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Serum- and glucocorticoid-regulated kinase 1 (SGK1) induction by the EWS/NOR1(NR4A3) fusion protein

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Abstract

The NR4A3 nuclear receptor (also known as NOR1) is involved in tumorigenesis by the t(9;22) chromosome translocation encoding the EWS/NOR1 fusion protein found in approximately 75% of all cases of extraskeletal myxoid chondrosarcomas (EMC). Several observations suggest that one role of EWS/NOR1 in tumorigenesis may be to deregulate the expression of specific target genes. We have shown previously that constitutive expression of EWS/NOR1 in CFK2 fetal rat chondrogenic cells induces their transformation as measured by growth beyond confluency and growth in soft agar. To identify genes regulated by the fusion protein in this model, we have generated a CFK2 cell line in which the expression of EWS/NOR1 is controlled by tetracycline. Using the differential display technique, we have identified the serum- and glucocorticoid-regulated kinase 1 (SGK1) mRNA as being up-regulated in the presence of EWS/NOR1. Co-immunocytochemistry confirmed over-expression of the SGK1 protein in cells expressing EWS/NOR1. Significantly, immunohistochemistry of 10 EMC tumors positive for EWS/NOR1 showed that all of them over-express the SGK1 protein in contrast to non-neoplastic cells in the same biopsies and various other sarcoma types. These results strongly suggest that SGK1 may be a genuine *in vivo* target of EWS/NOR1 in EMC.

Keywords: EWS/NOR1; Extraskeletal myxoid chondrosarcoma; SGK1; CFK2 cells

Extraskeletal myxoid chondrosarcoma (EMC; reviewed in ref. [1]) is a soft tissue tumor occurring primarily in the deep tissues of the extremities, most commonly the thigh and knee. EMC is most often found in patients over 35 years of age, and men are affected about twice as often as women. In general, it is a slow-growing tumor but it recurs and metastasizes in most cases. Radical local excision with or without adjunctive radiotherapy is the usual treatment. The incidence of EMC was found to be 2.3% among 603 soft tissue sarcomas [2]. In approximately 75% of EMC cases, a balanced t(9;22) (q22;q12) chromosome translocation is present [3]. This translocation results in the fusion of the EWS gene (for EWing's Sarcoma) on chromosome 22 to an orphan nuclear receptor gene on chromosome 9 called NOR1 (for Neuron-derived Orphan Receptor; ref. [4]; also named TEC, CHN, MINOR, and NR4A3; Nuclear Receptor Nomenclature Committee; ref. [5]). This gene fusion encodes a chimeric protein, EWS/NOR1, containing the amino-terminal domain of EWS fused in-frame to the complete amino acid sequence of NOR1 [4]. The EWS/NOR1 gene fusion is specific to EMC and is not detected in chondrosarcomas of skeletal origin or other sarcomas [4,6]. Three other gene fusions involving NOR1 have been characterized in EMC tumors negative for EWS/NOR1: TAF2N/NOR1, TFG/NOR1, and TCF12/NOR1. TAF2N and TFG both encode proteins homologous to EWS, and they are fused to NOR1 by a t(9;17) translocation for TAF2N [7] and a t(3;9) translocation for TFG [8]. TCF12 encodes a basic helix–loop–helix protein and is fused to NOR1 by a t(9;15) translocation [9]. The presence

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of a NOR1-containing fusion gene in more than 95% of EMC cases suggests that aberrant expression of the entire NOR1 coding region in the context of a fusion gene may be a necessary step in the development of these tumors.

Within the nuclear receptor superfamily NOR1 is most homologous to two other orphan receptors, NGFI-B and NURR1. All three receptors have been characterized as immediate-early gene products induced by a variety of mitogenic stimuli such as growth factors and liver regeneration [10–13]. They are expressed in many tissues but predominantly in the central nervous system, with both overlapping and distinct expression patterns [14–16]. In functional terms NOR1 appears involved in T-cell receptor-mediated apoptosis of immature thymocytes [17] and vascular smooth muscle cell proliferation [18]. In addition, a gene knock-out mouse model suggests that it plays a role in the development of the semicircular canals of the inner ear [19], and in neuronal survival and axonal guidance in the developing hippocampus [20]. NOR1 can bind to and activate transcription from a DNA response element called the NBRE (NGFI-B Response Element) [21], which was initially characterized as a DNA response element for NGFI-B [22]. This suggests that NOR1 may activate the expression of genes whose products are involved in proliferation and/or apoptosis. We have shown that the EWS/NOR1 fusion protein is a much more potent transcriptional activator of NBRE-containing promoters than the native NOR1 nuclear receptor [21], suggesting that the fusion protein may over-activate NOR1 target genes in EMC. We have also shown that constitutive expression of EWS/NOR1 in CFK2 fetal rat chondrogenic cells induces their transformation as measured by growth beyond confluency and growth in soft agarose medium [23]. To identify genes regulated by the fusion protein in this model, we have developed an inducible system in which the expression of EWS/NOR1 in CFK2 cells is controlled by tetracycline. In this study we show that the serum- and glucocorticoid-regulated kinase 1 (SGK1) mRNA and protein are up-regulated upon induction of EWS/NOR1 in CFK2 cells, and present evidence strongly suggesting that SGK1 may be a genuine target gene of EWS/NOR1 in EMC.

Materials and methods

Cell lines, plasmids, and transfections. The CFK2 cell line [24], two CFK2 cell lines stably transfected with the empty pcDNA vector (CFK2(pcDNA), ref. [23]), and two CFK2 cell lines stably transfected with the pcDNA(EWS/NOR1) expression vector (CFK2(EWS/NOR1), ref. [23]) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. G418 (Geneticin, Invitrogen) was added at a concentration of 250 μg/ml to the stably transfected cell lines. To generate the EWS/NOR1-inducible cell line, the EWS/NOR1 cDNA [21] was cloned into the *Bam*H I site of the pJMF2 vector [25]. Stable transfections of CFK2 cells with the empty pJMF2 or the pJMF2(EWS/NOR1) vectors were carried out using the Lipofectamine Reagent (Invitrogen) according to the manufacturer's instructions. The day following transfection cells were split at various dilutions, and the next day G418 and tetracycline (Sigma) were added to the medium at 550 μg/ml and 1 μg/ml, respectively. After three weeks individual clones were randomly picked and expanded in the same medium except that the concentration of G418 was lowered to 250 μg/ml. CFK2[pJMF2(EWS/NOR1)] clones were screened in duplicate in 24-well plates by transient transfection of 100 ng of the p(B1a)[8] vector containing the luciferase reporter gene under the control of a NBRE-containing promoter [22], which is activated by EWS/NOR1. One hundred nanograms of a normalizing pCMV/Gal vector, containing the β-galactosidase coding sequence under the control of the CMV promoter, was co-transfected with p(B1a)[8] to correct for transfection efficiencies. Following transfection, cells were incubated in medium containing 10 μg/ml tetracycline to repress EWS/NOR1 or in medium without tetracycline to induce expression of EWS/NOR1. After 48 h, cell extracts were prepared, and luciferase and β-galactosidase activities were determined using a Luciferase Assay System from Promega, a Beta-Gal Assay Kit from Clontech, and a Berthold MiniLumat LB 9506 Luminometer. Luciferase activities were divided by β-galactosidase activities and the results are expressed as relative light units. For the analysis of the SGK1 promoter, CFK2 cells were seeded in 6-well plates, and the next day transfected with 200 ng of the normalizing pCMV/Gal vector, 200 ng of the pGL3/SGK1 luciferase reporter construct or the empty pGL3 vector, and increasing amounts of a pcDNA/EWS/NOR1 expression vector. After 72 h cell extracts were prepared, and luciferase and β-galactosidase activities were measured as described above.

Western blotting and immunocytochemistry. For Western blot analyses, total protein extracts were prepared from uninduced (10 μg/ml tetracycline) and induced (no tetracycline) cultures, separated by SDS–PAGE, transferred onto an Immobilon-P membrane (Millipore), and reacted with an affinity-purified antibody prepared in our laboratory and directed against the amino-terminal portion of NOR1. A goat anti-rabbit antibody (Zymed) coupled to horseradish peroxidase and a Western Lightning Chemiluminescence kit (Perkin-Elmer) were used to detect the primary antibody. For Fig. 1B, the same membrane was reacted with the mAb1C3 monoclonal antibody specific for the Fragile X Mental Retardation Protein (FMRP) [26] as a loading control. For immunocytochemistry, cells were plated on Falcon culture slides and the next day tetracycline was removed from half the samples and the other half was incubated in 10 μg/ml tetracycline. After 72 h cells were fixed in acetone/methanol (7:3) 10 min at −20 °C and washed in PBS. For Fig. 1E, cells were reacted first with the NOR1 antibody described above and second with a goat anti-rabbit antibody (Invitrogen) coupled to the Alexa Fluor 488 fluorochrome. For Fig. 2C, cells were reacted first with a mouse NOR1 monoclonal antibody from R&D Systems (Cat. No. 2ZH7833 H) and a goat SGK1 antibody from Santa Cruz Biotechnology (Cat. No. sc-15885), and second with a goat anti-mouse antibody coupled to Alexa Fluor 594 and a rabbit anti-goat antibody coupled to Alexa Fluor 488 (both from Invitrogen). Photomicrographs were taken with a Nikon Eclipse TE300 microscope, a Hamamatsu ORKA-ER camera, and the SimplePCI (Compix, Inc.) imaging system.

Differential display analyses. The Delta Differential Display Kit from Clontech was used according to the manufacturer's instructions with the following minor modifications. Prior to the display, the RNA (5 μg) was digested with 10 U RNAse-free DNAse I (Amersham Biosciences) for 1 h at 37 °C, and the enzyme was inactivated by incubation at 75 °C for 10 min. The first-strand cDNA synthesis reaction was performed with the PowerScript reverse transcriptase (Clontech). The differentially expressed bands were reamplified with Qiagen *Taq* DNA polymerase, and the PCR products were cloned with the T/A Cloning Kit from Invitrogen, sequenced, and analyzed with the Basic Local Alignment Search Tool (BLAST) program against GenBank.

Northern blot and RT-PCR analyses. Total RNA extracts were prepared from induced and uninduced cultures and differentiated CFK2 cell lines [23] using the Trizol reagent (Invitrogen). The RNA (5 μg) was fractionated in a formaldehyde/MOPS agarose denaturing gel, transferred onto a Biodyne B membrane (Pall Corporation), and hybridized with a radioactive EWS, SGK1, or 18S ribosomal RNA probe using the ULTRAhyb hybridization solution (Ambion) and following the manufacturer's instructions. RT-PCR analyses of EWS/NOR1 (5′ oligo: 5′-cccactagttacccacccca-3′, 3′ oligo: 5′-ggctgagagtgtaggagga-3′) and GAPD (5′ oligo: 5′-tgcaccaccaactgcttag-3′, 3′ oligo: 5′-ggatgcagggatgatgttc-3′) mRNAs were carried out with the

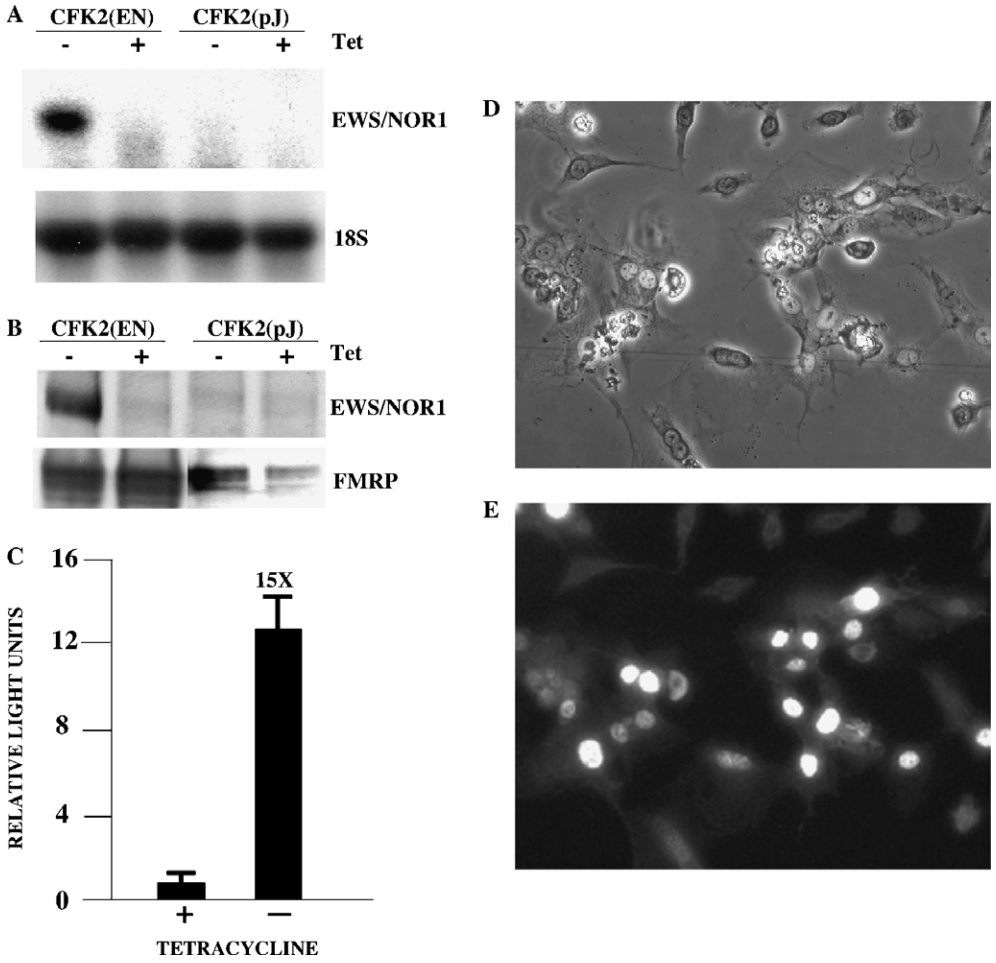

Fig. 1. Expression, activity, and localization of EWS/NOR1 in the inducible CFK2(EWS/NOR1) cell line and a control CFK2(pJMF2) cell line. (A) Northern blot analyses of total RNA prepared from uninduced and 72 h-induced cultures and hybridized with an EWS probe to detect EWS/NOR1. The membrane was rehybridized with an 18S probe for loading control. (B) Western blot analyses of total protein extracts prepared from uninduced and 72 h-induced cultures and reacted with a NOR1 antibody which recognizes EWS/NOR1. The FMRP antibody was used as a loading control. (C) Transient transfections of the inducible CFK2(EWS/NOR1) cell line with the NBRE-containing reporter vector and a normalizing β-galactosidase vector. After transfection, EWS/NOR1 expression was uninduced or induced for 48 h, and cell extracts were prepared and assayed for luciferase and β-galactosidase activities. (D,E) Immunocytochemical analysis of the 72 h-induced CFK2(EWS/NOR1) cell line with a NOR1 antibody which recognizes EWS/NOR1. (D) Phase contrast photomicrograph, (E) immunofluorescence photomicrograph of the same field. Magnification: 200×. CFK2(EN), CFK2(EWS/NOR1); CFK2(pJ), CFK2(pJMF2); Tet, tetracycline.

Omniscript RT kit and the TAQ polymerase from Qiagen. Products were resolved in 1.5% agarose gels and visualized by ethidium bromide staining.

Immunohistochemistry. Formalin-fixed paraffin-embedded sections of 10 EMC tumors were immunostained with a SGK1 polyclonal antibody from Santa Cruz Biotechnology (Cat. No. sc-15885) at 1:400 dilution with EDTA pre-treatment. A tissue microarray (TMA) section consisting of 0.6 mm triplicate cores of several other types of primitive sarcomas was used to evaluate the expression of SGK1 in selected other tumor types, as well as multiple cores of skeletal muscle, cardiac muscle, and smooth muscle. The sarcomas represented on this TMA section included 18 alveolar rhabdomyosarcomas, 11 embryonal rhabdomyosarcomas, 13 Ewing's sarcomas, 12 synovial sarcomas, 9 desmoplastic small round cell tumors, and 9 alveolar soft part sarcomas.

Results

Characterization of an EWS/NOR1-inducible CFK2 cell line

To design an inducible model for EWS/NOR1, we used the pJMF2 vector constructed by Lang and Feingold [25]. This vector contains two elements allowing for the tetracycline-regulated expression of a cloned cDNA: the coding sequence of the tetracycline-repressed transactivator tTA-VP16 placed under the control of a constitutive CMV promoter, and a minimal tTA-VP16-responsive promoter followed by a multiple cloning site and the SV40 polyadenylation signal. Since pJMF2 encodes both the tTA-VP16 transactivator and the regulated sequences, only one round of transfection is required to obtain cell lines expressing the inducible protein of interest. We have isolated a CFK2 cell line in which expression of EWS/NOR1 is tightly regulated by tetracycline. Fig. 1A shows that 72 h following removal of tetracycline, there is a significant induction of the EWS/NOR1 mRNA as determined by Northern blot analyses. Western blot analyses show a concomitant induction of the fusion protein (Fig. 1B). Transient transfections of the induced cells with the $p(B1a)^8$ reporter vector indicate that the induced fusion

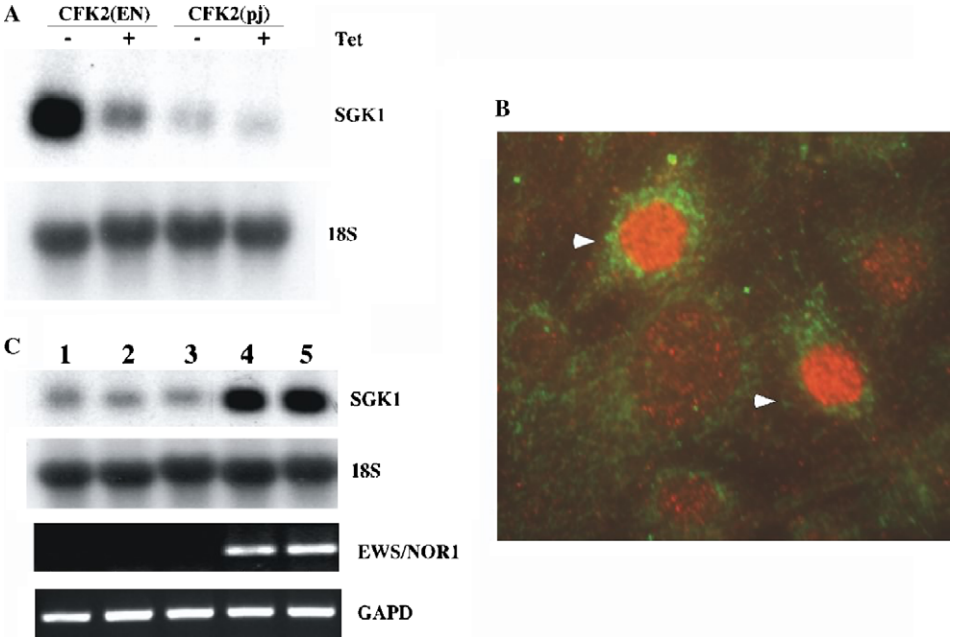

Fig. 2. Expression of SGK1 in CFK2 cell lines. (A) Northern blot analyses of total RNA from the inducible CFK2(EWS/NOR1) cell line and the CFK2(pJMF2) control cell line after a 72 h induction period hybridized with a SGK1 probe. The membrane was subsequently hybridized with an 18S probe. (B) Co-immunocytochemistry of the 72 h-induced CFK2(EWS/NOR1) cell line with a NOR1 antibody which recognizes EWS/NOR1 and a SGK1 antibody. The two white arrows identify cells in which EWS/NOR1 is induced in the nucleus (red) and SGK1 in the cytoplasm (green). Magnification: 400×. (C) Upper two panels: Northern blot analyses of total RNA from the original differentiated CFK2 cell line (lane 1), two control differentiated CFK2(pcDNA) cell lines (lanes 2 and 3), and two constitutive differentiated CFK2(EWS/NOR1) cell lines (lanes 4 and 5) hybridized first a SGK1 probe and subsequently with an 18S probe. Lower two panels: RT-PCR analyses of the same total RNA samples with PCR primers for EWS/NOR1 and GAPD. CFK2(EN), CFK2(EWS/NOR1); CFK2(pJ), CFK2(pJMF2); Tet, tetracycline. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

protein is transcriptionally active in CFK2 cells (Fig. 1C). Immunocytochemical analyses of the induced cultures show that approximately 75% of the cells express the fusion protein, and that the protein is in the nucleus, as expected for a transcription factor (Fig. 1D and E).

Up-regulation of SGK1 mRNA is upon EWS/NOR1 induction

To identify genes regulated by EWS/NOR1 in this model, a differential display analysis was performed using the induced and uninduced total RNA samples analyzed in Fig. 1A. Five bands were found to be differentially expressed between the two samples: four were up-regulated and one was down-regulated in the presence of EWS/NOR1. The bands were amplified and used as probes in Northern blot analyses of the induced and uninduced total RNA samples used for the display. Only one up-regulated band, corresponding to the SGK1 mRNA, was found to be significantly differentially expressed in these analyses (Fig. 2A). This induction was not related to the use of tetracycline since it was absent in the induced control CFK2(pJMF2) cell line (Fig. 2A). Two differential display bands gave signals too weak in the Northern blot analyses to allow unequivocal conclusions to be drawn, and two bands were found to be differentially expressed in both the induced CFK2(EWS/NOR1) and CFK2(pJMF2) cell lines, suggesting that this differential expression may be related to the use of tetracycline. We therefore conclude that SGK1 was the sole robust and valid candidate to emerge from the differential display analyses. To determine if the SGK1 protein was also over-expressed in the induced CFK2(EWS/NOR1) cell line, we performed co-immunocytochemistry using a monoclonal antibody directed against NOR1 and a goat antibody directed against SGK1. The results clearly show that cells in which EWS/NOR1 is induced in the nucleus contain greater amounts of SGK1 in the cytoplasm (Fig. 2B).

Expression of SGK1 in CFK2 cell lines and EMC tumors

As mentioned in the introduction, we have previously shown that constitutive expression of EWS/NOR1 in CFK2 cells induces their transformation as determined by growth beyond confluency and growth in soft agarose medium [23]. To determine if SGK1 is up-regulated in differentiated CFK2 cell lines constitutively expressing EWS/NOR1 and showing the transformation phenotype, Northern blot analyses were performed using total RNA from these cell lines. Fig. 2C shows that whereas the original CFK2 cell line and two CFK2 cell lines transfected with the empty pcDNA expression vector express low levels of SGK1 mRNA, two CFK2 cell lines constitutively expressing EWS/NOR1 and showing the transformation

phenotype express high levels of SGK1 mRNA. RT-PCR analyses of the total RNA samples used for the Northern blot analyses confirm that the two CFK2(EWS/NOR1) cell lines express the EWS/NOR1 fusion mRNA (Fig. 2C). To determine if EMC expressing EWS/NOR1 also express the SGK1 protein, immunohistochemistry was performed with a SGK1-specific antibody in 10 EMC, all of which contained the EWS/NOR1 fusion as confirmed by RT-PCR. Significantly, in all 10 cases, the tumor cells were positive for SGK1 (Fig. 3). The staining was cytoplasmic and generally of moderate intensity. The pattern of the cytoplasmic staining was granular in some cases (Fig. 3A) and diffuse in most cases (Fig. 3B). In some tumors, only focal areas were positive. Non-neoplastic cells in the EMC specimens, such as blood vessels and fibroblasts, were completely negative. Taken together, these results strongly suggest that SGK1 may be a genuine *in vivo* target of EWS/NOR1 in EMC tumors. We also stained a TMA section containing several other sarcoma types, including multiple cases of Ewing's sarcomas, rhabdomyosarcomas, synovial sarcomas, desmoplastic small round cell tumors, and alveolar soft part sarcomas. Four of 9 alveolar soft part sarcomas showed moderate to strong cytoplasmic positivity for SGK1 (data not shown), whereas the other tumor types were uniformly negative, except for one case of Ewing's sarcoma. Thus, expression of SGK1 is a characteristic and consistent finding in EMC, but is not entirely restricted to this tumor. Additional non-neoplastic tissues represented on the TMA section included skeletal muscle, cardiac muscle, and smooth muscle, and of these only cardiac muscle showed granular cytoplasmic immunostaining in a paranuclear location (data not shown).

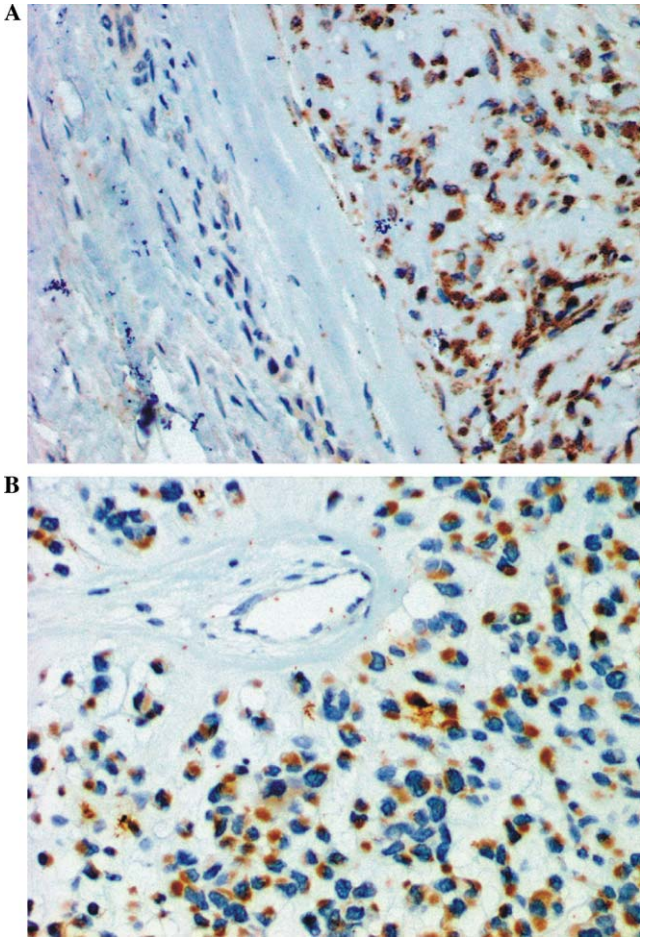

Fig. 3. Immunohistochemistry of two EMC tumors with a SGK1 antibody. Strong cytoplasmic immunoreactivity is observed in tumor cells but not in non-neoplastic cells [(A) see compressed adjacent tissue in left part of the image; (B) see blood vessel near center of image]. The cytoplasmic staining is granular in case (A) and more diffuse in case (B). (A) and (B): 200×.

EWS/NOR1 does not appear to directly activate the SGK1 promoter

The observation that EWS/NOR1 is a highly potent transcriptional activator raises the possibility that it may up-regulate the SGK1 mRNA by directly activating its promoter. To test this possibility, we have obtained from Dr. Christie P. Thomas a reporter construct containing a DNA fragment of 3.1 kb corresponding to the proximal region of the human SGK1 promoter cloned upstream a luciferase gene in the pGL3 vector [28]. Transient transfections of CFK2 cells with this vector indicate that although there is a significant increase in the activity of the reporter gene when the SGK1 sequences are present, increasing

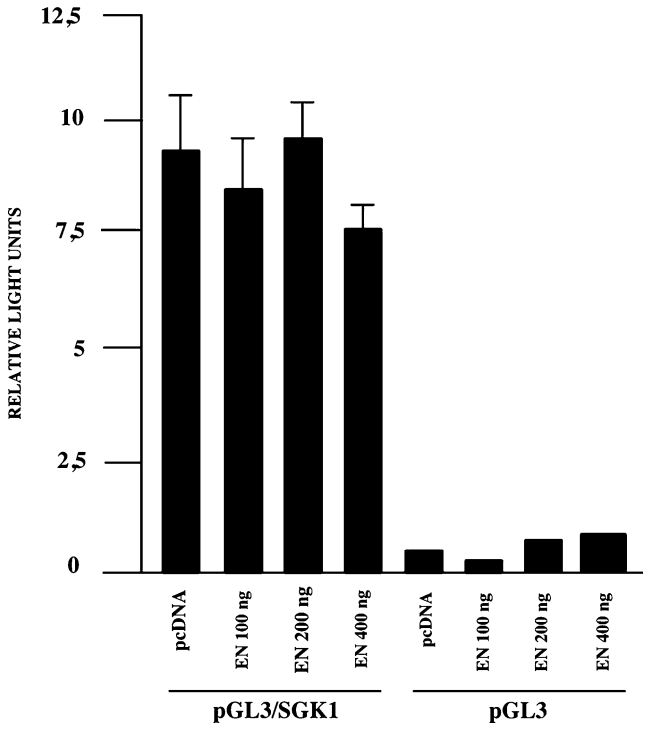

Fig. 4. Analysis of the human SGK1 promoter. CFK2 cells were co-transfected with the empty pGL3 or pGL3\SGK1 reporter vectors, and the empty pcDNA vector or the indicated amounts of an EWS/NOR1-expression vector. Transfections were performed in triplicate and the mean value is plotted with the standard deviation (vertical bars). EN, EWS/NOR1.

amounts of an EWS/NOR1-expressing vector does not alter the activity of the reporter gene (Fig. 4), suggesting that EWS/NOR1 cannot activate this promoter from the cloned sequences. In addition, we have performed a search for putative NBRE sites 10 kb upstream the transcriptional initiation point of the SGK1 gene using the TESS search program and the TRANSFAC database, and the results were negative. Finally we have performed short induction time points of the fusion protein in the inducible CFK2 (EWS/NOR1) cell line, and we have observed that although the induced fusion protein is clearly detected by Western blot 4 h after induction (Fig. 5A), there is no increase in the amount of the SGK1 mRNA 12 h after induction as determined by Northern blot (Fig. 5B). The transient increase in the SGK1 mRNA observed at the 2 h time point is due to the change of medium, as it is observed in both the EWS/NOR1-inducible cell line (Fig. 5B) and the control CFK2(pJMF2) cell line (Fig. 5C). Taken together, these results strongly suggest that EWS/NOR1 does not directly bind the SGK1 promoter to activate transcription.

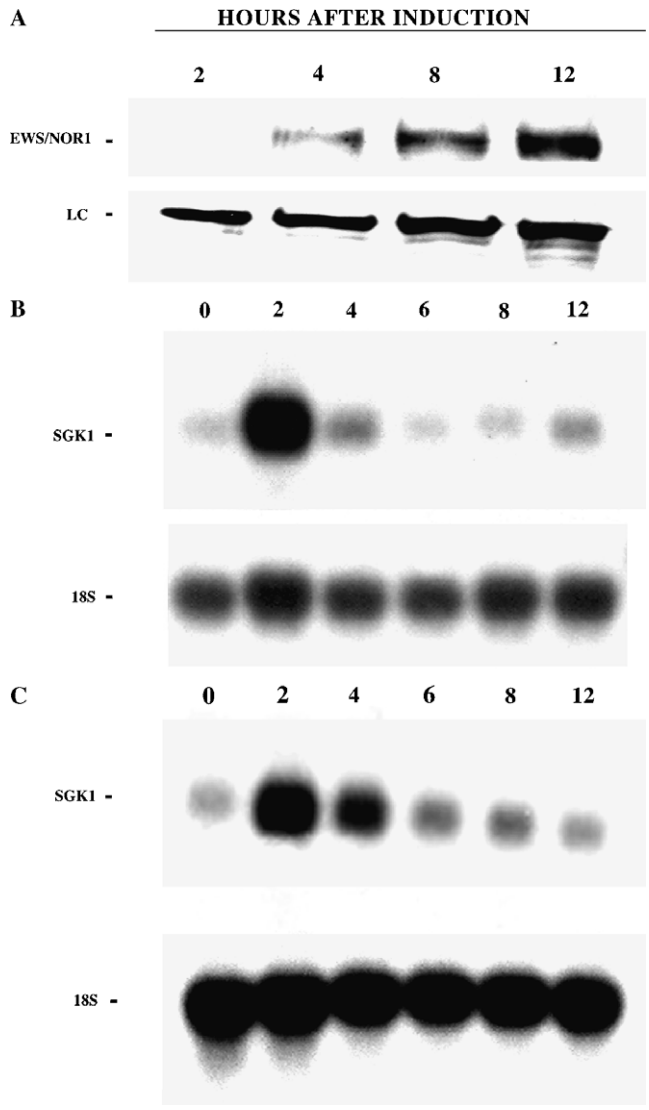

Fig. 5. Short induction time points of EWS/NOR1. The inducible EWS/NOR1 cell line and the control CFK2(pJMF2) cell line were induced in the absence of tetracycline for the indicated time points, and total protein and RNA extracts were prepared. (A) Western blot analysis of the inducible EWS/NOR1 cell line with the NOR1 antibody which recognizes EWS/NOR1. An unspecific band recognized by the antibody was used as a loading control (LC). (B) Northern blot analysis of the inducible EWS/NOR1 cell line with a SGK1 probe followed by an 18S probe as a loading control. (C) Northern blot analysis to the control CFK2(pJMF2) cell line with a SGK1 probe followed by an 18S probe as a loading control.

Discussion

SGK1 was originally identified as an up-regulated mRNA in a differential screen designed to uncover hormone-regulated genes involved in the control of tumor cell growth [27]. SGK1 encodes a serine/threonine kinase showing 54% homology in its catalytic domain with the well-described anti-apoptotic kinase AKT [27]. The enzymatic activity of SGK1 is controlled by the phosphoinositide (PI) 3-kinase-stimulated growth factor signaling pathway [28]. Unlike most kinases, which are primarily controlled at the enzymatic level by post-translational phosphorylation and dephosphorylation, SGK1 is also acutely controlled at the transcriptional level, as its mRNA is rapidly induced in response to a variety of stimuli including serum, glucocorticoids, follicle stimulating hormone, osmotic shock, and mineralocorticoids (reviewed in ref. [29]). Many studies have shown that SGK1 is an important regulator of epithelial sodium transport (reviewed in ref. [30]). In addition, it appears to play a key role in cell proliferation as its subcellular localization varies during the cell cycle of rat mammary tumor cells: it is nuclear in the S and G_2/M phases and cytoplasmic in the G_1 phase [31]. The cytoplasmic localization of SGK1 appears to be also associated with its cell survival properties. It was shown that glucocorticoid-mediated protection from apoptosis induced by growth factor deprivation or chemotherapeutic treatment of breast cancer cell lines is dependent on the induction of SGK1 [32,33], and that glucocorticoid-induced SGK1 localizes primarily to the cytoplasm [31]. Our immunohistochemistry results suggest that SGK1 is primarily or exclusively cytoplasmic in EMC tumor cells, suggesting that its role in the development of these tumors may be related to its anti-apoptotic properties. Recently, it was shown that the SGK1 protein is highly expressed in human breast cancer samples, and that it may contribute to prevent apoptosis in breast cancer cell lines by increasing NF-κB transcriptional activity [34], which is known to stimulate the expression of genes whose products block apoptosis [35]. It may be that the same mechanism occurs in EMC tumors cells expressing high levels of SGK1 protein.

The SGK1 mRNA was found to be up-regulated in hepatocellular carcinomas [36] and in ductal breast carcinoma *in situ* [37], and down-regulated in ovarian tumors [38], however these studies did not measure SGK1 protein

levels. This is an important issue as it was shown that the SGK1 protein has a half-life of less than 30 min in epithelial cells, due to rapid polyubiquitination and subsequent degradation by the 26S proteasome [39]. Therefore in addition to transcriptional up-regulation and reversible phosphorylation, ubiquitin modification may play a role in regulating the levels of active SGK1.

Our limited immunohistochemistry screen of other primitive sarcomas confirms that SGK1 has a restricted expression among this class of tumors, while being expressed in all EMC tested. This suggests that immunostaining for SGK1 could be a sensitive marker for the confirmation of a histologic diagnosis of EMC in specific settings. However, a more systematic immunohistochemistry analysis of histologic mimics of EMC is needed to determine the clinical utility of SGK1 immunostaining, because its detection in some alveolar soft part sarcomas indicates that it is not completely restricted to EMC among sarcomas.

The lack of transactivation of the SGK1 promoter construct by EWS/NOR1 and the observation that SGK1 is not up-regulated at short induction time points suggest that either EWS/NOR1 indirectly activates this promoter, or that the fusion protein may up-regulate the SGK1 mRNA through a post-transcriptional mechanism. This last possibility is raised by the observations that EWS/NOR1 modulates pre-mRNA splicing and interacts with the splicing protein U1C [40].

The observation that SGK1 is up-regulated in CFK2 cell lines constitutively expressing EWS/NOR1 and showing the transformation phenotype suggested to us that over-expression of SGK1 alone could possibly induce transformation. We have thus generated several clonal CFK2 cell lines over-expressing SGK1, however none of these cell lines showed a transformed phenotype (data not shown). Therefore, the fusion protein appears to regulate other pathways besides SGK1 in CFRK2 cells to induce transformation.

Acknowledgments

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