Ets and intact SRF binding sites termed mut-wt as well as an oligonucleotide with intact Ets and mutated SRF binding sites termed wt-mut. These oligonucleotides were incubated with the cell nuclear extracts and the in vitro translated proteins described above. The Ets binding site was shown to be important for the ternary complex formation (lanes 1–7), whereas the SRF binding site was essential for the formation of any protein-DNA complex related to SRF (lanes 8–14).

Western blot analysis using nuclear extracts showed that SRF protein expression slightly increased in the papilloma and the squamous carcinoma cell lines (P1 and B9, respectively) compared with the immortalized C50 cells. The amount of the SRF protein present in the spindle A5 and CarB cells was increased by ~10-fold compared with C50 cells (Fig. 1C).

Composition Analysis of the SRE Complex Formed in A5 Cells—The fact that the ternary complex was only formed in the spindle cell lines led us to investigate the expression levels of the TCF family members. Western blot analysis using nuclear extracts of the C50, P1, B9, A5, and CarB cell lines showed that the expression levels of Elk-1, Sap1a, and Net (upper bands) proteins did not change in tumor progression (Fig. 2A). To investigate the contribution of each factor to the ternary complex formation, the composition analysis of the protein complex formed in A5 cells was carried out by electrophoretic mobility shift assay (Fig. 2B). Incubation with anti-SRF, anti-Elk-1, anti-Sap1a, and anti-Net antibodies showed that SRF was the main component of the complex and that Elk-1, Sap1a, and mostly Net were participating in the formation of the ternary complex (Fig. 2B, lanes 2–5). The bottom
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**Fig. 1.** SRE binding activity is enhanced in spindle cell carcinomas. A, protein-DNA binding on the c-fos SRE. Electrophoretic mobility shift assays were performed as described under “Experimental Procedures,” using 5 μg of nuclear extracts from the cell lines C50, P1, B9, A5, and CarB. In vitro translated protein SRF and the mixture consisting of SRF and Elk-1 proteins were used as controls. The proteins were incubated with a radiolabeled oligonucleotide containing the intact Ets and SRF binding sites of the c-fos SRE promoter element (wt-wt). B, protein-DNA binding on mutant SRE oligos. Electrophoretic mobility shift assays were performed with two different mutated SRE oligos using the same nuclear extracts and in vitro translated proteins as above. The oligos were mutated either at the Ets binding site (lanes 1–7, mut-ets) or at the SRF binding site (lanes 8–14, mut-srf). These oligos were incubated with extracts from the cell lines C50 (lanes 1 and 8), P1 (lanes 2 and 9), B9 (lanes 3 and 10), A5 (lanes 4 and 11), and CarB (lanes 5 and 12) and the in vitro translated proteins SRF (lanes 6 and 13) and SRF mixed with Elk-1 (lanes 7 and 14). C, the transcriptionally autoregulated SRF protein is overexpressed in A5 and CarB cell lines. 15 μg of nuclear protein extracts from C50, P1, B9, A5, and CarB cells were loaded on a 10% SDS-polyacrylamide gel, and Western blot analysis was carried out.

**Fig. 2.** Composition analysis of the SRE complex formed in A5 cells. A, expression levels of TCFs. 15 μg of nuclear protein extracts of the C50, P1, B9, A5, and CarB cell lines were loaded on a 10% SDS gel. Western blot analysis showed the expression levels of Elk-1, Sap1a, and Net proteins, respectively, using the corresponding antibodies. B, detection of TCFs in the SRE complex. Electrophoretic mobility shift assays were carried out. 0.5 μg of each antibody recognizing the SRF, Elk-1, Sap1a, and Net proteins was incubated with 5 μg of nuclear extracts from the A5 cell line (lanes 2–5) or with 3 μl of in vitro translated proteins from the A5 cell line (lanes 2–5) before the addition of the oligonucleotide containing both the SRF and Ets binding sites (wt-wt). A5 nuclear extracts without any antibody added (lane 1) with the addition of anti-SRF antibody (lane 2), anti-Elk-1 antibody (lane 3), anti-Net antibody (lane 4), and anti-Sap1a antibody (lane 5) are shown. As controls, in vitro translated SRF protein without the addition of any antibody (lane 6) or with the addition of anti-SRF antibody (lane 7) or mixture of SRF and Elk-1 in vitro translated proteins without (lane 8) or with (lane 9) the addition of anti-Elk-1 antibody are also shown.

*band* corresponds to an SRE-interacting polypeptide not related to SRF and TCFs. In vitro translated proteins with the corresponding antibodies were used as controls (Fig. 2B, lanes 6–9).

These experiments showed that the ternary complex formation depends on the amount (Fig. 1C) and the DNA binding activity of the SRF protein rather than the expression levels of the protein members of the TCF family and that only at the late stages of carcinogenesis, target genes can potentially have maximal transcriptional induction through SRE.

RhoA Signaling Regulates the DNA Binding Activity of SRF—It has been reported that SRF is a nuclear target of an ill-defined RhoA-mediated signaling pathway. To investigate the RhoA effect, we selected B9 and A5 cells that represent the two distinct stages of epithelial to mesenchymal transition and show dramatic changes in SRF DNA binding activity. To de-
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SRF Target Genes Are Overexpressed in Spindle Tumor Cells—The expression levels of SRF target genes like SRF itself (Fig. 1C), vinculin, and actin were analyzed by Western blot assay. The expression of vinculin increased dramatically by 10-fold in mesenchymal tumor cells compared with the immortalized C50 cell line (Fig. 5A). The expression of actin also showed a slight increase in spindle tumors (Fig. 5B). These data together with the previously reported increase in the expression of c-fos gene (16) show that SRF target genes are overexpressed in A5 and CarB cell lines.

DISCUSSION

SRF function is important for cellular physiology and development. However, little is known for its implication in tumor-
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SRF is an important mediator of RhoA signaling to the nucleus, and its activity on target genes is dependent on cytoskeletal changes in several cell types (18). As described in Fig. 3, the regulation of SRF activity in mouse keratinocytes was RhoA-dependent. Alterations in actin dynamics mediated by the actin regulator LIM kinase-1 appear to be necessary and sufficient for the activation of SRF target genes. More specifically, it is the ability of both LIM kinase-1 and signals through Rho GTPases that regulate the actin treadmilling cycle, which in turn regulates SRF activity (19). Also, in REF52 cells, active RhoA induces actin stress fibers and focal adhesions that contain vinculin and causes transcriptional activation through the SRE element (20). High SRF activity was detected in A5 spindle tumor cells, which are mesenchymal cells with an altered organization of actin cytoskeleton compared with squamous B9 cells. Remarkably, B9 and A5 clones were isolated from the same primary tumor, and they represent the two extreme stages of the epithelial to mesenchymal transition (30). It is probable that in A5 cells, additional factors with a similar function to the recently described muscle activator striated muscle activator of Rho signaling, (35) might be important in activating RhoA signaling and SRF-dependent transcription. Our study has shown that actin stress fiber formation was also regulated by RhoA signaling in these cells and that this effect is mediated by SRF function. These results could be related to the finding that oncogenic Ras down-regulates Rac and up-regulates Rho activity, although sustained activation of the Raf/ERK pathway leads to epithelial-mesenchymal transition of Madin-Darby canine kidney cells (36).

The involvement of RhoA in the induction of the spindle phenotype is important in view of the recent demonstration that transforming growth factor β (TGF-β) can stimulate cell motility and actin cytoskeletal reorganization by a RhoA-dependent mechanism (37). It has previously been demonstrated

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**Fig. 4.** Increased actin stress fiber formation in A5 cells is a RhoA- and SRF-dependent process. A, photograph after phalloidin staining of exponentially growing B9 cells. B, B9 cells were transfected with 1 μg of activated RhoA and 48 h later were stained with phalloidin. C, B9 cells were co-transfected with activated RhoA and dominant negative SRF and 48 h later were stained with phalloidin. D, phalloidin staining of exponentially growing A5 cells. E, A5 cells were transfected with 1 μg of dominant negative RhoA and 48 h later were stained with phalloidin. F, A5 cells were co-transfected with dominant negative RhoA and SRF and 48 h later were stained with phalloidin. In all cases, the cells were attached on glass coverslips and were incubated with rhodamine-conjugated phalloidin in 1:350 concentration for 45 min at room temperature.

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**Fig. 5.** SRF target genes are overexpressed in the spindle cancer cells. A, vinculin is highly expressed in spindle cell carcinomas. 30 μg of total cell extracts from C50, P1, B9, A5, and CarB cells were loaded on a 10% SDS-polyacrylamide gel, and Western blot analysis followed. B, actin expression levels are increased in A5 and CarB cells. 30 μg of total cell extracts from C50, P1, B9, A5, and CarB cells were loaded on a 10% SDS-polyacrylamide gel, and the actin expression levels were detected by Western blot analysis.

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In this study, we have analyzed the role of the SRF transcription factor in the unraveling of neoplastic processes using the mouse skin multistage carcinogenesis model. Our results demonstrate for the first time that SRF is implicated in cancer progression, specifically at the epithelial-mesenchymal transition of carcinoma cells. In addition, the expression and activity of SRF are associated with the activation of RhoA and actin dynamics.

The high SRF DNA binding activity at the SRE in the spindle tumor cell lines could be attributed to elevated SRF protein levels and post-translational events. High protein expression levels of SRF results in the activation of target genes, the SRF gene itself included, which is regulated by the binding sites of two SRF, a Ets, and a Sp1/Egr-1 located in its promoter sequence. The SRF binding sites are targeted by Rho-dependent pathways, and the Sp1 binding site is targeted by a Ras-dependent pathway (32). Remarkably, Ras and Rho signaling pathways cross-talk during malignant cell transformation (33). It is probable that the high expression levels of SRF protein observed in A5 and CarB cell lines result from the transcriptional regulation of the SRF gene by activated Ras and RhoA signaling. Remarkably, RhoA has been found to be overexpressed and more active in the mesenchymal A5 cells compared with the epithelial B9 cells.

It has been shown in many studies that SRF activity depends on the interaction with factors like TCFs to regulate SRE-responsive genes. The TCF phosphorylation status might well have a role in the late stages of tumor progression in this system, because TCFs have been shown to be regulated by c-Jun NH2-terminal kinase and ERK pathways and c-Jun NH2-terminal kinase (22) as well as ERK signals are highly active in A5 and CarB cells. We detected the SRF-TCF complex formation only in cells with high SRF activity. It has been shown recently that differential usage of signal transduction pathways defines at least two types of SRF target genes (34) depending on the functional interaction of SRF with TCFs.
that some features of the epithelial-mesenchymal transition can be induced in squamous carcinoma cells by treatment with TGF-β and that this transition can be inhibited by the expression of dominant interfering TGF-β receptor mutants (38). The induction of the spindle phenotype by TGF-β is compatible with earlier results showing that the overexpression of this growth factor in the epidermis of transgenic mice leads to an increase in the rate of tumor progression and a higher incidence of spindle tumors (39). Taken together, these observations suggest that the activation of Rho A is a consequence of up-regulation of TGF-β signaling that is associated with the development of the spindle phenotype. The results presented here establish an important further step in this process by demonstrating that RhoA activation leads directly to the activation of the DNA binding capacity of SRF transcription factor complex and to the consequent induction of their downstream target sequences.

It is not clear whether the overexpression of SRF itself or the regulation of one or more of its target genes is responsible for progression to mesenchymal tumors. We have presented evidence that SRF target genes like vinculin, actin, c-fos (22), and SRF gene itself are highly expressed in mesenchymal cells, indicating an important role for SRF in the formation of spindle tumor phenotype. It will be of interest to further investigate the regulation of SRF targets in this cell system as well as the effects of long term inhibition of SRF function, which may lead to phenotypic reversion.

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