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Experimental Neurology 191 (2005) S45–S59

Experimental Neurology

www.elsevier.com/locate/yexnr

Truncated N-terminal mutants of SV40 large T antigen as minimal immortalizing agents for CNS cells

William J. Freed*, Peisu Zhang, Joseph F. Sanchez, Ora Dillon-Carter, Mark Coggiano, Stacie L. Errico, Brian D. Lewis, Mary Ellen Truckenmiller

Cellular Neurobiology Research Branch, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, Baltimore, MD 21224, USA

Received 12 April 2004

Abstract

Immortalized central nervous system (CNS) cell lines are useful as in vitro models for innumerable purposes such as elucidating biochemical pathways, studies of effects of drugs, and ultimately, such cells may also be useful for neural transplantation. The SV40 large T (LT) oncoprotein, commonly used for immortalization, interacts with several cell cycle regulatory factors, including binding and inactivating p53 and retinoblastoma family cell-cycle regulators. In an attempt to define the minimal requirements of SV40 T antigen for immortalizing cells of CNS origin, we constructed T155c, encoding the N-terminal 155 amino acids of LT. The p53 binding region is known to reside in the C-terminal region of LT. An additional series of mutants was produced to further narrow the molecular targets for immortalization, and plasmid vectors were constructed for each. In a p53 temperature sensitive cell line model, T64-7B, expression of T155c and all constructs having mutations outside of the first 82 amino acids were capable of overriding cell-cycle block at the non-permissive growth temperature. Several cell lines were produced from fetal rat mesencephalic and cerebral cortical cultures using the T155c construct. The E107K construct contained a mutation in the Rb binding region, but was nonetheless capable of overcoming cell cycle block in T64-7B cell and immortalizing primary cultured cells. Cells immortalized with T155c were often highly dependent on the presence of bFGF for growth. Telomerase activity, telomere length, growth rates, and integrity of the p53 gene in cells immortalized with T155c did not change over 100 population doublings in culture, indicating that cells immortalized with T155c were generally stable during long periods of continuous culture.
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Keywords: Immortalization; SV40 large T antigen; T155g; T155c; Plasmid; Telomerase; Telomere; Growth arrest; T64-7b; Cell line; Cell cycle; Oncogene

Introduction

Cell lines are very useful for many applications in cell biology, ranging from in vitro studies of viral propagation, developmental mechanisms, and drug effects, to potential clinical uses in transplantation. The concept of a cell line, a homogeneous cell population which can be indefinitely propagated in culture is, however, at odds with the normal development and function of an organism. Normal somatic cells exert lateral influences upon their neighbors to adopt different fates, and most cells in the mature organism are not continuously cycling. Thus, a homogeneous and perpetual population of identical somatic vertebrate cells does not normally exist in nature, except in the aberrant situation where mutations accumulate to produce tumors (e.g., Nowak et al., 2002). Thus, the creation of such cells requires either intervention or extraction from tumors.

Although tumor cell lines are valuable as in vitro models, for example, PC12 cells, SH-SY5Y cells and many others, tumor cells have abnormalities, are usually unstable, and of course are not useful for transplantation. Numerous cell lines have been created by the introduction of oncogenes

* Corresponding author. Cellular Neurobiology Research Branch, NIDA Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Department of Health and Human Services, 5500 Nathan Shock Drive, Baltimore MD 21224. Fax: +1 410 550 1621.
E-mail address: wfreed@intra.nida.nih.gov (W.J. Freed).

0014-4886/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.expneurol.2004.08.019

into primary cultures, and these cells are not tumorigenic, at least in some cases, in animals (e.g., Anton et al., 1994; Gray et al., 2000; Isono et al., 1992; Lundberg et al., 2002; Okoye et al., 1994; Whittemore and Onifer, 2000). It is widely recognized that tumor formation requires a number of mutational events, and some viral oncogenes apparently have the capacity to stimulate cell division without entirely converting the target cells into tumor cells. The concept of producing cell lines in the laboratory for the ultimate purpose of transplantation into human subjects is, therefore, an attempt to create cells which behave like tumor cells in some but not all respects.

In concept, a transplantable cell line would have the capacity for context-dependent unlimited growth, while under other contexts retaining the capacity for integration into an organism as a normal somatic cell. There are a number of strategies through which this might be achieved. One important element of this aim is to alter a cell sufficiently to allow for unlimited growth in vitro, while minimally interfering with normal cellular functions. Thus, the purpose of the present experiment is solely to focus on properties of the oncogene itself, to explore the minimum requirements for obtaining unlimited growth of cell lines derived from primary CNS rodent primary cultures.

One of the most commonly used immortalizing agents is the product of the simian virus 40 large tumor oncogene, SV40 large T (LT) (Bryan and Reddel, 1994; Cepko, 1989, 1992; Snyder, 1995). Cells immortalized with LT, either wild type or temperature sensitive variants, typically are not tumorigenic after transplantation into the brain (Giordano et al., 1996; Isono et al., 1992; Okoye et al., 1994, 1995; Renfranz et al., 1991), but are often phenotypically immature and genetically abnormal (Cepko, 1992); reviewed in Bryan and Reddel (1994).

In previous experiments, we identified T155, now called T155g, which is a 155-amino acid N-terminal fragment of SV40 large T antigen which also encodes the small t antigen. This agent was able to immortalize primary cells from rat mesencephalon (Truckenmiller et al., 1998), and cells produced by T155g were stable over long-term culture (Dillon-Carter et al., 2002; Truckenmiller et al., 2002). The entire p53 binding region, which may promote chromosomal rearrangements and prevent apoptosis, is deleted from T155g. In the present study, we further explored oncogene fragments derived from T155g to identify a minimal immortalizing agent for primary rodent CNS cultures.

Methods

Construction of T155c plasmid vector

To construct a plasmid containing the cDNA sequence for the truncated SV40 large T gene, a fragment containing the coding sequence for the first 155 amino acids of the T antigen was generated by polymerase chain reaction (PCR) using the A7 cell line (Geller and Dubois-Dalcq, 1988) as a template. The A7 cell line contains the full-length cDNA of SV40 large T integrated into its genomic DNA. The PCR oligonucleotide primers, custom synthesized by Gibco Life Technologies (Gaithersburg, MD), were SV40-5226 (5′tattccagaagtagtgaggagg) and SV40-4352-Not (5′cgcgcgaagcttaatgttctattactaaacacagcatgctc). The latter primer contains an added stop codon and *Not*1 restriction endonuclease site at the 5′ end. PCR amplification was carried out using Perkin-Elmer GeneAmp reagents (Foster City, CA). The amplification conditions were: 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s The PCR products were agarose gel purified using kit reagents from Qiagen Inc, (Chatsworth, CA), and ligated into a pCRII TA cloning vector (Invitrogen, San Diego, CA). The insert was isolated from positive colonies by *Hin*dIII and *Not*1 digestion and subcloned into a pRc/RSV expression vector (Invitrogen), which includes the Rous sarcoma virus LTR to drive expression and the neomycin resistance gene for selection. The region of the plasmid insert was sequenced in both directions to verify the T155 cDNA sequence (Johns Hopkins DNA Sequencing Facility, Baltimore MD).

Construction of mutant T155c vectors

A series of deletion and substitution mutants of the T155c plasmid were generated by site-directed mutagenesis (Fig. 1) using a two-side overlap extension PCR method (McPherson, 1991). The pRc/RSV/T155c plasmid was used as the template. The PCR primers for each mutant were two complementary mutagenic primers and two flanking primers (PREP forward primer and pcDNA3.1/BGH reverse primer, Invitrogen). The mutagenesis was accomplished by combining each mutagenic primer with a flanking primer and carrying out the first two PCR reactions (95°C for 2 min, then 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 68°C for 30 s, followed by a final elongation step at 72°C for 5 min). The resulting two PCR products were combined and annealed, and a third PCR reaction was carried out with only the two flanking primers. The final PCR product, which contained the mutation or deletion, was cloned into a PCR TA vector (Invitrogen), and then shuttled into pRc/RSV/T155c vector, replacing the T155c insert. The inserts of each mutant plasmid were sequenced in both forward and reverse directions to confirm the success of the mutagenesis (Johns Hopkins DNA Sequencing Facility).

Cell cultures

T64-7B cells were provided by Dr. Arnold J. Levine, Princeton University. These cells were derived from rat embryo fibroblasts transformed by activated ras and a temperature-sensitive mutant of p53 (Martinez et al., 1991; Michalovitz et al., 1990). The cells actively divide at 37–

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