Down-regulation of Lysyl Oxidase-induced Tumorigenic Transformation in NRK-49F Cells Characterized by Constitutive Activation of Ras Proto-oncogene*

Received for publication, February 23, 2001, and in revised form, April 12, 2001
Published, JBC Papers in Press, April 25, 2001, DOI 10.1074/jbc.M101695200

Monia Giampuzzi, Gerardo Botti, Michele Cilli‡, Rosanna Gusmano, Agnès Borel§, Pascal Sommer¶, and Armando Di Donato†
From the Department of Nephrology, Istituto G. Gaslini, Largo G. Gaslini, 5, 16147 Genova, Italy, the §Istituto Nazionale per la Ricerca sul Cancro, 16136 Genova, Italy, and the ¶Institute de Biologie et Chimie des Protéines, UPR 412-CNRS, 69367 Lyon, France

Several investigations have suggested a putative tumor suppressor role for lysyl oxidase because it is down-regulated in many human and oncogene-induced tumors. To address this issue we down-regulated the enzyme in normal rat kidney fibroblasts by stable transfection of its cDNA in an antisense orientation. The selected clones revealed an absence of lysyl oxidase and dramatic phenotypic changes, interpretable as signs of transformation. The antisense lysyl oxidase clones showed, indeed, loose attachment to the plate and anchorage-independent growth and were highly tumorigenic in nude mice. Moreover, we found an impaired response of the PDGF and IGF-1 receptors to their ligands. In particular, the transformed cells showed a down-regulation of both PDGF receptors and expressed the 105-kDa isoform of the IGF-1β receptor, which was not present in the normal control cells. The lack of response to PDGF-BB has been described as a feature of ras-dependent phenotypes. Therefore, we looked at the status of the p21ras. Indeed, we found a significantly higher level of active p21ras both during steady-state growth and prolonged starvation. Our data reveal new evidence for a tumor suppressor activity of lysyl oxidase, highlighting its particular role in controlling Ras activation and growth factor dependence.

Lysyl oxidase (LOX) is the key enzyme that controls collagen and elastin maturation. Indeed, it catalyzes the oxidative deamination of peptidyl lysine and hydroxylsine to peptidyl-α-aminoacidic-δ-semialdehyde into elastin and collagen chains. The consequent aldehydes lead to a spontaneous condensation forming inter- and intrachain cross-links. This post-translational modification of extracellular matrix molecules seems to have a very important role both for collagen and elastin structural aspects and for triggering still unknown signal transduction pathways. Several reports have suggested a clear association between organ fibrosis and increased LOX activity (3–9).

The most intriguing aspect regarding LOX activity refers to its putative cell phenotype control and/or tumor suppressor activity. In many naturally occurring and oncogene-induced tumors, LOX is down-regulated, while, in contrast, LOX is one of the main genes induced in concomitance with the reversion process (10–14). In particular it seems that LOX was down-regulated in cells transformed by ras or ras-dependent oncogenes, so that it was first identified as a "ras recision gene" (trg) (10, 11, 13). In particular, Friedman and co-workers (10, 11) showed that H-ras-transfected NIH-3T3, induced to revert by interferon β/γ, would return to their transformed phenotype upon transfection with an antisense LOX vector. The reversion or the re-transformation did not affect the level of p21ras although other possible mechanisms or parameters were not studied (10).

The localization of the enzyme is mainly extracellular, although recently it has been confirmed that processed LOX is localized intracellularly and inside the nucleus (16–18). Our recent finding that LOX can enhance the transcriptional activity of the COL3A1 promoter (15) seems to suggest a direct function for LOX in the nucleus. Therefore, LOX may have an intracellular substrate(s) that mediates its ability to control the cell phenotype. Despite these intriguing findings, there are no hypotheses to date about the mechanism through which LOX might actually work as a tumor suppressor. In the present study we have addressed this issue by studying the effects of the down-regulation of LOX in normal rat kidney fibroblast cells (NRK-49F).

MATERIALS AND METHODS

Antisense Vector and Transfection—NRK-49F cells were stably transfected with pCLO3 vector, a pcDNA3 plasmid carrying the fragment from −33 to +985 of the mouse LOX coding sequence (19) in antisense orientation subcloned in KpnI and XbaI restriction sites. A control transfection on the same cells was performed with the pcDNA3 vector alone. Both transfections were performed in quadruplicates. Several pCLO3 clones (as-LOX) were isolated after extensive selection with G418 and designated as DDM-A/B/C/D for those derived from the antisense LOX-transfected plates. The controls were named DDM-A/B/C/D. From the clones A/B/C/D of both transfections further clones were selected and designated by a number following the letter of the original clone (e.g. A1, A2, A-C).

Cell Culture—NRK-49F were grown in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum (FCS), 1% glutamine, 1% nonessential amino acids, and antibiotics at 37 °C, 5% CO2 in a humidified incubator. The clones derived from the transfection with pcDNA3 and pCLO3 plasmids were selected by adding 400 μg/ml G418 to the above medium for at least a month. K-NRK, normal rat kidney fibroblasts transformed by K-ras (American Tissue and Cell Culture (ATCC), Manassas, VA), were grown in the same medium as above. LP8–3 cells, NIH-3T3 fibroblasts transformed by Ha-Rasval-52, were grown in the same medium as above, but with 1% pyruvate and without nonessential amino acids.

This paper is available on line at http://www.jbc.org
acids (kindly provided by Dr. Juan Carlos Lacal, Instituto de Investigaciones Biomédicas, CSIC, Madrid, Spain). The MCF-7 cells are derived from a mammary carcinoma tumor (ATCC) and were grown in the same medium as for NRK-49F, but without nonessential amino acids.

Anchorage-independent Colony-forming Assay—
About 5 \times 10^3 cells/35-mm plate were seeded in 0.35% top agarose and poured over a layer of 0.5% agarose. Both agarose layers were prepared to contain 1/3 of the medium required for the indicated cells. The plates were incubated at 37 °C, 5% CO₂ under humidified conditions.

Tumorigenicity of as-LOX Cells in Nude Mice—
5-week-old nude mice clone crcl:cd1-nuBR (Charles River, Lecco, Italy) were injected subcutaneously with 10^6 cells from the following cell lines: controls, NRK-49F, and DDM-C4 (NRK-49F transfected with pCDNA3.1 alone); as-LOX, DDM-AL-A4 and -C6; positive control, LP8–3. The mice were kept under standard sterile conditions and followed for the indicated times.

The three dimensions, height (h), length (l), and width (w), of each tumor were measured at the indicated times, and the volumes were calculated according to the following formula: volume = \( \frac{\pi \times h (h^2 + 3a^2)}{6} \), where a = \( (w + \sqrt{w^2 - 4})/2 \). To test the recidivism of the primary neoplasias, tumors from one control individual and two from the as-LOX groups were excised. Then the animals were followed up for 3 weeks.

Protein Analysis—
Total cell lysates were prepared in radioimmune

FIG. 1. A, phenotype comparison between the control cells, NRK-49F transfected with vector alone (upper panels) and as-LOX cells (lower panels). In the left panels control-transfected NRK-49F cells (DDM-C4) and pCLO3/as-LOX clone DDM-AL-A4 were grown in their normal medium containing 10% FCS. In the right panels the same cells were grown for 48 h without 10% FCS (starvation). Notice the increase in the cell density for as-LOX even after starvation (compare lower panels, left and right). B, graph showing the growth in control cells (DDM-C4) and as-LOX cells (DDM-AL-A4). About 20,000 of the indicated cells were seeded in 35-mm plates in their normal culture conditions. After adhesion to the plate (24 h), the cells were deprived of FCS for 48 h. Subsequently the growth medium was re-integrated with 10% FCS, and the cells were grown for 3 more days. The same results are shown in the inset, but using a logarithmic scale to express the number of cells. This allows the detection of the dramatic cell growth arrest and death in the control cell line during the 48 h of starvation. The results expressed are the average \( \pm \) S.E. of a typical experiment performed in triplicate.

FIG. 2. LOX and LOL protein expression in control and as-LOX cells.
30 μg of total cell lysates from the indicated cell lines were analyzed by Western blot with anti-LOX (A, Ref. 7) and anti-LOL (B, Ref. 22) rabbit polyclonal. In the figure, the bands corresponding to LOX and LOL precursors are indicated. The as-LOX clone used in this experiment was DDM-AL-A4, but similar results were obtained with almost all the tested as-LOX clones.

FIG. 3. PCR detects the insertion of pCLO3 in as-LOX clones.
The figure shows that the expected 1.6-kilobase band is amplified only from the genomic DNA of the as-LOX cells, whereas the control cell DNA produced only a smear. Lane 2 shows the expected amplification product using 10 ng of pCLO3 as template.
Chemicals) 5% dimethylsulfoxide was added.

9 °C for 30 s, and the extension step was at 72 °C for 1 min. In the bases. The samples were amplified for 32 cycles with an annealing at. 9- GCGCGTAACCACCACA-3

antisense LOX sequence and designed in position 2304: 5- GTCACGCT-

GGGCGGTAGGCGTGTAC-3; the reverse primer was specific for the primer, specific for the pCDNA3 vector, was in position 707: 5- GCAAAT-

primer, specific for pCDNA3 vector, was in position 707: 5- GCAAAT- applying the plasmid and the antisense LOX sequence. The forward primers were designed to amplify a fragment overlapping the plasmid and the antisense LOX sequence. The forward primer, specific for pcDNA3.1 vector, was in position 707: 5- GCAAAT-

as-LOX clones. The primers were designed to amplify a fragment over-lapping the plasmid and the antisense LOX sequence. The forward primer, specific for pcDNA3 vector alone. The as-LOX clones

carried the expression vector, we performed PCR to detect the plasmid, further Western blot analysis. Results and Discussion

The lysyl oxidase antisense clones (as-LOX) clones showed striking phenotypic changes when compared with the control clones (Fig. 1A). The Western blot analysis (Fig. 2A) showed a dramatic down-regulation of LOX in the as-LOX clone (DDM-AL-A4), whereas the control clones showed a typical suffer-ance evident from the reduction of the cytosolic compartment and elongation of the cell body (Fig. 1A). These observations prompted us to look for the typical features of tumorigenicity. Fig. 4, A and B shows a soft-agar colony-forming assay that tests the ability of the cells to grow under anchorage-independent conditions. The experiment also included two positive controls, K-NRK cells, K-ras oncogene-transformed homologous to NRK-49F, and a mammary tumor cell line, MCF-7. In Fig. 4A, a macroscopic comparison of the colony-forming ability of two control clones versus two as-LOX clones selected from two independent transfections is shown. It is clear that as-LOX can grow in soft-agar forming a great number of large colonies, whereas the control cells formed fewer and smaller colonies, almost undetectable to the naked eye. As expected, K-NRK and MCF-7 cells also formed colonies, although K-NRK to a lesser extent. Fig. 4B shows a microscopic image of the colonies formed by the tested cell lines. It appears that as-LOX clones and MCF-7 produced comparable size colonies. As a further approach to define the transformed status of as-LOX cells, we tested their tumorigenicity when injected in nude mice. We injected subcutaneously $10^{-6}$ cells/mouse in a total of 14 ani-
The significance of the differences between as-LOX and control cells was evaluated by Student's t test and is indicated in the graph.

In an attempt to characterize the as-LOX cells at a molecular level, we investigated the response to several growth factors and the levels of their respective receptors. Among them, we found that the treatment with PDGF-BB did not produce tyrosine autophosphorylation of the PDGF-β receptor. Fig. 6A (upper panel) shows the anti-phosphotyrosine immunoblot of the immunoprecipitated PDGF-β receptor from control and as-LOX cells after 5 and 10 min of PDGF-BB treatment. It clearly appears that the tyrosine phosphorylation of the receptor occurs only in the control cells, whereas the signal is completely absent in the as-LOX cells. Surprisingly, when we challenged the same immunoprecipitate with a specific anti-PDGF-β receptor to control the efficiency of the immunoprecipitation, we could not detect a band in the as-LOX clones (Fig. 6A, lower panel). Thus, the absence of response seems to be due to a dramatic down-regulation of the receptor itself, rather than to a biochemical defect. These results prompted us to test the response of the PDGF-α receptor as well, to verify if our finding was isolated to the β receptor. Fig. 6B shows that also in this case there was no autophosphorylation of the receptor upon PDGF-BB triggering (upper panel), because of its dramatically inhibited expression (lower panel). The same results were obtained challenging the cells with PDGF-AA (data not shown). Moreover, we analyzed the response to another important growth factor, IGF-1, often implicated in transformation and tumorigenesis as well as in differentiation processes (28–31). Surprisingly, we detected an abnormal expression of the IGF-1β receptor, appreciably different in molecular weight and amount. Fig. 7A shows that...
while NRK-49F and control-transfected cells exhibited the normal 95-kDa receptor, the as-LOX showed a higher expression of the 105-kDa isoform. Interestingly, in the same Western blot it can be observed that the LP8–3 cell line (NIH-3T3 expressing activated Ha-ras) predominantly displayed the same receptor isoform. This 105-kDa variant seems to be tissue-specific and, according to some investigators, expressed during fetal development (32–35) or even in leukemic cells (36). A functional analysis of the IGF-1β receptor showed that again in as-LOX cells there was no autophosphorylation upon IGF-1 challenging (Fig. 6, B and C), although this isoform has been described as fully functional.

The down-regulation and/or absence of PDGF receptor autophosphorylation are a recurrent feature of some of the ras-transformed cell lines (37–39). Therefore, we analyzed the functional state of p21ras by using the Raf-1 Ras-Binding Domain (RBD) pull-down assay, which specifically recognizes the GTP-bound form of p21ras. Fig. 8A shows that the active fraction of p21ras is highly increased in the two as-LOX clones that we used to induce tumors in the athymic mice. The same blot showed that the difference in the activated fraction of p21ras is not because of an increase in its total pool. Moreover, we studied the status of p21ras under different growth conditions: after 48 h of starvation, after 24 h of 10% FCS exposure following starvation, or a steady-state confluent status. Fig. 8B shows that the activated form of the protein is overall much higher in the as-LOX cells when compared with the control cells. Indeed, the active p21ras was between 25 and 11 times higher than the control cells (Fig. 8C), depending on their growth status. As expected, the difference was highest after 48 h of starvation, when normal cells do not show any activated p21ras whereas it was minimum (but still very high) after growth in 10% FCS, certainly as a result of the serum mitogenic stimulus.

Our findings prove that LOX can act as a tumor suppressor, at least in our cell model. Previous attempts to antagonize the tumorigenicity of ras-transformed cells by overexpressing LOX...
reversion by interferon did not affect the level of H-ras product, which, moreover, was under a heterologous LTR promoter. Our study confirms Friedman’s findings, but in a more physiological model. Indeed, we used normal fibroblasts that did not show abnormal levels of active p21ras and seemed normally regulated by the main growth factors. We also showed that as-LOX cells were unresponsive to some growth factors, the meaning of which is more difficult to explain. As mentioned above, this is not a new feature for a transformed cell line. The most obvious and rational physiological meaning of this finding might be that the pathway downstream of the growth factor receptor is already activated. Indeed, this was the case, when we look at the constitutive activation of p21ras in the as-LOX cells. As elsewhere suggested, our results also reinforced the idea of a control of LOX on ras proto-oncogene. From our data it cannot be determined if the activation of p21ras is a direct consequence of a de-regulation depending on LOX absence or is rather the indirect result of the cell transformation. Certainly, Ras activation must play an important role in the described tumorigenic process, probably enhanced by the loss of response to two important growth factors. Likely, other elements of the mitogenic pathway are activated as well, which might have triggered a negative feedback down-regulating the level of the receptors, at least for PDGF. A very recent study (40) suggests that the PDGF-β receptor can be down-regulated in an ubiquitin-dependent fashion as a consequence of loss of attachment to the substrate. We don’t know yet, but a similar mechanism might be active in our as-LOX cells, because these cells display a looser attachment to the plate and, as a consequence, an elevated sensitivity to the trypsin.

Regarding the IGF-1β receptor, the meaning of the 105-kDa isoform is not clear. We believe that it is related to a sort of de-differentiation or transformation into a different cell type. Nevertheless, in this case the signal from the growth factor seems abolished. All together, our data suggest that the absence of LOX determined a sort of growth factor independence. It should be recalled that growth factors are not synonymous to mitosis, but they are rather controllers of the cell cycle, maintaining the correct equilibrium with other extracellular signals. In our case, at least two of these important controllers are lost and this, by itself, might account for the observed transformation. More studies need to be performed to analyze other elements of the signal transduction in the as-LOX cells, mainly involving the cell-substrate and cell-cell attachment, which also seem to be altered. More challenging is the goal of determining the mechanisms by which the absence of LOX induced the described alterations. New insights will certainly be revealed by the identification of putative intracellular LOX targets or substrates, one of our next goals.

REFERENCES
1. Pimmel, S. R., and Martin, G. R. (1968) Proc. Natl. Acad. Sci. U. S. A. 61, 705–716
2. Kagan, H. M., and Trackman, P. C. (1991) Am. J. Respir. Cell Mol. Biol. 3, 296–310
3. Murawaki, Y., Kusakabe, Y., and Hirayama, C. (1991) Hepatology 14, 1167–1173
4. Siegel, R. C., Chen, K. H., Greenspan, J. S., and Aguilar, J. M. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 2945–2949
5. Riley, D. J., Kerr, J. S., Berg, R. A., Iamu, B. D., Pietra, G. G., Edelman, N. H., and Prockop, D. (1982) Science 215, 125–129
6. Di Donato, A., Ghiggeri, G. M., Di Duca, M., Jivotenko, E., Acinni, R., Campolo, J., Ginevi, P., and Gusmano, R. (1997) Nephron 76, 192–200
7. Sommer, P., Gleyzal, C., Racuret, M., Delbourg, M., Serraz, M., Joazeiro, P., Peyrol, S., Kagan, H., Trackman, P. C., and Grimaud, J. A. (1993) Lab. Invest. 69, 460–470
8. Jourdan-Le Saux, C., Gleyzal, C., Garnier, J. M., Peraldi, M., Sommer, P., and Grimaud, J. A. (1994) Biochem. Biophys. Res. Commun. 209, 587–592
9. Chanoki, M., Ishii, M., Kobayashi, H., Fushida, H., Yashiro, N., Hamada, T., and Ooshima, A. (1995) Br. J. Dermatol. 133, 710–715
10. Contente, S., Kenyon, K., Raimondi, D., and Friedman, R. M. (1990) Science 249, 796–798
11. Kenyon, K., Contente, S., Trackman, P. C., Tang, J., Kagan, H. M., and

---

2 A. Donato, personal observations.
Friedman, R. M. (1991) *Science* **253**, 802

12. Hämaäinen, E. R., Kemppainen, R., Kuivaniemi, H., Tromp, G., Vaheri, A., T., P., and I., K. K. (1995) *J. Biol. Chem.* **270**, 21590–21593

13. Kryzosiak, W. J., Shindo-Okada, N., Teshima, H., Nakajima, K., and Nishimura, S. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4879–4883

14. Hajnal, A., Klemenz, R., and Schäfer, R. (1993) *Cancer Res.* **53**, 4670–4675

15. Giampuzzi, M., Botti, G., Di Duca, M., Arata, L., Ghiggeri, G., Gusmano, R., Ravazzolo, R., and Di Donato, A. (2000) *J. Biol. Chem.* **275**, 36341–36349

16. Li, W., Nellaiappan, K., Strassmaier, T., Graham, L., Thomas, K., and Kagan, H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12817–12822

17. Di Donato, A., Lacal, J. C., Di Duca, M., Giampuzzi, M., Ghiggeri, G., and Gusmano, R. (1997) *FEBS Lett.* **419**, 63–68

18. Nellaiappan, K., Risitano, A., Liu, G., Nicklas, G., and Kagan, H. M. (2000) *J. Cell. Biochem.* **79**, 576–582

19. Contente, S., Csiszar, K., Kenyon, K., and Friedman, R. M. (1993) *Genomics* **16**, 395–400

20. Taguchi, A., Blood, D. C., del Toro, G., Canet, A., Lee, D. C., Qu, W., Tanji, N., Lu, Y., Lalla, E., Fu, C., Hofmann, M. A., Kielinger, T., Ingram, M., Lu, A., Tanaka, H., Hori, O., Ogawa, S., Stern, D. M., and Schmidt, A. M. (2000) *Nature* **405**, 354–360

21. Laemmli, U. K. (1970) *Nature* **227**, 680–685

22. Decitre, M., Gleyzl, C., Raceur, M., Peyrol, S., Aubert-Foucher, E., Csiszar, K., and Sommer, P. (1998) *Lab. Invest.* **78**, 143–151

23. Kenyon, K., Modi, W. S., Contente, S., and Friedman, R. M. (1993) *J. Biol. Chem.* **268**, 18435–18437

24. Kim, Y., Boyd, C. D., and Csiszar, K. (1995) *J. Biol. Chem.* **270**, 7176–7182

25. Saito, H., Papaoconstantinou, J., Saito, H., and Goldstein, S. (1997) *J. Biol. Chem.* **272**, 8157–8160

26. Jourdan-Le Saux, C., Tronzecker, H., Bagic, L., Bryant-Greenwood, G. D., Boyd, C. D., and Csiszar, K. (1999) *J. Biol. Chem.* **274**, 12939–12944

27. Carnero, A., Cuadrado, A., del Peso, L., and Lacal, J. C. (1994) *Oncogene* **9**, 1387–1395

28. Garroute, F. L., Remacle-Bonnet, M. M., Lehmann, M. M., Marvaldi, J. L., and Pommier, G. J. (1997) *Endocrinology* **138**, 2021–2032

29. Freeman, J. W., Mattingly, C. A., and Strodel, W. E. (1995) *J. Cell. Physiol.* **165**, 155–163

30. Scottlandi, K., Benini, S., Sarti, M., Serra, M., Lollini, P. L., Maurici, D., Pieci, P., Manara, M. C., and Baldini, N. (1996) *Cancer Res.* **56**, 4570–4574

31. Perez-Juste, J. G. and Aranda, A. (1999) *Oncogene* **18**, 5393–5402

32. Hainaut, P., Kowalski, A., Giorgetti, S., Baron, V., and Van Obberghen, E. (1991) *Biochem. J.* **273**, 673–678

33. Moss, A. M., and Livingston, J. N. (1993) *Biochem. J.* **294**, 685–692

34. Barenton, B., Domeyne, A., Garandel, V., and Garofalo, R. S. (1993) *Endocrinology* **133**, 651–660

35. Garofalo, R. S., and Rosen, O. M. (1989) *Mol. Cell. Biol.* **9**, 2806–2817

36. Kellerer, M., Obermaier-Kusser, B., Ermer, B., Wallner, U., Haring, H. U., and Petrides, P. E. (1990) *J. Biol. Chem.* **265**, 9340–9345

37. Rake, J. B., Quinones, M. A., and Faller, D. V. (1991) *J. Biol. Chem.* **266**, 5348–5352

38. Paasinen-Sohns, A., and Holtta, E. (1997) *Oncogene* **15**, 1953–1966

39. Rice, P. L., Porter, S. E., Koski, K. M., Ramakrishna, G., Chen, A., Schrump, D., Kazlauskas, A., and Malkinson, A. M. (1999) *Mol. Carcinog.* **25**, 285–294

40. Baron, V., and Schwartz, M. (2000) *J. Biol. Chem.* **275**, 39318–39323
Down-regulation of Lysyl Oxidase-induced Tumorigenic Transformation in NRK-49F Cells Characterized by Constitutive Activation of Ras Proto-oncogene

Monia Giampuzzi, Gerardo Botti, Michele Cilli, Rosanna Gusmano, Agnès Borel, Pascal Sommer and Armando Di Donato

J. Biol. Chem. 2001, 276:29226-29232.
doi: 10.1074/jbc.M101695200 originally published online April 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101695200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 20 of which can be accessed free at http://www.jbc.org/content/276/31/29226.full.html#ref-list-1