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Identification of 12-Lipoxygenase Interaction with Cellular Proteins by Yeast Two-Hybrid Screening†

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ABSTRACT: The platelet isoform of 12-lipoxygenase (12-LOX) is expressed in a variety of human tumors. 12-LOX metabolizes arachidonic acid to 12(*S*)-hydroxyeicosateraenoic acid (12(*S*)-HETE), which induces a number of cellular responses associated with tumor progression and metastasis. Little is known about 12-LOX regulation and no direct regulators of 12-LOX activity have been identified. To identify potential regulators of 12-LOX, we isolated cDNAs encoding 12-LOX interacting proteins using the yeast two-hybrid system. We screened a yeast two-hybrid interaction library from human epidermoid carcinoma A431 cells and identified four cellular proteins that interact specifically with 12-LOX. We identified type II keratin 5, lamin A, the cytoplasmic domain of integrin β4 subunit and a phosphoprotein C8FW as 12-LOX interacting proteins. Here, we demonstrated that keratin 5, a 58 kD protein required for formation of 8 nm intermediate filaments, binds to 12-LOX in human tumor cells and may contribute to the regulated trafficking of 12-LOX. We also showed that lamin A binds 12-LOX in human tumor cells. These proteins provide the first candidate regulators of 12-LOX.

12-lipoxygenase (EC 1.13.11.31), one of at least three lipoxygenases, metabolizes arachidonic acid (AA) to 12-hydroperoxyeicosatetraenoic acid (12-HPETE),[1] which is subsequently converted to 12(S)-HETE and hepoxilins (*1*). Enzymological, immunological, and molecular biological evidence indicates that the 12-LOX proteins expressed in leukocytes, platelets, and tracheal epithelium are three distinctly different enzymes (*2*−*3*). Platelet-type 12-LOX is expressed normally in platelets, megakaryocytes, umbilical vein endothelial cells, and a wide variety of human and rodent tumor cells including HEL (human erythroleukemia) cells and A431 cells (human epidermoid carcinoma) (*4*). Platelet 12-lipoxygenase is a dual-function enzyme that posseses both oxygenase and lipoxin synthase activity (*5*). Platelet 12-LOX metabolizes only AA (but not c-18 fatty acids, such as linoleic acid) to form exclusively 12(S)-HETE (*6*) and utilizes leukotriene A_4 to generate lipoxin A_4 and B_4 during platelet−leukocyte interactions (*5*). 12(S)-HETE induces a plethora of responses in tumor cells and is linked to tumor progression and metastasis (*7*). For example, 12-(S)-HETE has been reported to stimulate integrin expression and secretion of proteinases, enhance tumor cell motility and invasion, and induce angiogenesis (*7*−*8*). Leukotrienes and

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[1] Abbreviations: A431 cell, human epidermoid carcinoma; 12-LOX, 12-lipoxygenase; 12(*S*)-HETE, 12(*S*)-hydroxyeicosateraenoic acid; AA, arachidonic acid; HEL, human erythroleukemia; Cdk, cyclin-dependent kinase; FLAP, 5-lipoxygenase activating protein.

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lipoxins participate in multicellular events including thrombosis, inflammation, immunity, and atherosclerosis. For instance, lipoxin A_4 down-regulates leukocyte responses and trafficking and neutrophil−endothelial interaction (*9*−*10*), while lipoxin B_4 regulates human neutrophil adherence and motility (*11*).

Human platelet-type 12-LOX is believed to play a role in cancer and other pathological conditions, such as psoriasis, atherosclerosis, and arthritis. In a clinical study of 137 prostate cancer patients, platelet-type 12-LOX expression levels were determined in cancer tissue and compared with the levels in matching normal tissue from each patient. Approximately 38% of the patients studied exhibited elevated levels of platelet-type 12-LOX in the cancer tissues. This elevated 12-LOX correlated positively with tumor stage and grade and positivity for prostate cancer cells in the surgical margins (*12*). In addition 12-LOX activity is up regulated in tumor cells following γ radiation (*13*). Clearly, understanding how the 12-LOX enzyme is regulated will be an important step in elucidating the role of this enzyme in a variety of human cancers.

The enzyme activity of 12-LOX and 5-LOX is regulated by a translocation process following cell stimulation. It has been shown that Ca^{2+} and thrombin increase 12-LOX activity and 12(S)-HETE production by the translocation of 12-LOX from cytosol to membrane in human platelets, HEL cells, and A431 cells (*14*−*15, 33, 41*). A recent report demonstrates that an anti-platelet agent inhibits the 12-LOX activity and 12(S)-HETE production by blocking the translocation of 12-LOX (*15*). However, to date there is no diret evidence for protein(s) that could mediate 12-LOX translocation, and no direct regulator/interacting protein of 12-LOX has been found. In constrast, for leukocyte 5-LOX, there is evidence of complex protein−protein interactions in its nuclear membrane translocation, activation, and substrate acquisition in intact cells (*27*). For example, FLAP (5-lipoxygenase-activating protein) assocation with 5-LOX after its translocation acts as an arachidonic acid transfer/docking protein that "presents" the substrate to 5-LOX on the leukocyte nuclear membrane (*16*). This finding was the first indication that a protein directly associates with and regulates the activity of a member of the lipoxygenase family. Recently, three additional 5-LOX-interacting proteins that may be involved in regulation and/or nuclear localization were identified using the yeast two-hybrid system (*17*).

In this study, we used a yeast two-hybrid approach to identify proteins that interact with 12-LOX and that may regulate its activity. We constructed a two-hybrid library from A431 cells and identified from it four distinct cellular proteins that interacted specifically with 12-LOX. They are human type II keratin K5, nuclear envelope protein lamin A, integrin β4 cytoplasmic domain, and human C8FW phosphoprotein. The identification of these 12-LOX-interacting proteins may help us to understand the complex ways in which 12-LOX is involved in tumor progression and metastasis.

MATERIALS AND METHODS

Yeast Strains and Manipulation. *Saccharomyces cerevisiae* yeast strains used were RFY231 (MATa *his*3 *ura*3-1 *trp*1Δ::*his*G *leu*2::3Lexop-*LEU*2) (*19*) and RFY206 (MATa *his*3Δ200 *leu*2-3 *lys*2Δ201 *ura*3-52 *trp*1Δ::*his*G) (*21*). Yeast were grown using standard microbiological techiques and media (*22*−*23*). Media designations are as follows: YPD is YP (yeast extract plus peptone) medium with 2% glucose. Minimal dropout media are designated by the component that is left out (e.g., -ura -his -trp -leu medium lacks uracil, histidine, tryptophan, and leucine). Minimal media contained either 2% glucose (Glu) or 2% galactose plus 1% raffinose (Gal). X-Gal minimal dropout plates contained 40 mg/mL X-Gal and phosphate buffer at pH 7.0. DNA was introduced into yeast by LiOAc-mediated transformation as described (*24*).

Human A431 Cell cDNA Library Construction. RNA was isolated from human epidermoid carcinoma A431 cells, and mRNA was purified using Oligo-tex Beads mRNA kit (QIAGEN Inc, Valencia, CA). cDNA was synthesized with the Stratagene cDNA synthesis kit, essentially according to the manufacturer's instructions. Briefly, poly(A)+ mRNA derived from human epidermoid carcinoma A431 cells was used to direct the synthesis of first-strand cDNA by reverse transcriptase with an XhoI-oligo d(T) primer. Following second-strand synthesis, the cDNA was ligated to an EcoRI adaptor, digested with XhoI, and subsequently size selected over cDNA Size Fractionation Columns from Gibco BRL. Fractions containing cDNAs ranging from 500 bp to > 3 kb in length were pooled and subcloned into the yeast library plasmid pJG4-5 (*25*). The average insert size was evaluated by purifying plasmid DNA from 48 random clones and digesting with EcoRI+XhoI. The size of each product was determined by gel electrophoresis. The A431 cDNA had 3.0×10^6 independent clones, and 90% of the plasmids had cDNA inserts of 0.3−3.8 kb (average size, 1.2 kb). The resultant cDNA library contains 3.0×10^6 primary recombinant clones.

Plasmids. Bait plasmids expressing LexA fusion proteins were derivatives of the *HIS*3 2 μm plasmid, pEG202 (*20*). The full-length cDNA encoding human platelet-type 12-LOX was provided by Dr. Colin Funk (University of Pennsylvania). The entire coding region of the cDNA was amplified by the polymerase chain reaction (PCR) with a 5′ *Bam*HI site introduced in the upper primer (CCGGGGATCCG-T*ATG*GGCCGCTACCGCATC) and the lower primer corresponding to an *Xho*I site from the original cDNA downstream of the stop codon (GCCGGCGAGCTCAGTCTACCA-CTGTGACAA). The PCR product was digested with *Bam*HI and *Xho*I and inserted into the *Bam*HI/*Xho*I sites of pEG202 (*20*) to generate the bait plasmid pLexA-12-LOX; the plasmid encodes the entire 663 amino acid human platelet 12-LOX protein. Additional bait plasmids used in the specificity test are described elsewhere (*21, 25*−*26*).

Yeast Two-Hybrid Screen. The yeast two-hybrid procedures were conducted as described (*28*−*29*). Strain RFY231 containing pSH18-34 and pLexA-12-LOX was transformed with A431 interaction library DNA and a total of 6.5×10^6 independent colonies were collected. The selection for interacting clones was performed in media containing galactose and lacking leucine. 400 Leu+ yeast colonies were further tested for lacZ expression by plating them on medium containing galactose and X-Gal. 40 of the Leu+ positive clones demonstrated galactose-dependent activation of both reporters, suggesting interaction between the galactose-inducible cDNA protein and 12-LOX. Library plasmids from yeast colonies, expressing the putative 12-LOX-interacting proteins, were rescued by transformation of yeast plasmid

DNA into KC8 *E. coli* followed by selection on minimal medium lacking tryptophan (*29*). To determine which clones were unique, PCR products generated with primers flanking the cDNA insertion site in pJG4-5 (BC01 and BC01, ref *29*) were digested with *Alu*I and *Hae*III and analyzed on a 2% agarose gel. The specificity of the unique interactors was tested using the interaction mating assay as described (*21*). Briefly, rescued library plasmids were introduced into RFY231 and the transformants were mated with various derivatives of RFY206 that each contained pSH18-34 and a bait plasmid expressing a LexA fusion. 12 library plasmids encoded proteins that interacted with the 12-LOX bait but not unrelated baits. These 12 cDNAs were sequenced and evaluated by the Basic Local Alignment Search Tool (BLAST) through the National Center for Biotechnology Information Internet site (www.ncbi.nlm.nih.gov).

Immunoprecipitation. Cells were lysed in a cold lysis buffer consisting of 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 0.5% NP-40, 0.1% SDS. The lysate was clarified by centrifugation at 10000g for 10 min. Isolation of nuclear extracts was according to the standard protocol (*30*). The supernatants and soluble nuclear extracts were immunoprecipitated with 4−6 μL (1−2 μg)antibody against human platelet-type 12-LOX or anti-5LOX and anti-15-LOX antibodies (Oxford Biomedical Research, Oxford, MI) or the anti-keratin (Chemicon International INC., Temecula, CA) or anti-lamin A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 h, followed by 40 μL Sepharose 4B-conjugated protein G at 4 °C overnight. Immune complexes were washed three times in the lysis buffer, and the pellets were suspended in SDS sample buffer for SDS-PAGE electrophoresis. Aliquots of total cell lysate were mixed with 1 vol of SDS sample buffer (85 mM Tris-HCl, pH 6.8, containing 1.4 (w/v) SDS, 14% (v/v) glycerol, 5% (v/v) mercaptoethanol and a trace of bromophenol blue, boiled for 5 min and subjected to SDS-PAGE on 4−20% acrylamide gel. Proteins were electrophoretically transferred to nitrocellulose membranes. After transfer, nonspecific sites were blocked with 5% (w/v) nonfat-dry milk in TTBS (0.1% Tween-20, 20 mM Tris base, 137 mM NaCl, 3.8 mM HCl, pH 7.6) for 2 h at 25 °C followed by probing primary antibody. After the blot was washed three times in TTBS, the membranes were incubated for 1 h at 25 °C with horseradish peroxidase-conjugated secondary anti- IgG (dilution: 1:4500. Amersham, Arlington Heights, IL). The blot was washed again in TTBS, then developed using ECL according to the manufacture's instructions (Amersham, Arlington Heights, IL).

Immunofluorescent Staining. Intracellular 12-LOX and keratin were localized using a modification (*49*) of the general immunocytochemical methodologies described by Willingham (*50*). 2×10^4 Cells were grown in 4-well Lab-Tek chamber (Nalge Nunc Intl, Naperville, IL) in 0.5 mL media to 60−80% confluence and the media changed to serum free media the night before the experiment. Cells were washed with PBS 3× and then fixed with 3.7% formaldehyde in phosphate-buffer saline, pH 7.4 for 10 min. Fixation and subsequent steps were performed at 25 °C for intracellular labeling. After the sample was washed with PBS, the cells were blocked with 2 mg/mL BSA in PBS. All subsequent antibody and wash solution contained 0.1% saponin. Cells were incubated with primary antibody (rabbit anti-human 12-LOX, mouse anti-human keratin) for 2 h and washed. In controls, preimmune serum (rabbit or mouse) was substituted for the primary antibody (1:100 dilution). Cells were incubated in secondary antibody (FITC-conjugated AffiniPure Goat anti-mouse IgG or Rhodamine Red-X-conjugated AffiniPure-Goat anti-Rabbit IgG) and secondary blocking reagent for 1 h. Secondary antibody was diluted in PBS-0.1% saponin (1:100 dilution) and 5% normal goat serum was added to this solution. After the resultant was washed, the coverslips were mounted upside-down on slides with SlowFade anti-fade reagent and observed with a Zeiss LSM 310 laser confocal microscope.

Transfection. The full-length cDNA encoding human platelet-type 12-LOX from pCMV-12-LOX (provided by Dr. Colin Funk, University of Pennsylvania) was cloned into the *Eco*RI/*Xba*I sites of pcDNA3.1 (Invitrogen), which uses the neomycin-resistance gene as the selectable marker. Cells grown in 6-well plates were transfected with 3−12 μg of pcDNA-12-LOX by the FuGENE 6 Transfection Reagent (Boehringer Mannheim Co., Indianapolis, IN) following the manufacture's instructions. Neomycin-resistant cells were selected in 300 μg/mL Geneticin (G418; LifeTechnologies, Inc., Grand Island, NY). The cells were passaged and tested by immunoprecipitation and Western Blotting assays for enhanced 12-LOX protein expression. Cells transfected with vector alone were selected and analyzed in parallel. While in culture, cells were fed with a G418-containing medium to prevent outgrowth of revertant cells. All of the cells were frozen at early passage for subsequent study.

RESULTS AND DISCUSSION

A431 Interaction Library. To identify proteins that may interact with 12-lipoxygenase, we constructed a yeast two-hybrid interaction library from A431 cells. This cell line has been widely used to study 12-LOX in tumor cells, since it expresses enzymatically active platelet-type 12-LOX protein, but not the leukocyte-type isoform (*33*−*35*). In A431 cells, the predominant amount of 12-LOX protein resides in the cytosol (*33, 36*). In contrast, 12-LOX enzyme activity is mainly localized in the membrane fraction (*33, 37*). EGF (TPA, Ca^{2+}) increases total cellular 12-LOX protein and enhances the association of 12-LOX protein with perinuclear or cytoplasmic membrane (*33, 37*−*41*). In addition, EGF stimulates 12-LOX activity and generation of 12(S)-HETE from cellular arachidonate (*33, 36*−*37, 41*). These features make A431 cells an ideal model to study the regulation of 12-LOX activity.

To construct the cDNA library from A431 cells, we isolated poly(A)+ mRNA from 80% confluent cultured A431 cells, used it to synthesize unidirectional cDNA, and inserted the cDNA into the yeast two-hybrid vector pJG4-5 (see Materials and Methods). This vector allows conditional expression (induced by galactose and repressed by glucose) of cDNA-encoded proteins with a transcription activation domain at their amino termini. The resulting library had 3.0×10^6 independent clones, of which 90% had inserts with sizes ranging from 0.3 to 3.8 kb (average size 1.2 kb).

12-Lipoxygenase Interacting Proteins. To identify potential regulators of 12-LOX, we screened the A431 library for clones that encoded 12-LOX-interacting proteins (see Ma-

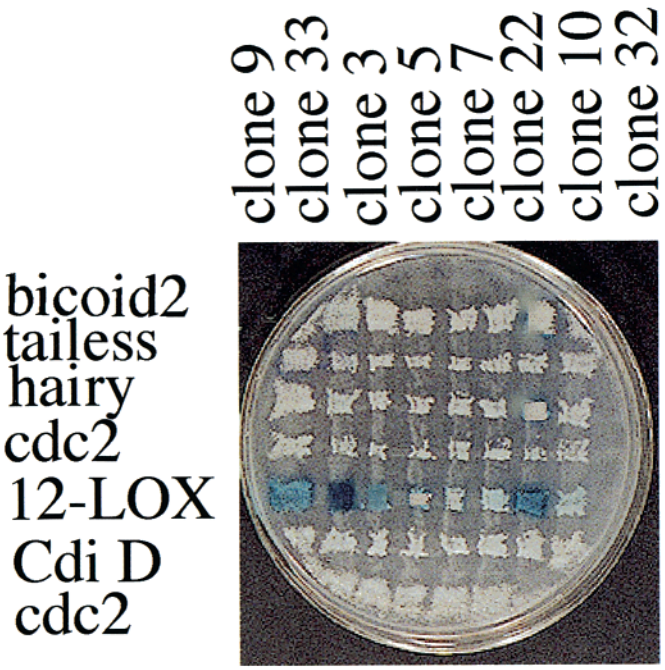

FIGURE 1: Specificity of 12-LOX interactors in the yeast two-hybrid assay. Representative plate of a mating assay to test the specificity of 12-LOX interactors. This plate demonstrates the interactions between seven bait proteins (horizontal lines of yeast) and eight prey proteins (vertical lines) on a Gal/Raf X-Gal plate (urp-his-trp- drop out). The strength of the interaction is suggested by the level of activation of lacZ reporter as indicated by blue color, as summaried in Table 1.

terials and Methods). Yeast expressing the full-length platelet isoform of 12-LOX fused to the LexA DNA-binding domain were transformed with the A431 interaction library. From 6.5×10^6 yeast transformants, we identified 12 independent clones in which the LexA-driven reporters were active only in galactose, indicating that they contained cDNA-encoded proteins that interacted with LexA-12-LOX. We isolated the library plasmids from these clones and determined that they represented four different cDNAs. Six identical clones encoded the carboxy-terminal 330 amino acid residues of type II keratin K5. Two clones encoded the carboxy-terminal 202 residues of the nuclear envelope protein lamin A. Another two clones encoded the cytoplasmic domain of integrin β4. The final two clones encoded a phosphoprotein, C8FW, with some similarity to protein kinases. To further determine the specificity of the four 12-LOX interactors, we conducted a two-hybrid mating assay to test for interactions with LexA-12-LOX and with other unrelated LexA fused proteins. As shown in Figure 1 and Table 1, all four clones interacted specifically with 12-LOX, but not with several other proteins including *Drosophila* cyclin D, Cdk1, hairy, tailess, or bicoid.

Human Keratin K5 Interacts with 12-LOX in A431 Cells. Keratins are major components of intermediate filaments. In epithelial cells, cytoskeletal intermediate filaments contain type I and type II keratins. The 58 kD K5 protein is essential for formation of 8-nm intermediate filaments, disruption of which may reduce tumor invasion and metastasis (*42*−*43*). For example, disruption of intermediate filaments has been shown to inhibit the expression of integrins on the surface of tumor cells (*44*), decrease their interaction with platelets and endothelial cells (*45*), and reduce lung colonization in

Table 1: Specific 12-LOX Interactors[a]

		bait panel					
clone	sequence	12-LOX	biciod2	tailess	hairy	cdc2	Cdi D
1	keratin K5	++++	−	−	−	−	−
2	β4 integrin	+++	−	−	−	−	−
3	C8FW	++	−	−	−	−	−
9	β4 integrin	+++	−	−	−	−	−
10	lamin A	+++	±	−	−	−	−
12	C8FW	++	±	−	−	−	−
15	keratin K5	++++	−	−	−	−	−
33	keratin K5	++++	−	−	−	−	−
34	keratin K5	++++	−	−	−	−	−
35	lamin A	+++	±	−	−	−	−
37	keratin K5	++++	−	−	−	−	−
38	keratin K5	++++	−	−	−	−	−

[a] Summary of 12-LOX interactions with the panel of baits. Interaction mating was performed as described in Materials and Methods and shown in Figure 1. Level of interaction lac Z (reporter gene activation) as determined by blue color on X-Gal indicator plates: ++++ indicates dark blue, +++ blue, ++ light blue, + very light blue, ± almost white, and − white.

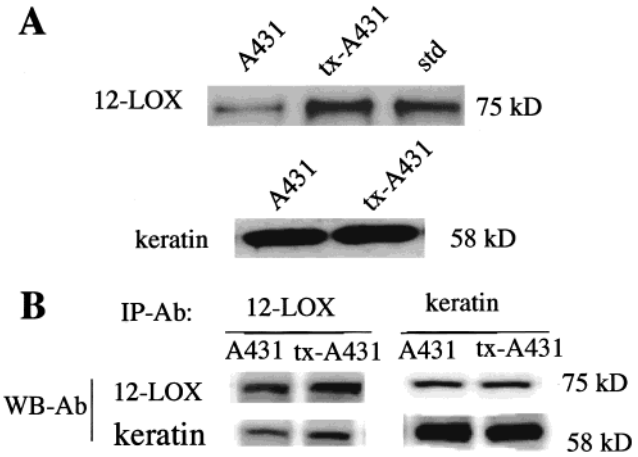

FIGURE 2: Interaction of 12-lipoxygenase and keratin in vitro. (A) Western blotting: 12-LOX (upper panel) and keratin (lower panel) protein in untransfected A431 cells (A431) and 12-LOX transfectants (tx-A431). Human recombinant platelet-type 12-LOX was used as standard (std). (B) Immunoprecipitation: 12-LOX and keratin coimmunoprecipitated from untransfected A431 cells (A431) and 12-LOX transfectant (tx-A431). Aliquots of cell lysates from either cell type were immunoprecipitated either with anti-12-LOX or anti-keratin 5 antibodies. After the samples were washed, precipitated pellets were resolved by SDS-PAGE, and proteins were detected by immunoblotting with anti-keratin 5 or anti-12-LOX antibodies. Positions of 12-LOX and keratin are indicated. The experiment was repeated three times. For each experiment, mouse, or rabbit IgG and Sepharose 4B-conjugated protein G beads alone were used as controls.

an experimental metastasis assay (*46*). Interestingly, treatment of tumor cells with a selective 12-LOX inhibitor (e.g., BHPP; see ref *47*) also has been demonstrated to inhibit integrin expression, tumor cell platelet, and tumor cell endothelial cell interactions (*48*) and experimental metastasis in vivo (*49*). To confirm the interaction of 12-LOX with keratin in human cells, we conducted immunoprecipitation assays and confocal immunofluorescent staining in A431 cells. Meanwhile, A431 cells were transfected with a pcDNA 3.1 expression construct, containing human platelet-type 12-lipoxygenase cDNA. The overexpression of 12-LOX in A431 cells was confirmed by Western Blotting (Figure 2A). 12-LOX antibody co-immunoprecipitated keratin with 12-LOX in A431 cells and transfectants. Similarly, keratin antibody

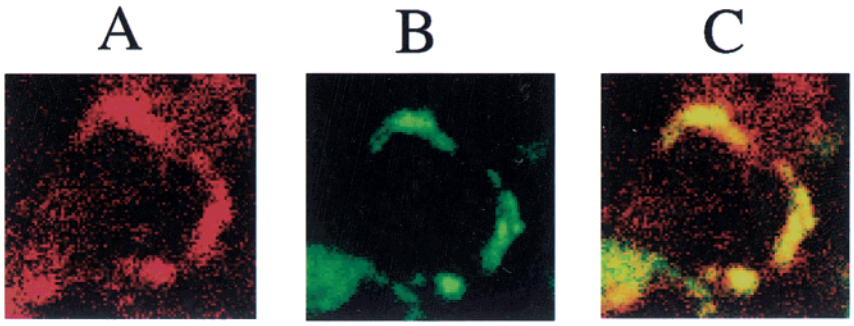

FIGURE 3: 12-LOX colocalization with keratin by laser cofocal immunofluoresence images. Cells were fixed, permeabilized, and incubated first with anti-12-LOX (A) or anti-keratin (B) antibodies, then with FITC-conjugated AffiniPure goat anti-mouse IgG (A, green) or Rhodamine Red-X-conjugated AffiniPure goat anti-rabbit IgG (B, red). Merged image in C (yellow) superimposes image from the first two images. Yellow color indicates overlap between 12-LOX (green) and keratin (red).

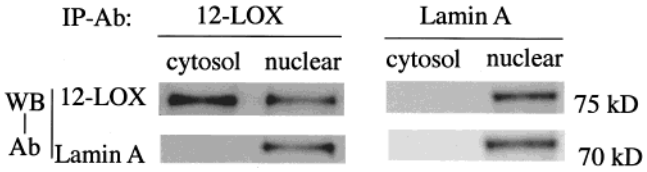

FIGURE 4: Interaction of 12-LOX and lamin A in A431 cells by immunoprecipitation assay: 12-LOX and lamin A coimmunoprecipitated from nuclear extracts of untransfected A431 cells. After the centrifugation at 25000*g*, the supernatants and pellets from 5 $\times$ 10^6 cells were designated as either subcellular fraction of cytosol or nuclear. The supernatnants and resuspended pellets were immunoprecipitated either with anti-12-LOX or anti-lamin A antibodies. After the samples were washed, precipitated pellets were resolved by SDS-PAGE and proteins were detected by immunoblotting with anti-lamin A or anti-12-LOX antibodies. Positions of 12-LOX and lamin A are indicated. The experiment was repeated three times. For each experiment, mouse or rabbit IgG and Sepharose 4B-conjugated protein G beads alone were used as controls.

co-immunoprecipitated 12-LOX with keratin in A431 cells and transfectants (Figure 2B). We also co-immunoprecipitated 12-LOX with lamin A using 12-LOX antibody and co-immunoprecipitated lamin A with 12-LOX using lamin A antibody (Figure 4). We were able to use immunofluorescent staining in A431 cells to show that 12-LOX co-localized with keratin in the cytoplasm, mostly around the cell nucleus forming a ring-like structure (in yellow), as seen in Figure 3. Combined, our results indicate that 12-LOX physically interacts with keratin in the cytoplasm and with lamin A in the nucleus of human tumor cells.

Our findings are consistent with a recent report that 12-LOX activity is detected predominantly in the particulate fractions in murine keratinocytes (*50*). These particulate fractions were found by ultrastructural analysis to contain mainly insoluble proteins such as keratin but not membrane structures (*50*). Taken together, these findings suggest that human keratin may be a novel regulator of 12-LOX activity by its effect on 12-LOX subcellular localization. The mechanism whereby keratin may affect the subcellular localization of 12-LOX is unknown. However, there are studies suggesting that intermediate filaments participate in intracellular trafficking of proteins to the plasma membrane. For example, it has been demonstrated that intermediate filaments associate and facilitate the transport of single vesicles and lipoprotein droplets in CHO, adipose and steroidogenic cells (*51*−*52*). Another study has shown that 5-LOX binding to certain cytoskeletal proteins including α-actin and that actin may mediate compartmentalization and translocation of 5-LOX in myeloid cells (*53*). Interestingly, we recently observed that the translocation of 12-LOX from cytosol to membrane upon stimulation in A431 cells was blocked after disrupting the keratin component of intermediate filaments (data not shown). It is tempting to speculate that keratin may be similarly involved in the transport of 12-LOX from the cytoplasm to a membrane-bound site. Hagmann et al. (*41*) have demonstrated 12-LOX activity in isolated nuclei, and our laboratory has demonstrated that, upon stimulation, 12-LOX associates with membrane structures and that this association results in an increase in 12-LOX activity (*36*, *41*). We are currently attempting to further characterize the interaction between keratin and 12-LOX.

In addition to keratin, we demonstrated that 12-LOX associates with lamin A, phosphoprotein C8FW, and integrin β4 subunit by yeast two-hybrid screen. Like type II keratin K5, lamin A is also an intermediate filament protein and has been identified as a component of the nuclear lamin A, a meshwork of intermediate filaments on the inner surface of nuclear membranes. The association of 12-LOX with lamin A is consistent with the finding of 12-LOX activity in isolated nuclei, as mentioned above. Since keratin and lamin A both are components of cytoskeletal intermediate filaments, we hypothesize that keratin and/or lamin A may contribute to the regulated trafficking of 12-LOX. As will be described elsewhere, we also have shown by coimmunoprecipitation and confocal immunofluorescent colocalization that the β4 integrin interacts specifically with 12-LOX in A431 and CHO cells and that this interaction increases enzymatic activity of 12-LOX with increased 12(S)-HETE production (Tang et al., unpublished observation). When the same experiment was performed and probed with antibodies to 5-LOX or 15-LOX no association between the β4 integrin and 5-LOX or 15-LOX was observed (data not shown) demonstrating the specificity of 12-LOX interaction with the β4 integrin. The β4 integrin is expressed on carcinoma cells and is linked to tumor cell motility and invasion (*54*). Another 12-LOX interactor as determined by yeast two-hybrid analysis is the phosphoprotein C8FW. The human full-length has not been sequenced and no antibody is available. Therefore, we have not yet verified its interaction with 12-LOX in human cancer cells. A similar protein, called C5FW, has been cloned from dog thyroid cells and shares 95% amino acid identity with its human counterpart. These novel proteins can be phosphorylated after mitogenic stimulation, and meanwhile, they themselves can function as a kinase (*18*). It will be interesting to further characterize the

interaction between 12-LOX and these other proteins and how they may contribute to 12-LOX regulation.

In summary, we have isolated four 12-LOX interacting proteins: type II keratin K5, Lamin A, cytoplasmic domain of β4 integrin, and phosphoprotein C8FW. Thus far we have verified that three of these (keratin, lamin A, and β4 integrin) are bona fide 12-LOX binding proteins in human cancer cells. We further demonstrated 12-LOX and keratin physically interact and colocalize around the nuclear membrane. Further characterization of the individual interaction between 12-LOX and each of these four proteins will provide insights into the regulation of 12-LOX activity. Ultimately, a clearer understanding of 12-LOX regulation may permit therapeutic intervention in tumor progression and metastasis.

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