Transcriptional regulation of the Ras-related protein TC21/R-Ras2 in endothelial cells

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Abstract Applying an RNA display strategy to identify genes of autocrine activated endothelial cells, we identified among others the Ras-related protein TC21/R-Ras2 as a differentially expressed gene of bovine aortic endothelial cells (BAEC). Migrating BAEC express prominently upregulated steady state levels of TC21/R-Ras2 mRNA (Northern blot, in situ hybridization) and protein (Western blot). Growth factor stimulation identified TC21/R-Ras2 as aFGF, bFGF, and EGF inducible molecule of BAEC. Exposure to actinomycin D revealed a half life time of TC21/R-Ras2 mRNA of >2 h. These results strongly suggest that transcriptional regulation of Ras molecules contributes to their signal transduction capacity and a possible role of TC21/R-Ras2 in the signal transduction of autocrine activated endothelial cells.

Key words: Endothelial cell; TC21; R-Ras2; Ras molecule

1. Introduction

Members of the superfamily of Ras GTPases play critical roles as regulators of normal cellular growth, differentiation, and development. Acting as molecular switch by cycling between that active GTP bound state and the inactive GDP bound state, they regulate a plethora of cellular functions that range from cellular proliferation (Ras GTPases), cytoskeletal organization (Rac/Rho GTPases), vesicle trafficking (ARF and Rab GTPases), and nuclear transport (Ran GTPases) [1].

The activity of Ras proteins is controlled by guanine nucleotide exchange factors (GEFs) that catalyze the conversion of Ras to the active GTP bound state and by GTPase activating proteins (GAPs) that accelerate the hydrolysis of bound GTP to GDP [2,3]. Activated Ras binds to multiple proteins in a GTP-dependent manner to exert its diverse effector functions [4]. Of these, the binding of the ser/thr kinase Raf-1 and downstream signaling through the ubiquitous mitogen activated protein (MAP) kinase cascade has been studies most extensively.

Despite the enormous progress that has been made in the identification of factors that regulate the activity of Ras molecules surprisingly little is known about the transcriptional regulation of Ras proteins themselves. In an effort to identify factors that regulate effector functions of autocrine activated endothelial cells, we analyzed gene expression of quiescent, resting and subconfluent, migrating bovine aortic endothelial (BAE) cells by differential RNA display [5]. The rationale of the experiments was directed by the hypothesis that the subtle difference in growth configuration between subconfluent, migrating and resting, quiescent endothelial cells reflects some of specific properties of angiogenic endothelial cells in vivo in comparison to the phenoytpe of the mature, quiescent vascular endothelium. The phenotypic properties of subconfluent endothelial cells are under autocrine control and our screen consequently led to the identification of follistatin as a differentially expressed endothelial cell gene that regulates autocrine endothelial cell activity during angiogenesis [6]. Among other potentially relevant candidate genes that may regulate angiogenesis, we identified the Ras-related protein TC21/R-Ras2 [7] as a differentially expressed gene of subconfluent, migrating BAE cells. Based on this findings we have studied the transcriptional regulation of TC21/R-Ras2 in endothelial cells, which was found to be exclusively expressed by subconfluent, migrating not by quiescent, resting BAE cells. Based on the established functions of TC21/R-Ras2 in mediating mitogenic signal transduction including the possible transforming capacity of normal TC21/R-Ras2 upon overexpression [8], transcriptional regulation of TC21/R-Ras2 may play an important role in the mitogenic signal transduction of autocrine activated endothelial cells.

2. Materials and methods

2.1. Cytokines, antibodies and reagents

bFGF, aFGF, TGFβ_1, and EGF were obtained from Promega (Madison, WI). Rabbit polyclonal anti TC21/R-Ras2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). DMEM and other cell culture media were from Life Technologies (Eggenstein, Germany). Fetal bovine serum was obtained from Biochrom (Berlin, Germany).

2.2. Cell culture

Bovine aortic endothelial (BAE) cells were isolated from thoracic aortas of healthy cattle by collagenase digestion following standard protocols. Cells were cultured at 37°C in 75 cm^2 tissue culture dishes in Dulbecco's modified Eagle medium (DMEM) containing 10% heat inactivated fetal bovine serum (FBS) and frozen in liquid nitrogen at passage 2 or 3. Cells were routinely split at a 1:5 ratio and cultured up to 50 passages. Using these culture conditions, subconfluent BAE cells express high levels of endogenous bFGF up to passage 20 which will then gradually decline to undetectable levels during in vitro senescence [9]. Subconfluent migrating BAE cells were harvested at approximately 50% confluence 48 h after seeding. Quiescent, postconfluent BAE cells were reefed once and harvested three days after growing to confluence.

2.3. Differential RNA display

A comparative differential RNA display analysis of gene expression of migrating, subconfluent and quiescent, resting bovine aortic endothelial cells (BAEC) was performed as described previously [5]. In brief, cytoplasmic RNA was reverse transcribed and the differential RNA display PCR was performed for 40 cycles using a 10mer random oligonucleotide and a T11XY 3′ anchoring primer. Display products

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were resolved on a denaturing 6% PAA sequencing gel. Differentially expressed fragment cDNAs were excised, reamplified by PCR, cloned and sequenced applying standard protocols.

2.4. RNA isolation and Northern blot analysis

Cells were washed twice with PBS, harvested with a cell scraper. RNA was isolated according to the single step guanidium thiocyanate-phenol-chloroform extraction procedure using Trizol (Life Technologies, Eggenstein, Germany). For Northern blot analysis of TC21/R-Ras2 expression, 20 μg of total RNA was electrophoresed through a 1% agarose gel, capillary transferred onto nylon membranes (Hybond N, Amersham, Germany), and used for hybridization with the random prime labeled TC21/R-Ras2 cDNA probe (DNA labeling kit, Pharmacia, Uppsala, Sweden). Hybridization with an 18S rRNA oligonucleotide was performed to confirm equal loading of the different lanes [9]. All Northern blot experiments have at least been performed twice with similar results. To avoid any subjective bias that may result from unequal loading of different lanes, all Northern blot experiments were also analyzed densitometrically.

2.5. In situ hybridization

BAE cells were grown on glass coverslips as either confluent or subconfluent cells. Monolayers were fixed with 4% paraformaldehyde, washed, and treated with proteinase K (1 μg/ml, 37°C, 15 min). Prehybridization was carried out for 2 h at 55°C in 0.3 M NaCl, 20 mM Tris (pH 7.5), 5 mM EDTA, 1×Denhardt's solution, 10 mM dithiothreitol, and 50% formamide, after which the coverslips were incubated with ^{35}S-uridine 5′ triphosphate (UTP)-labeled sense and antisense TC21/R-Ras2 probes for 16 h. A 398 bp fragment of the 612 bp TC21/R-Ras2 cDNA was cloned in both directions into pCRIII (Invitrogen). Sense and antisense probes were generated with T7 RNA polymerase after linearization with EcoRV (Riboprobe System, Promega, WI). After hybridization (at 55°C overnight), the specimens were washed with 2×SSC, 10 mM β-mercaptoethanol, 1 mM EDTA (twice for 10 min each), treated with RNase A (20 μg/ml, 30 min, 37°C), and washed in 2×SSC (as above) followed by a high stringency wash at 55°C for 2 h (0.1×SSC, 10 mM β-mercaptoethanol, 1 mM EDTA). Slides were coated with autoradiographic emulsion (Kodak, NTB2), exposed for 3 weeks, and then developed (Kodak, D-19). Slides were evaluated and photographed by light microscopy using dark-field and bright-field illumination.

2.6. Western blot analysis

Cells were washed twice with PBS, harvested with a cell scraper, and lysed in a 1% NP40 buffer. Nuclei were removed by centrifugation and the lysate was subjected to reducing SDS-polyacrylamide gel electrophoresis (12% gel). Proteins were transferred to cellulose nitrate membranes (Schleicher und Schüll, Dassel, Germany) and blocked overnight in 2% BSA/PBS. TC21/R-Ras2 protein was detected using a polyclonal rabbit anti TC21/R-Ras2 antiserum (0.2 μg/ml). Bound antibody was visualized by incubation with a HRP-labeled anti rabbit Ig second antibody (1:5000), which was visualized by chemiluminescent detection (ECL Detection System, Amersham).

3. Results

3.1. Autocrine regulated differential expression of TC21/R-Ras2 in BAE cells

Comparative Northern blot analysis of migrating, subconfluent and resting, confluent bovine aortic endothelial (BAE) cells identified the Ras-related protein TC21/R-Ras2 as a differentially regulated gene of endothelial cells (Fig. 1A). Subconfluent BAE cells abundantly expressed TC21/R-Ras2 transcripts. In contrast, TC21/R-Ras2 expression was almost completely downregulated in resting confluent BAE cells.

Cultured BAE cells exhibit a considerable degree of phenotypic heterogeneity. We consequently confirmed the differential expression of TC21/R-Ras2 transcripts in BAE cells by in situ hybridization to trace expression levels of individual cells. These experiments revealed that TC21/R-Ras2 mRNA levels were homogeneously upregulated in subconfluent, migrating BAE cells and evenly downregulated in resting confluent BAE cells (Fig. 1C).

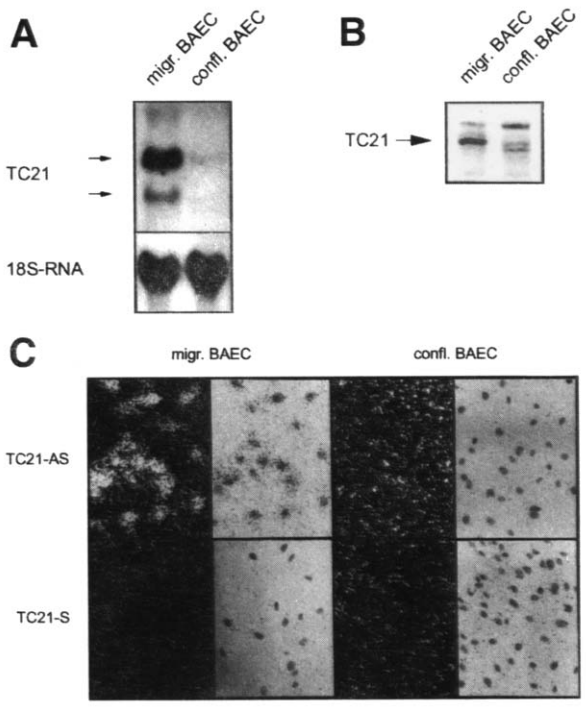

Fig. 1. Differential expression of the Ras-related molecule TC21/R-Ras2 in bovine aortic endothelial (BAE) cells. A: Subconfluent, migrating BAE cells express prominently upregulated levels of steady state TC21/R-Ras2 mRNA levels. Both TC21/R-Ras2 transcripts are upregulated with the larger 2.4 kb transcript being most prominently expressed in migrating BAE cells. B: Western blot analysis of migrating and resting BAE cells identifies TC21/R-Ras2 protein in migrating and not in resting BAE cells. C: Dark-field and bright-field images of in situ hybridization experiments with subconfluent, migrating (left) and confluent, resting (right) BAE cells using antisense (upper panel) and sense (lower panel) TC21/R-Ras2 probes. Prominent TC21/R-Ras2 expression is uniformly detectable in subconfluent BAE cells. In contrast, only background hybridization levels are detectable in confluent BAE cells.

Analysis of TC21/R-Ras2 protein expression confirmed the differential expression of TC21/R-Ras2 as determined by Northern blot and in situ hybridization. TC21/R-Ras2 protein was detected in subconfluent and not in resting monolayers of BAE cells (Fig. 1B).

3.2. Cytokine regulation of endothelial TC21/R-Ras2 expression

In the experiments performed so far, TC21/R-Ras2 expression in BAE cells was induced under autocrine control by releasing the cells from growth arrest. A number of endogenous growth factors have been implicated in regulating growth control of autocrine activated endothelial cells including the heparin-binding growth factor bFGF [10,11]. Stimulated resting BAE cells with different cytokines revealed that aFGF, bFGF, and EGF induced TC21/R-Ras2 expression, whereas $TGF\beta_1$ did not induce TC21/R-Ras2 expression (Fig. 2).

We next determined the time course of TC21/R-Ras2 expression following bFGF stimulation. Exposing BAE cells to bFGF for various time periods resulted in maximum TC21/R-Ras2 transcripts levels 8 h after the addition of bFGF (Fig. 3).

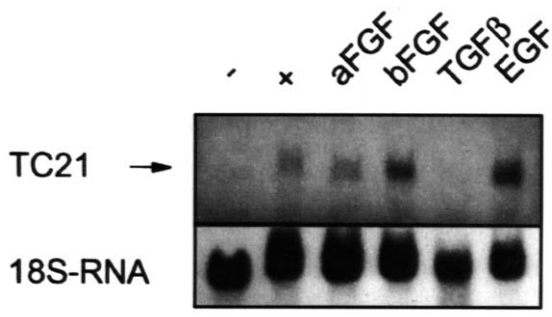

Fig. 2. Cytokine induction of TC21/R-Ras2 expression in BAE cells. Resting BAE cells were stimulated for 4 h with 5 ng/ml aFGF, bFGF, TGFβ, and EGF (−, resting control cells; +, serum stimulation of resting cells).

This surprisingly slow response to exogenously added bFGF strongly suggests that bFGF does not directly stimulate TC21/R-Ras2 expression.

3.3. Analysis of TC21/R-Ras2 mRNA stability

To determine TC21/R-Ras2 mRNA half life time in cultured BAE cells, we exposed subconfluent, migrating TC21/R-Ras2 expressing BAE cells to actinomycin D and determined TC21/R-Ras2 transcript levels by Northern blot analysis. As shown in Fig. 4, TC21/R-Ras2 transcript levels remain constant for 2 h after the addition of actinomycin D. At 4 h after actinomycin D treatment, TC21/R-Ras2 transcript levels have declined to barely detectable levels.

4. Discussion

Quiescent, resting endothelial cells line the inside of all blood vessels forming a structurally and functionally heterogeneous cell population [12]. Mitogenic activation of endothelial cells in the healthy adult occurs highly restricted and is limited to regeneration and repair processes, such as reendothelialization and angiogenesis. Angiogenic endothelial cells express a distinct repertoire of molecules that enables them to perform specific microenvironmental interactions during the angiogenic cascade. These include specific adhesive interactions involving $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins [13] as well as the ligands of endothelial cell specific growth factors such as the receptor tyrosine kinases flk-1/KDR and flt-1 [14].

Mechanisms of autocrine activation contribute to the angiogenic cascade by further stimulating endothelial cells that are initially activated by exogenous growth factors. In order to identify growth regulatory mechanisms of autocrine activated endothelial cells, we have analyzed gene expression of

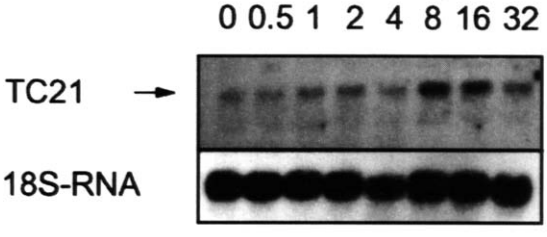

Fig. 3. Time course of bFGF induced TC21/R-Ras2 expression. Resting BAE cells were stimulated with 5 ng/ml bFGF for the time periods indicated. Maximum TC21/R-Ras2 expression is detectable at 8 h after stimulation.

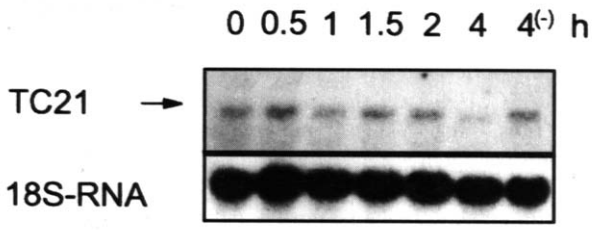

Fig. 4. Analysis of TC21/R-Ras2 transcript stability in BAE cells. Subconfluent, migrating BAE cells, expressing high levels of TC21/R-Ras2 transcripts, were exposed to actinomycin D for the time periods indicated after which cells were harvested for Northern blot analysis. TC21/R-Ras2 transcripts levels are unchanged for the first 2 h after actinomycin D exposure. After 4 h of actinomycin D treatment, TC21/R-Ras2 transcripts are barely detectable ($4^{(-)}$, control cell population after 4 h without actinomycin D treatment).

quiescent, resting and subconfluent, migrating endothelial cells by differential RNA display. Among the differentially expressed genes of migrating endothelial cells was the Ras-related protein TC21/R-Ras2. TC21/R-Ras2 was originally identified by homology cloning [7]. It displays 55% amino acid homology with $p21^{ras}$. Of the many different Ras molecules that have been identified in recent years, TC21/R-Ras2 is in addition to mutated Ras itself the only Ras family molecule that has been shown to exert transforming potential [8,15]. Interestingly, recent data even suggest that not just mutated TC21/R-Ras2, but also overexpression of the normal gene is capable of inducing neoplastic transformation [8]. These experiments suggest a similarly critical role of TC21/R-Ras2 as of Ras itself in transducing mitogenic signals. Little is known about the signal transduction cascade activated by TC21/R-Ras2. Experimental evidence suggests that the same upstream signals that activate Ras signal transduction also stimulate TC21/R-Ras2. In contrast to Ras, however, TC21/R-Ras2 appears to engage different downstream signaling pathways that do not involve Raf [16]. Differences between Ras and TC21/R-Ras2 functions are also supported by the observation that farnesyltransferase inhibitors act as inhibitors of Ras but not of TC21/R-Ras2 [17].

Little is known about TC21/R-Ras2 function in vivo. In contrast to the ubiquitous expression of Ras proteins, TC21/R-Ras2 protein expression is restricted (high levels in kidney, placenta, and ovaries; moderate levels in liver as well as cardiac and skeletal muscle; no expression in brain, testes, and lung) [16] suggesting that the function of TC21/R-Ras2 may be limited to certain tissues and/or cells. We were surprised to find a dramatic transcriptional regulation of TC21/R-Ras2 in cultured endothelial cells. Quiescent, resting BAE cells expressed barely detectable TC21/R-Ras2 mRNA and protein. In contrast, activating BAE cells just by releasing them from growth arrest resulted in a prominent upregulation of TC21/R-Ras2 mRNA and subsequently protein levels. Growth factor stimulation identified TC21/R-Ras2 as aFGF, bFGF, and EGF inducible gene. The time course of gene expression, however, would suggest that the induction of TC21/R-Ras2 gene expression was not a direct growth factor mediated early response event.

Ras molecules are important regulators of endothelial cell function. Transfection of SV40 large T antigen expressing murine endothelioma cells with oncogenic Ras leads to full neoplastic transformation of cells that are capable of forming angiosarcomas in vivo [18]. The structurally and functionally

related Ras family member TC21/R-Ras2 appears to have even higher transformation potential [8,15] and a more limited expression pattern in vivo. The observed differential expression of TC21/R-Ras2 in autocrine regulated endothelial would, thus, suggest that it may play an important role in mediating mitogenic signal transduction of endothelial cells during processes of endothelial cell activation, such as angiogenesis and reendothelialization. Furthermore, regulation of TC21/R-Ras2 expression in endothelial cells may be of more general relevance since the transcriptional regulation of Ras molecules has received little attention so far despite the fact that this may be an important regulator of Ras molecule function.

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References

[1] Macara, I.G., Lounsbury, K.M., Richards, McKiernan, C. and Bar-Sagi, D. (1996) FASEB J. 10, 625–630.
[2] Boguski, M.S. and McCormick, F. (1993) Nature 366, 643–654.
[3] Quilliam, L.A., Khosravi-Far, R., Huff, S.Y. and Der, C.J. (1995) BioEssays 17, 395–404.
[4] Katz, M.E. and McCormick, F. (1997) Curr. Opin. Genet. Dev. 7, 75–79.
[5] Kozian, D.H. and Augustin, H.G. (1995) Biochem. Biophys. Res. Commun. 209, 1068–1075.
[6] Kozian, D.H., Ziche, M. and Augustin, H.G. (1997) Lab. Invest. 76, 267–276.
[7] Drivas, G.T., Shih, A., Coutavas, E., Rush, M.G. and D'Eustachio, R. (1990) Mol. Cell. Biol. 10, 1793–1798.
[8] Clark, G.J., Kinch, M.S., Gilmer, T.M., Burridge, K. and Der, C.J. (1996) Oncogene 12, 169–176.
[9] Augustin-Voss, H.G., Voss, A.K. and Pauli, B.U. (1993) J. Cell. Physiol. 157, 279–288.
[10] Yayon, A. and Klagsbrun, M. (1990) Cancer Metastasis Rev. 9, 191–202.
[11] Ribatti, D., Urbinati, C., Nico, B., Rusnati, M., Roncali, L. and Presta, M. (1995) Dev. Biol. 170, 39–49.
[12] Augustin, H.G., Kozian, D.H. and Johnson, R.C. (1994) BioEssays 16, 901–906.
[13] Friedlander, M., Brooks, P.C., Shaffer, R.W., Kincaid, C.M., Varner, J.A. and Cheresh, D.A. (1995) Science 270, 1500–1502.
[14] Mustonen, T. and Alitalo, K. (1995) J. Cell Biol. 129, 895–898.
[15] Graham, S.M., Cox, A.D., Drivas, G., Rush, M.G., D'Eustachio, R. and Der, C.J. (1994) Mol. Cell. Biol. 14, 4108–4115.
[16] Graham, S.M., Vojtek, A.B., Huff, S.Y., Cox, A.D., Clark, G.J., Cooper, J.A. and Der, C.J. (1996) Mol. Cell. Biol. 16, 6132–6140.
[17] Carboni, J.M., Yan, N., Cox, A.D., Bustelo, X., Graham, S.M., Lynch, M.J., Weinmann, R., Seizinger, B.R., Der, C.J. and Barbacid, M. et al. (1995) Oncogene 18, 1905–1913.
[18] Arbiser, J.L., Moses, M.A., Fernandez, C.A., Ghiso, N., Cao, Y., Klauber, N., Frank, D., Brownlee, M., Flynn, E., Parangi, S., Byers, H.R. and Folkman, J. (1997) Proc. Natl. Acad. Sci. USA 94, 861–866.