

- 5'-triphosphate (ATP), 100 mM KCl, and 1 mM dithiothreitol (DTT). Samples were incubated at 37°C for 5 min, fixed with 0.5% glutaraldehyde, and squashed under cover slips.
- An Olympus VE-DIC equipped with Plan Apo 60×/1.4 numerical aperture DIC lens and Hamamatsu 2400 newvicon video camera was used. Images were enhanced with an Argus 20 (Hamamatsu). Centrosomes were first perfused into perfusion chamber on ice. Perfusion chamber surfaces were blocked by incubation with 5 mg/ml casein in BRB80 and washed with 0.5% NP-40 in BRB80. Microtubule assembly was initiated by perfusion of 33 μM tubulin in BRB80 containing 1 mM GTP, 1.5 mM ATP, 100 mM KCl, 1 mM DTT, and 0.5 mg/ml casein. Subsequently, tubulin with purified proteins or control buffer was perfused, and the fate of microtubules was monitored.
 - Modified from the perfusion assay described in (27). After absorption of centrosomes, blocking with casein, and washing with NP-40, 25 μM tubulin with purified

- proteins or control buffer in BRB80 containing 1 mM GTP, 1.5 mM ATP, 60 mM KCl, 1 mM DTT, 0.5% NP-40, and 0.5 mg/ml casein were perfused into the chamber on ice. The perfusion chamber was warmed to 30°C and sealed. Recording was performed at 30°C every 1 s for 10 min. Analysis of microtubule dynamics was done as described (70). Catastrophe frequency was calculated by dividing the total number of events by the duration of growth and pause phases.
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A Phosphatase Associated with Metastasis of Colorectal Cancer

Saurabh Saha, Alberto Bardelli,* Phillip Buckhaults, Victor E. Velculescu, Carlo Rago, Brad St. Croix, Katharine E. Romans, Michael A. Choti, Christoph Lengauer, Kenneth W. Kinzler,† Bert Vogelstein†

To gain insights into the molecular basis for metastasis, we compared the global gene expression profile of metastatic colorectal cancer with that of primary cancers, benign colorectal tumors, and normal colorectal epithelium. Among the genes identified, the *PRL-3* protein tyrosine phosphatase gene was of particular interest. It was expressed at high levels in each of 18 cancer metastases studied but at lower levels in nonmetastatic tumors and normal colorectal epithelium. In 3 of 12 metastases examined, multiple copies of the *PRL-3* gene were found within a small amplicon located at chromosome 8q24.3. These data suggest that the *PRL-3* gene is important for colorectal cancer metastasis and provide a new therapeutic target for these intractable lesions.

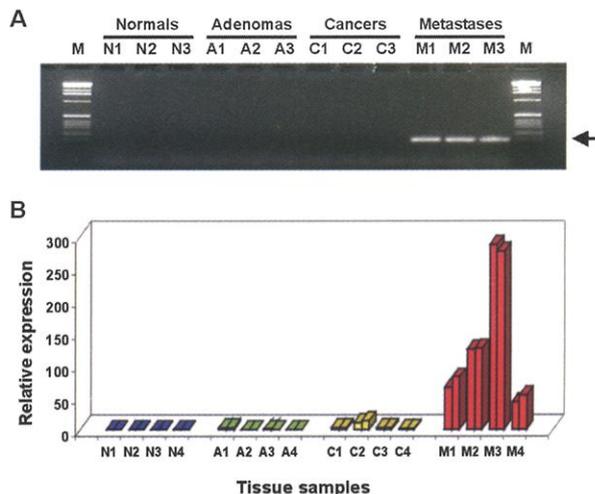
Metastasis is the neoplastic process responsible for most deaths from cancer because the primary tumors can usually be surgically removed. Metastatic cells undergo cytoskeletal changes, loss of adhesion, and enhanced motility and express proteolytic enzymes that degrade the basement membrane (1–3). However, much remains to be learned about this lethal process, and further progress is contingent upon identifying novel genes and pathways that are consistently and specifically altered in metastatic lesions.

In the case of colorectal tumorigenesis, the genes associated with initiation and progression to the invasive (cancerous) stage are well known (4). However, no gene has been shown to be consistently and specifically activated in liver metastases, the lesions that are

usually responsible for the deaths of colorectal cancer patients. To learn which genes might be involved in this process, we per-

formed global gene expression profiles of liver metastases using serial analysis of gene expression (SAGE) technology (5). We first prepared a SAGE library from microdissected metastases (6). Surprisingly, we found that many of the transcripts identified in these libraries were characteristic of normal hepatic or inflammatory cells, precluding quantitative analysis (7). To produce a more specific profile of metastatic epithelial cells, we developed an immunoaffinity fractionation procedure to purify colorectal epithelial cells from contaminating stromal and hepatic cells (8). A SAGE library was prepared from cells purified in this manner, yielding ~95,000 tags representing at least 17,324 transcripts (6). These tags were compared with ~4 million tags derived from diverse SAGE libraries, particularly those from normal and malignant (but nonmetastatic) colorectal epithelium (9). One hundred and forty-four transcripts were represented at significantly higher levels in the metastasis library than in the other libraries, while 79 transcripts were

Fig. 1. *PRL-3* expression in human colorectal tumors of different stage. The expression of *PRL-3* was evaluated by real-time PCR (8) and compared with that of the β -amyloid precursor protein (*APP*) gene, shown previously to be expressed at nearly identical levels in normal and neoplastic colorectal tissues (9). The metastases analyzed in this experiment were derived from patients other than the ones from whom the normal epithelium and other lesions were derived. Epithelial cells were purified as described (8). (A) Gel of RT-PCR products from normal colorectal epithelium (N1 to N3), adenomas (A1 to A3), primary cancers (C1 to C3), and metastases (M1 to M3). Real-time PCR was performed for 24 cycles, when RT-PCR products from the metastases were evident but before signals from the other lesions had appeared. Arrow indicates the *PRL-3* RT-PCR product of 198 bp. Lane M, molecular size markers. (B) Results are expressed as the ratio between *PRL-3* and *APP* expression and are normalized to the average expression in adenomas. Duplicates are shown for each analysis.



Howard Hughes Medical Institute, The Oncology Center, Department of Surgery, and Program in Cellular and Molecular Medicine, Johns Hopkins Medical Institutions, Baltimore, MD 21231, USA.

*On leave from the University of Torino, Institute for Cancer Research, 10060 Candiolo, Torino, Italy.
 †To whom correspondence should be addressed. E-mail: kinzlike@jhmi.edu (K.W.K.); vogelbe@welch.jhu.edu (B.V.)

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represented at significantly lower levels in the metastasis library (10).

Transcripts that were enriched rather than depleted in the metastasis library have obvious diagnostic and therapeutic potential. We therefore selected 38 of the most interesting enriched transcripts for further analysis (Table 1) (11). To confirm the SAGE data, we compared the expression of these transcripts in several microdissected metastases, primary cancers, pre-malignant adenomas, and normal epithelium by quantitative, real-time polymerase chain reaction (PCR) (8). Although all 38 of these transcripts were found to be elevated in at least a subset of the metastatic lesions tested, only one gene, *PRL-3*, was found to be consistently overexpressed.

PRL-3 (also known as *PTP4A3*) encodes a small, 22-kD tyrosine phosphatase that is lo-

cated at the cytoplasmic membrane when prenylated at its COOH-terminus and in the nucleus when it is not conjugated to this lipid (12). Among normal human adult tissues, it is expressed predominantly in muscle and heart (13). Although *PRL-3* had not been linked previously to human cancer, overexpression of *PRL-3* has been found to enhance growth of human embryonic kidney fibroblasts (13), and overexpression of *PRL-1* or *PRL-2*, close relatives of *PRL-3*, has been found to transform mouse fibroblasts and hamster pancreatic epithelial cells in culture and promote tumor growth in nude mice (14, 15). On the basis of this information, our preliminary expression data, and the importance of other phosphatases in cell signaling and neoplasia, we examined *PRL-3* in greater detail. We first investigated *PRL-3* expression in epithelial cells purified from colorectal tissues from

various stages of colorectal neoplasia using the procedure described above (8); this purification proved essential for accurate quantification of *PRL-3* expression. *PRL-3* was expressed at low levels in normal colorectal epithelium and epithelium from benign tumors (adenomas) and at intermediate levels in a subset of malignant stage I or II cancers (Fig. 1). In contrast, it was expressed at relatively high levels in each of 12 colorectal cancer metastases (examples in Fig. 1).

The metastases evaluated in Fig. 1 were not derived from the same patients from whom the earlier stage lesions were obtained. Previous analyses have revealed that expression patterns of many genes differ between individuals, and that comparisons of tissues from different patients could potentially be misleading (16). To address this concern, we purified epithelial cells from a liver metastasis

Table 1. Selected genes up-regulated in colon cancer metastasis. SAGE TAG refers to the 10-base pair (bp) sequence immediately adjacent to the *Nla* III restriction site (CATG) in each transcript. SAGE libraries were constructed from two normal colonic tissues (one library containing 48,479 tags, the other 49,610 tags), two primary colorectal cancers (one library containing 55,700 tags, the other 41,371 tags), and a colorectal metastasis (94,445 tags). Transcript description refers to the Unigene assignment of the SAGE TAG

(www.ncbi.nlm.nih.gov/SAGE/SAGetag.cgi). When tags matched two Unigene assignments, both are listed. The numbers listed in each column refer to the number of SAGE tags corresponding to the indicated gene that were observed in the library made from the indicated tissue. For example, 12 SAGE tags were observed for *PRL-3* in the SAGE library from the metastasis, but no *PRL-3* SAGE tags were observed in the other four libraries. EST, expressed sequence tag.

SAGE Tag	Normal 1	Normal 2	Cancer 1	Cancer 2	Metastasis	Description
TAGGTCAGGA	0	0	0	0	12	Protein tyrosine phosphatase type IVA (<i>PRL-3</i>)
TGAT TGGT T T	0	0	0	0	11	cDNA: FLJ23603
GGAATATGCA	0	0	0	1	18	LOC54675: hypothetical protein
AATCT TGT T T	0	0	0	0	9	Hypothetical gene ZD52F10
GTCT TCT TAA	0	0	1	0	16	DNAJ domain-containing
ATAATAAAAAG	0	0	1	1	30	GRO3 oncogene/T45117 hU1-70K protein
TAAATATGGA	0	0	0	0	7	Attractin
AT T T TTGTAT	0	0	1	0	14	Bcl-2 binding component 3
TAGCTGAAA	4	0	0	1	67	Nuclear receptor subfamily 4
GGAGGGCTGG	0	0	1	0	13	Mitogen-activated protein kinase 8 interacting protein 2/ESTs similar to S26689
GTCAGTCACT	1	0	0	0	13	Hairy (<i>Drosophila</i>) homolog
TTGAGTAGGA	1	0	1	0	23	LUC7 (<i>Saccharomyces cerevisiae</i>)-like
TGGGGCCGA	0	3	0	0	33	Transducin-like enhancer of split 2, homolog of <i>Drosophila</i> E (sp1)
TGGGCTGGGG	0	0	2	1	32	Adipose differentiation-related protein
TAGCTGGAAC	0	0	1	0	10	ESTs, no known homologies
CTTCCTTGCC	0	0	2	3	46	Keratin 17
GCGGCAGT TA	0	0	0	1	8	Casein kinase 2, alpha prime polypeptide
CTGCACT TAC	1	0	2	3	46	Minichromosome maintenance deficient 7 (<i>S. cerevisiae</i>)
AAGCTGT T TA	1	0	1	1	23	v-Jun avian sarcoma virus 17 oncogene homolog/LSFR2 gene 2/MGC2550 protein
GTGAGGGCTA	1	2	1	0	28	Plexin B1
GGGGCTGTAT	0	0	1	0	7	Transforming growth factor, beta 1 (TGF-β)
CTGGAGGCTG	1	1	0	1	20	ESTs, similar to GTP-rho binding protein 1 (rhophilin)
GGCTGGGT T T	1	1	1	5	42	H2.0 (<i>Drosophila</i>)-like homeobox 1
GGGGGTGGGT	1	0	0	1	10	Mago-nashi (<i>Drosophila</i>) homolog, proliferation-associated
CAGCATCTAA	1	1	1	1	19	Putative Rab5-interacting protein
T T TCCAATCT	0	0	0	4	18	Vascular endothelial growth factor
T T TCTAGGGG	0	1	2	2	22	PTD008 protein
AAAGTGAAGA	1	0	3	5	37	FLJ11328 protein/ribosomal protein L10
T T TGCAC T TG	3	1	0	0	15	wee1+ (<i>Schizosaccharomyces pombe</i>) homolog/protein x 013
CCTGGAATGA	1	0	0	3	15	cDNA: FLJ12683
GGAGGTAGGG	1	0	3	0	15	PTK7 protein tyrosine kinase 7
CTGTACT TGT	9	5	0	0	52	v-fos FBJ murine osteosarcoma viral oncogene homolog B
TCCT TGCT TC	1	1	3	1	22	FLJ20297 protein
TATCTGTCTA	0	0	2	5	25	SET translocation (myeloid leukemia-associated)
T TAGATAAGC	0	0	3	8	33	Chaperonin-containing TCP1, subunit 6A (zeta 1)
AGTGGAGGGA	1	0	1	1	9	Ataxin 2 related protein
TGCAGATATT	0	0	2	1	8	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual-specificity phosphatase)
GGGAGGGGTG	1	1	2	2	14	Matrix metalloproteinase 14 (membrane-inserted)

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sis, the advanced (stage III) colorectal cancer giving rise to the metastasis, and normal colorectal epithelium from each of six different patients. There was little or no expression in the normal epithelium from these cases, intermediate expression in the advanced primary cancers, and significantly higher expression in each of the matched metastatic lesions (Fig. 2).

The observation that *PRL-3* is expressed at relatively high levels in metastases is consistent with its playing a causative role in metastasis. However, the most definitive way to implicate a gene in human cancer is to identify genetic alterations of that gene (17). We therefore determined whether *PRL-3* was genetically altered through gene amplifica-

tion, a well-known mechanism for increasing the expression of growth-regulating genes in human cancers (18). Through radiation hybrid and syntenic mapping, we found that the *PRL-3* gene was located ~3 Mb from the telomere of chromosome 8q, at a position corresponding to chromosomal band 8q24.3 (19). Real-time PCR analyses of genomic DNA prepared from the purified epithelial cells of 12 metastatic lesions, each from a different patient, was performed to determine *PRL-3* gene content; as with expression analyses, such purification proved critical for reliable quantification. In each case, we determined the genomic content of *PRL-3* sequences relative to that of a sequence near the centromere of chromosome 8q (8). This com-

parison allowed us to distinguish true amplification from simple increases in chromosome number due to aneuploidy (18). We found that 3 of the 12 metastases studied exhibited amplification, to levels of ~25 copies, 26 copies, and 37 copies per diploid genome.

We next compared the genomic content of sequences distributed throughout chromosome 8q to define those that were amplified in these three metastases (8). The availability of the nearly complete human genomic sequence considerably facilitated this mapping effort (20, 21). Although the *PRL-3* gene was not found in public databases generated through the Human Genome Project, it was found on a Celera scaffold located ~145 Mb from the telomere of chromosome 8p and ~3 Mb from the telomere of chromosome 8q, consistent with our radiation hybrid mapping data (19, 22) (Fig. 3). In all cases, amplification was confined to a very small region of chromosome 8q that included *PRL-3* (Fig. 3) (22). The only known or predicted genes within this small amplicon were *PRL-3* and a hypothetical gene homologous to a TATA-binding protein. No expression of the latter gene was detectable in metastatic lesions when assessed by reverse transcription (RT)-PCR. However, the region of the Celera scaffold on which *PRL-3* maps (22) contains gaps estimated to be <20 kb in size. If these gaps were in fact larger than expected, the total size of the amplicon would be larger than the 100 kb indicated in Fig. 3 and could contain additional genes.

In summary, we have identified a gene encoding a tyrosine phosphatase that is consistently overexpressed in metastatic colorec-

Fig. 2. *PRL-3* expression in matched metastases and advanced primary cancers. Colonic epithelial cells were purified from a liver metastasis, a primary cancer giving rise to the metastasis, and normal colorectal epithelium from each of six patients. (A) Gel of RT-PCR products from matched normal, primary cancer, and metastasis from four patients. Real-time PCR was performed for 24 cycles, when RT-PCR products from the metastases were evident but before signals from the other lesions had appeared. Arrow indicates the *PRL-3* RT-PCR product of 198 bp. Lane M, molecular size markers. (B) Relative levels of expression were determined as in Fig. 1.

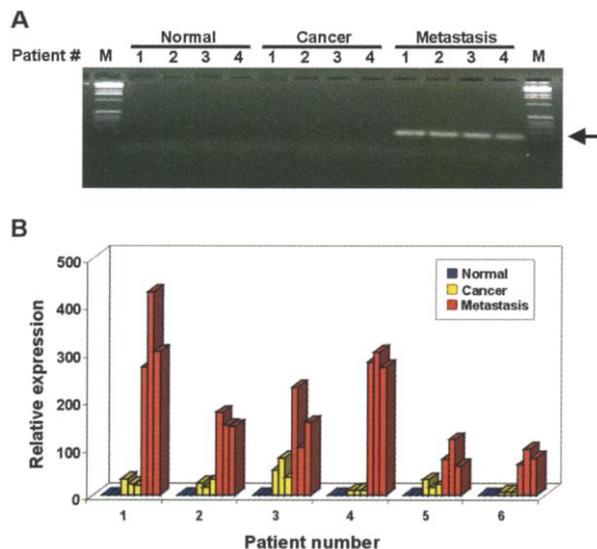
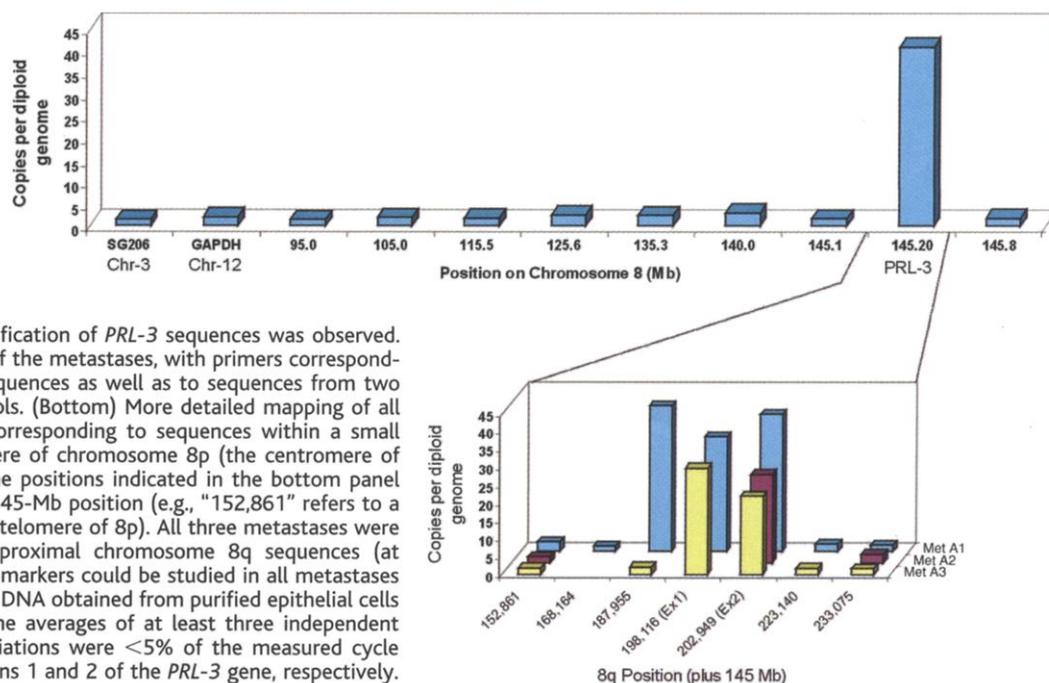


Fig. 3. Amplicon mapping of *PRL-3*. The number of copies of sequences corresponding to each of the indicated loci was determined by real-time PCR. In each case, the number of copies per diploid genome was determined by comparison with equal amounts of DNA from normal cells (8). Genomic DNA from purified epithelial cells of metastases from three different patients was used for these experiments; in another nine patients, no amplification of *PRL-3* sequences was observed. (Top) Results obtained from one of the metastases, with primers corresponding to several chromosome 8q sequences as well as to sequences from two other chromosome arms as controls. (Bottom) More detailed mapping of all three metastases, with primers corresponding to sequences within a small region ~145 Mb from the telomere of chromosome 8p (the centromere of chromosome 8 is at ~45 Mb). The positions indicated in the bottom panel refer to nucleotides distal to the 145-Mb position (e.g., "152,861" refers to a position 145,152,861 bp from the telomere of 8p). All three metastases were shown to lack amplification of proximal chromosome 8q sequences (at positions 95 and 135 Mb). Not all markers could be studied in all metastases because of the limited amounts of DNA obtained from purified epithelial cells of these lesions. Bars represent the averages of at least three independent determinations, and standard deviations were <5% of the measured cycle numbers. Ex1 and Ex2 refer to exons 1 and 2 of the *PRL-3* gene, respectively.



tal cancers. The confinement of the gene to a small amplicon in a subset of these tumors provides important, complementary evidence for the role of this gene in metastasis; the major genes previously shown to be amplified in naturally occurring cancers are all oncogenes (18, 23, 24). Extra copies of chromosome 8q DNA sequences have been observed in the advanced stages of many different tumor types, including advanced colon cancers (25–28). It has been suggested that the *c-MYC* gene on chromosome 8q24.12 is the target of such 8q overrepresentation. In the three metastatic lesions we examined, *c-MYC* was not amplified and was in fact located ~14 Mb from the boundaries of the *PRL-3* amplicon. It will, therefore, be of interest to evaluate the expression and genomic representation of *PRL-3* in the metastases of other cancer types.

Further experiments will be required to determine the biochemical mechanisms through which *PRL-3* influences neoplastic growth and to establish its causative role in the metastatic process. However, one of the most important ramifications of the work described here concerns its potential therapeutic implications. Most of the previously described genetic alterations in colorectal cancers involve inactivation of tumor suppressor genes. The proteins produced from these genes are difficult to target with drugs, because they are inactive or absent in the cancer cells (29). In contrast, enzymes whose expression is elevated in cancer cells, like that encoded by *PRL-3*, provide excellent targets for drug discovery purposes.

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7. Examples of the nonepithelial transcripts identified in the initial libraries are vitronectin and lysozyme. These genes are transcribed in stromal cells and accounted for 0.4 and 0.1%, respectively, of the tags in SAGE libraries prepared from unpurified metastatic lesions but were not found in the SAGE library derived from purified metastatic epithelial cells. Similarly, transcripts made in hepatocytes, like apolipoprotein C-III, accounted for 0.3% of the tags from unpurified metastasis libraries but were not found in the libraries from purified cells. These differences in vitronectin, lysozyme, and apolipoprotein C-III expression among SAGE libraries derived from purified and unpurified epithelial cells were statistically significant ($P < 0.0001$, χ^2 test).
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rectal epithelial libraries (30). Monte Carlo simulations were used to identify transcripts that were expressed in the metastasis library at levels 10-fold higher or 10-fold lower than in the other libraries, with P -chance < 0.0001 , as described (31). A complete listing of the SAGE tags and corresponding transcripts identified in this study is available at www.sagenet.org.

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32. Supported by the National Colorectal Cancer Research Alliance and NIH grants CA57345, CA 62924, and CA43460. K.W.K. received research funding from Genzyme Molecular Oncology (Genzyme). Under a licensing agreement between the Johns Hopkins University and Genzyme, the SAGE technology was licensed to Genzyme for commercial purposes, and B.V., K.W.K., and V.E.V. are entitled to a share of the royalties received by the university from the sales of the licensed technology. B.V., K.W.K., B.S.C., and V.E.V. are consultants to Genzyme. The university and researchers (B.V., K.W.K., V.E.V.) own Genzyme stock, which is subject to certain restrictions under university policy. The terms of these arrangements are being managed by the university in accordance with its conflict-of-interest policies. The SAGE technology is freely available to academia for research purposes.

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Kinetic Stabilization of the α -Synuclein Protofibril by a Dopamine- α -Synuclein Adduct

Kelly A. Conway,* Jean-Christophe Rochet,* Robert M. Bieganski, Peter T. Lansbury Jr.†

The substantia nigra in Parkinson's disease (PD) is depleted of dopaminergic neurons and contains fibrillar Lewy bodies comprising primarily α -synuclein. We screened a library to identify drug-like molecules to probe the relation between neurodegeneration and α -synuclein fibrilization. All but one of 15 fibril inhibitors were catecholamines related to dopamine. The inhibitory activity of dopamine depended on its oxidative ligation to α -synuclein and was selective for the protofibril-to-fibril conversion, causing accumulation of the α -synuclein protofibril. Adduct formation provides an explanation for the dopaminergic selectivity of α -synuclein-associated neurotoxicity in PD and has implications for current and future PD therapeutic and diagnostic strategies.

A central role for α -synuclein fibrilization in PD (1, 2) is supported by the fact that fibrillar α -synuclein is a major component of Lewy bodies (3, 4) and that the α -synuclein gene has been linked to autosomal dominant PD (FPD) (5, 6). Although both FPD α -synuclein mutations [Ala⁵³ → Thr (A53T) and Ala³⁰ → Pro (A30P)] accelerate the formation of nonfibrillar,

oligomeric protofibrils in vitro, A30P inhibits the conversion of protofibrils to fibrils (7). This finding suggests that protofibrils may be pathogenic and fibrils inert, or even protective (2). Transgenic mice that express wild-type human α -synuclein (8) exhibit PD-like dopaminergic deficits and motor dysfunction, and they develop neuronal inclusions that, in contrast to those