contributes to βARK translocation, the receptor also participates in the membrane association of this enzyme. The enhanced rate of βARK-mediated receptor phosphorylation observed in the presence of βγ would thus appear to be a consequence of the membrane localization of the kinase through formation of a receptor–βARK-βγ ternary complex.

Unlike receptor phosphorylation, the receptor-facilitated membrane localization of the βARK-βγ complex was stimulus independent (Fig. 5, A and B). Thus, the βARK-βγ complex binds to either activated or inactivated receptor. In vivo, G proteins undergo a cycle of guanine nucleotide exchange during which they exist in two distinct states, an inactive conformation in which $G_{\alpha\text{-guanosine diphosphate}}$ ($G_{\alpha\text{-GDP}}$) is complexed to βγ, and an active state in which $G_{\alpha\text{-guanosine triphosphate}}$ ($G_{\alpha\text{-GTP}}$) subunits, capable of interacting with and activating various effectors (*29*), are dissociated from βγ. Because the exchange of GDP for GTP is stimulated by the ligand-activated receptor, uncomplexed βγ subunits should be available in vivo only in the presence of agonist-occupied receptor. Thus, in this sense, translocation of βARK through formation of the βARK-βγ–receptor complex is predicted to be agonist dependent in vivo. Indeed, addition of α subunits to βγ, a condition that favors formation of the heterotrimeric G protein, inhibited the enhancement of βARK-mediated receptor phosphorylation observed on addition of βγ alone.

RK, unlike βARK, ends with a CAAX sequence and is farnesylated and carboxylmethylated in vivo. Furthermore, the posttranslationally modified form of this enzyme is about four times as active in phosphorylating rhodopsin as its unfarnesylated counterpart (*26*). The enhanced rate of rhodopsin phosphorylation accompanying farnesylation is caused by light-dependent translocation of RK to rhodopsin-containing membranes (*30*). Both βARK and RK thus appear to translocate to membranes in a prenylation-dependent fashion.

βγ subunits have been implicated in regulation of a K^+ channel (*31*) and activation of phospholipase A2 (*32*). βγ also stimulates type II adenylyl cyclase in the presence of activated α_s, thus acting as a conditional activator of cAMP synthesis (*33*). The results presented in this study suggest another role of the βγ dimer, the enhancement of agonist-stimulated receptor phosphorylation and desensitization.

REFERENCES AND NOTES

1. J. L. Benovic, M. Bouvier, M. G. Caron, R. J. Lefkowitz, *Annu. Rev. Cell. Biol.* **4**, 405 (1988).
2. H. Kuhn, *Prog. Retinal Res.* **3**, 123 (1984); H. G. Dohlman, J. Thorner, M. G. Caron, R. J. Lefkowitz, *Annu. Rev. Biochem.* **60**, 653 (1991).
3. J. L. Benovic, R. H. Strasser, M. G. Caron, R. L. Lefkowitz, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2797 (1986).
4. U. Wilden, S. W. Hall, H. Kuhn, *ibid.*, p. 1174.
5. M. J. Lohse, M. G. Caron, R. J. Lefkowitz, J. L. Benovic, *ibid.* **86**, 3011 (1989).
6. J. L. Benovic *et al.*, *J. Biol. Chem.* **262**, 17251 (1987).
7. M. M. Kwatra, J. L. Benovic, M. G. Caron, R. J. Lefkowitz, M. M. Hosey, *Biochemistry* **28**, 4543 (1989).
8. J. L. Benovic, F. Mayor, R. L. Somers, M. G. Caron, R. J. Lefkowitz, *Nature* **322**, 869 (1986).
9. R. H. Strasser, J. L. Benovic, M. G. Caron, R. J. Lefkowitz, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6362 (1986); H. Kuhn, *Biochemistry* **21**, 4389 (1978).
10. K. Haga and T. Haga, *FEBS Lett.* **268**, 43 (1990); *J. Biol. Chem.* **267**, 2222 (1992).
11. J. L. Benovic, R. G. L. Shorr, M. G. Caron, R. J. Lefkowitz, *Biochemistry* **23**, 4510 (1984).
12. J. L. Benovic, A. DeBlasi, W. C. Stone, M. G. Caron, R. J. Lefkowitz, *Science* **246**, 235 (1989).
13. R. A. Cerione *et al.*, *Biochemistry* **23**, 4519 (1984).
14. P. J. Casey, M. P. Graziano, A. G. Gilman, *ibid.* **28**, 611 (1989).
15. W. J. Phillips, S. Trukawinski, R. A. Cerione, *J. Biol. Chem.* **264**, 16679 (1989).
16. P. Gierschik, C. Simons, C. Woodward, R. Somers, A. M. Spiegal, *FEBS Lett.* **172**, 321 (1984).
17. J. J. Onorato *et al.*, *Biochemistry* **30**, 5118 (1991).
18. J. A. Pitcher, unpublished data.
19. D. J. Kelleher and G. L. Johnson, *Mol. Pharmacol.* **34**, 452 (1988).
20. W. L. Lorenz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8715 (1991).
21. D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988).
22. N. Gautam, M. Baetscher, R. Aebersold, M. I. Simon, *Science* **244**, 971 (1989); J. D. Robishaw, V. K. Kalman, C. R. Moomaw, C. A. Slaughter, *J. Biol. Chem.* **264**, 15758 (1989); J. B. Hurley, H. K. Fong, D. B. Teplow, W. J. Dreyer, M. I. Simon, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 6948 (1984); N. Gautam, J. Northup, H. Tamir, M. I. Simon, *ibid.* **87**, 7973 (1990).
23. R. K. Lai, D. Perez-Sala, F. J. Canada, R. R. Rando, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7673 (1990); Y. Fukada *et al.*, *Nature* **346**, 658 (1990).
24. H. K. Yamane *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5868 (1990); S. M. Mumby, P. J. Casey, A. G. Gilman, S. Gutowski, P. C. Sternweis, *ibid.*, p. 5873.
25. W. F. Simonds, J. E. Butrynski, N. Gautam, C. G. Unson, A. M. Spiegel, *J. Biol. Chem.* **266**, 5363 (1991).
26. J. Inglese, J. F. Glickman, W. Lorenz, M. G. Caron, R. J. Lefkowitz, *ibid.* **267**, 1422 (1992).
27. U. Wilden and H. Kuhn, *Biochemistry* **21**, 3014 (1982); H. Shichi and R. L. Somers, *J. Biol. Chem.* **253**, 7040 (1978).
28. J. A. Pitcher, unpublished data.
29. L. Birnbaumer, *Annu. Rev. Pharmacol. Toxicol.* **30**, 675 (1990).
30. J. Inglese, W. J. Koch, M. G. Caron, R. J. Lefkowitz, *Nature*, in press.
31. D. E. Logothetis, Y. Kurachi, J. Galper, E. J. Neer, D. E. Clapham, *ibid.* **325**, 321 (1987).
32. C. L. Jelsema and J. Axelrod, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3623 (1987).
33. W.-J. Tang and A. G. Gilman, *Science* **254**, 1500 (1991); A. D. Federman, B. R. Conklin, K. A. Schrader, R. R. Reed, H. R. Bourne, *Nature* **356**, 159 (1992).

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Requirement for the Adenovirus Type 9 *E4* Region in Production of Mammary Tumors

Ronald Javier,* Karel Raska, Jr., Thomas Shenk†

Oncogenic viruses demonstrating a strict tropism for the mammary gland provide special opportunities to study the susceptibility of this tissue to neoplasia. In rats, human adenovirus type 9 (Ad9) elicits mammary fibroadenomas that are similar to common breast tumors in women, as well as phyllodes-like tumors and mammary sarcomas. By constructing recombinant adenoviruses between Ad9 and Ad26 (a related nontumorigenic virus), it was shown that the Ad9 *E4* region was absolutely required to produce these mammary tumors. This indicates that an adenovirus gene located outside the classic transforming region (*E1*) can significantly influence the in vivo oncogenicity of an adenovirus. Consistent with a direct role in mammary gland oncogenesis, the Ad9 *E4* region also exhibited transforming properties in vitro. Therefore, the Ad9 *E4* region is a viral oncogene specifically involved in mammary gland tumorigenesis.

Human adenoviruses are classified as DNA tumor viruses because of their ability to induce tumors in rodents or to transform rodent cells in culture, and the E1 region (*E1A* and *E1B* genes) encodes the proteins responsible for the oncogenic properties of these viruses (*1*). The E1A proteins alone are capable of immortalizing primary rodent cells in culture (*2*) and, in cooperation with the E1B proteins (*3*), produce fully transformed cells. The transforming properties of these viral oncoproteins result, at least in part, from an ability to complex with important cellular proteins (*4–8*). Such complexes between viral oncoproteins and cellular proteins are believed to perturb the normal functions of the targeted host proteins, most of which appear to regulate important control points of the cell cycle.

Among the oncogenic adenoviruses, the subgroup D adenovirus Ad9 is unique because it elicits exclusively estrogen-depen-

dent mammary tumors in female rats (9–11). In contrast, a typical oncogenic adenovirus produces sarcomatous tumors at the site of virus inoculation in both male and female animals (12). These observations suggest that Ad9 encodes a novel protein that targets tumorigenesis to the mammary gland.

The unique oncogenicity of Ad9 is mirrored by an unusual pattern of viral gene expression in mammary tumors: Ad9 E1A mRNA expression, but not Ad9 E1B mRNA expression, is detected in the tumors (10). Because two adenovirus oncogenes are normally required to transform a normal cell, it seemed possible that, in order to produce a mammary tumor, another Ad9 gene besides *E1A* may be necessary to augment or substitute for the presumed deficiency of E1B function in the cells. The work presented below was initiated to identify such a viral gene.

In contrast to Ad9, most subgroup D viruses are not able to produce tumors of any type in Wistar/Furth (W/Fu) rats. Specifically, among eight selected subgroup D viruses, two (Ad9 and Ad10) produced mammary tumors in female animals, whereas six others (Ad15, Ad17, Ad24, Ad25, Ad26, and Ad30) did not (13). This provided an opportunity to construct recombinant viruses between Ad9 and one of these nononcogenic subgroup D viruses because members of this subgroup share greater than 90% DNA homology (14).

We arbitrarily chose Ad26 as the nononcogenic partner and initially constructed four different recombinant viruses between Ad9 and Ad26 with the use of the overlap recombination technique (15). In addition, because these recombinant viruses were assembled entirely from cloned viral DNA (16), we also generated Ad9 and Ad26 viruses from cloned DNA (17) as appropriate controls for the recombinant viruses (Fig. 1). The viruses were then amplified to high titer and inoculated into female W/Fu rats.

Mammary tumor induction by virus AC-9 immediately established that the oncogenic difference between Ad9 and Ad26 mapped outside the left 7.5% of the genome (*E1A* and part of *E1B*), whereas tumor induction by virus AB-9 suggested that the difference more precisely localized to the right 30% of the viral genome (Fig. 1). The nontumorigenic phenotype of the recombinant viruses with reciprocal structures (AB-26 and AC-26) provided additional support for this conclusion. Therefore, in contrast to previous studies with human adenoviruses that identified the left 10% of the genome as the only region essential for oncogenicity (1), the results presented here suggest that a gene encoded on the right 30% of the Ad9 genome was critical for mammary gland oncogenesis.

The right 30% of the adenovirus genome contains four candidate genes (*L4*, *E3*, *L5*, and *E4*) that could be necessary for Ad9 tumorigenesis (Fig. 2A). In order to determine which of these right-end–encoded viral genes was essential for mammary tumor production, we constructed six additional recombinant viruses between Ad9 and Ad26 (18). The left 70% of these viral genomes consisted of Ad26 DNA, and the

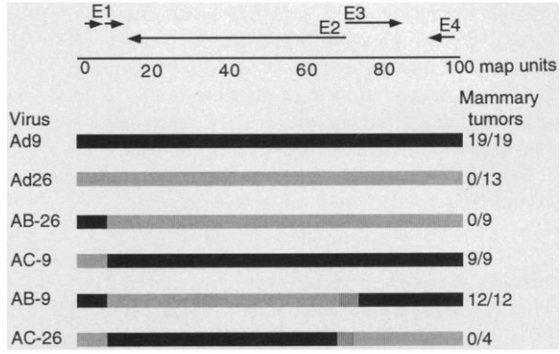

Fig. 1. The genomic structures and mammary gland oncogenicities of Ad9 and Ad26 plasmid-generated viruses (17) and first-generation recombinant viruses between Ad9 and Ad26 (16). The adenovirus genome and its early transcription units are shown above the viral genomic structures. Black areas represent Ad9 DNA and gray areas represent Ad26 DNA. Vertical-lined regions denote where the DNA origin (Ad9 DNA versus Ad26 DNA) is uncertain. To assay oncogenicity, we obtained near-term pregnant W/Fu rats from Sprague-Dawley (Indianapolis, Indiana), and newborn rats (24 to 48 hours old) were injected subcutaneously over both the left and right shoulders with a total of 5×10^7 plaque-forming units (PFU) of virus in 0.4 ml of Dulbecco's modified Eagle medium supplemented with 10% calf serum. At 4 to 6 weeks old, infected juvenile rats were weaned and separated by sex. The formation of mammary tumors in these animals was monitored by palpation and visual inspection for 7 months to 1 year. Mammary tumors began to arise when the rats were approximately 3 months old and by 5 months old most tumors were detectable.

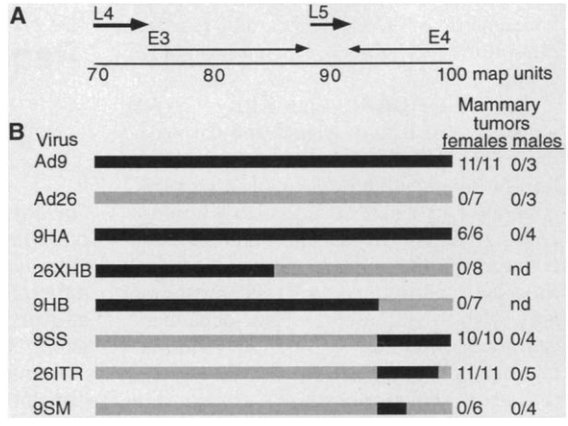

Fig. 2. (**A**) The right-end 30% of the adenovirus genome and transcription units contained therein. (**B**) The genomic structures and mammary gland oncogenicities of Ad9 and Ad26 plasmid-generated viruses (17) and second generation recombinant viruses between Ad9 and Ad26 (18). Except for Ad9, the viruses shown have Ad26 DNA for the left genomic 70%, which is not shown. Black areas represent Ad9 DNA and gray areas represent Ad26 DNA. Tumorigenicity was determined as described in Fig. 1.

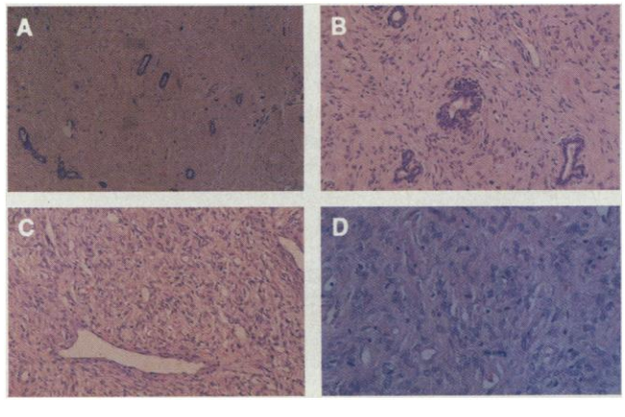

Fig. 3. Histological analyses of mammary tumors induced by recombinant virus 9SS. (**A**) Mammary fibroadenoma. (**B**) Benign phyllodes-like tumor. (**C**) Malignant phyllodes tumor. (**D**) Solid sarcoma. The sarcoma is displayed at a higher magnification than the other tumors to facilitate observation of mitotic cells characteristic of the tumor.

R. Javier and T. Shenk, Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, NJ 08544.
K. Raska, Jr., Department of Pathology, University of Medicine and Dentistry of New Jersey—Robert Wood Johnson Medical School, and St. Peter's Medical Center, New Brunswick, NJ 08903.

*Present address: Division of Molecular Virology, Baylor College of Medicine, Houston, TX 77030.
†To whom correspondence should be addressed.

remaining right 30% consisted of varying amounts of Ad9 and Ad26 DNA (Fig. 2B).

These experiments demonstrated that both the 9SS and 26ITR viruses elicited mammary tumors. The genome of virus 9SS consisted almost entirely of Ad26 DNA except that the extreme right 2300 bp were replaced with Ad9 sequences, whereas the genome of virus 26ITR was identical to virus 9SS except that the extreme right 300 bp were derived from Ad26 sequences (Fig. 2B). These results indicated that the Ad9 function essential for mammary gland oncogenesis was entirely encoded within the Ad9 *E4* region. Consistent with an oncogenic role, Ad9 *E4* region mRNA is expressed in all Ad9-induced mammary tumors (*10*). The nontumorigenic behavior of viruses 26XHB and 9HB, which possess entirely Ad26-derived *E4* regions, strengthened the conclusion that the *E4* region encoded the essential right-end function. In addition, because histological examination of the tumors elicited by virus 9SS (Fig. 3) and 26ITR (*19*) showed them to be identical to Ad9-induced mammary tumors, it could be concluded that the oncogenic properties of Ad9 were completely transferred to Ad26 by these Ad9 *E4* region sequences.

Conceivably, the essential E4 gene product could function to facilitate the spread of Ad9 virus in test animals from the site of inoculation to the target tissue. However, because Ad9 E1B mRNA is not detected in mammary tumors (*10*), we preferred the idea that the *E4* region functioned as a viral oncogene that either supplements or replaces an E1B deficiency. To test this hypothesis directly, we examined the ability of *E4* to cooperate in a transformation assay with the Ad9 *E1* region. A representative experiment is shown in Table 1. These experiments were performed on the established rat embryo fibroblast line CREF (*20*) because Ad9 does not transform primary rat embryo fibroblasts or baby rat kidney cells in culture (*21, 22*). In contrast to previous studies (*21*), we were unable to transform CREF cells with a clone containing the Ad9 *E1* region (Table 1). The likely explanation for this result is that Ad9 E1B mRNA expression is deficient in CREF cells, as has been demonstrated in Ad9-induced mammary tumors (*10*). A clone containing the Ad9 *E4* region produced some foci on CREF cells, indicating that E4 was expressed in the absence of E1A and revealing a transforming potential for this region. In addition, when Ad9 *E1* region– and Ad9 *E4* region–containing clones were cotransfected, a ninefold increase in transformants was produced above that of the Ad9 *E4* region clone alone (Table 1). Consistent with these results, the *E4* region of oncogenic virus Ad12 confers an ability to grow in soft agar to Ad12 *E1* region–transformed 3Y1 cells (*23*). Therefore, the Ad9 *E4* region possesses transforming properties in vitro, suggesting that this viral gene functions directly in mammary gland oncogenesis.

Like Ad9, Ad26 also transforms CREF cells (*24*), so it was not surprising that the Ad26 *E4* region also cooperated with the Ad9 *E1* region to transform these cells (Table 1). The Ad26 *E4* region is competent for transformation in CREF cells but defective for tumorigenesis in the rat mammary gland. Clearly, transformation in vitro does not always correlate with oncogenicity in vivo. Ad5 transforms primary rat cells in culture more efficiently than Ad12 (*25*), but only Ad12 virions or Ad12-transformed primary rat cells are tumorigenic in immunocompetent rats (*1*). Although no single adenoviral gene has been identified to explain completely the oncogenic differences between Ad5 and Ad12 (*26, 27*), it has been established that Ad12 E1A proteins, but not Ad5 E1A proteins, inhibit transcription of the major histocompatibility complex (MHC) class I gene, resulting in a stronger evasion of Ad12-transformed cells from the immune system (*28–32*). For human papillomaviruses, the difference in oncogenicity among various isolates has been attributed to at least two viral proteins. Here, the E6 and E7 proteins of oncogenic human papillomaviruses complex better with p53 (*33*) and pRB (*34, 35*), respectively, than do the same proteins derived from nononcogenic papillomaviruses. Because Ad9 and Ad26 E4 mRNAs appear identical in both infected A549 cells (a human cell line that supports the replication of both Ad9 and Ad26) and transformed CREF cells (36), it appears that an inherent protein difference is also responsible for the oncogenic difference between the *E4* regions of these viruses.

The *E4* regions of subgroup D viruses Ad9 and Ad26, similar to those of subgroup C virus Ad2 (*37*) and subgroup A virus Ad12 (*38*), are complex transcription units containing multiple open reading frames (ORFs; Fig. 4A). One deviation of sub-

Table 1. Transformation of CREF cells by cooperation between the Ad9 *E1* region and either the Ad9 or Ad26 *E4* region. Transformation assays with CREF cells were performed similarly to the method used by Babiss *et al.* (*60*). Approximately 10^6 CREF cells on a 100-mm cell culture dish in Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (FCS) were transfected by the calcium phosphate method with a total of 20 μg of supercoiled DNA (10 μg of each plasmid). When the *E1* or *E4* region plasmids were used alone, supercoiled pUC19 was added to bring the total DNA content to 20 μg. Twenty-four hours after transfection, the cells were washed twice with phosphate-buffered saline solution, trypsinized, and passaged onto three 100-mm culture dishes. After another 24 hours, the culture medium was changed to a low-calcium medium (suspension-culture minimum essential medium) supplemented with 5% FCS and Nystatin (100 U/ml, Gibco). Cells were fixed in methanol, stained with Giemsa, and foci were scored 8 weeks after transfection. Ad9 *E1* region plasmid, Spe I–Hind III DNA fragment (0 to 17 mu) in pUC19; Ad9 and Ad26 *E4* region plasmids, Xho I–Spe I DNA fragments (87 to 100 mu) in pUC19.

Plasmids	Number of foci			Total
	Plate 1	Plate 2	Plate 3	
Ad9 E1	0	0	0	0
Ad9 E4	0	1	1	2
Ad26 E4	1	2	0	3
Ad9 E1 + Ad9 E4	6	6	7	19
Ad9 E1 + Ad26 E4	16	19	13	48

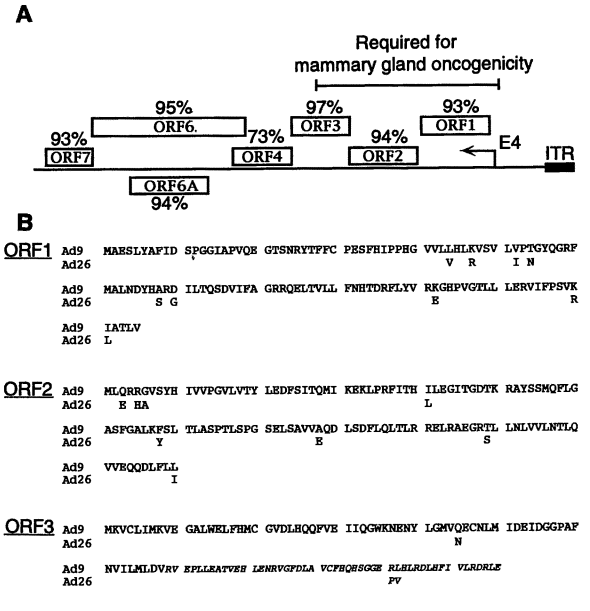

Fig. 4. (**A**) Illustration of the E4 open reading frame (ORF) organization for both Ad9 and Ad26. The boxes show the location of each E4 ORF with the percentage values denoting the amino acid homology between Ad9 and Ad26 for the designated ORF. The region of *E4* defined as being required for mammary gland oncogenesis (nt 305 to nt 1449 from the right end) is also indicated. (**B**) Comparison of the predicted amino acid sequences of Ad9 and Ad26 E4 ORF1, ORF2, and ORF3. The portion of the ORF3 protein encoded outside the defined essential segment of *E4* is shown in italics.

group D from the other subgroups, however, was the presence of an additional E4 ORF, ORF6A, which overlapped internally with ORF6; the significance of this novel ORF remains to be determined. Comparison of the seven predicted Ad9 E4 proteins with those of Ad26 demonstrated that a high degree of similarity existed between most of the E4 ORFs (Fig. 4A). The one exception was ORF4; however, the nontumorigenicity of virus 9SM (see Fig. 2B), which encoded an Ad9 E4 ORF4, argues that this dissimilarity was not responsible for the oncogenic difference between Ad9 and Ad26.

Taking into account both the tumorigenicity of virus 26ITR and the nontumorigenicity of virus 9SM (Fig. 2B), we have mapped the relevant genetic difference between Ad9 and Ad26 to a DNA segment of ~1 kb within the *E4* region (Fig. 4A). This segment extends from nucleotide (nt) 305 to nt 1449 (1144 bp) from the right end and contains a portion of the E4 upstream promoter region, ORF1, ORF2, and a portion of ORF3. The 170-nt E4 upstream promoter region displayed only a single nucleotide difference between Ad9 and Ad26 (*39*), and the 125-residue ORF1, 130-residue ORF2, and 117-residue ORF3 exhibited a nine–, eight–, and one–amino acid difference, respectively (Fig. 4B).

A search of the protein database with Ad9 and Ad26 ORF1, ORF2, and ORF3 amino acid sequences yielded no striking protein sequence homologies (aside from the corresponding E4 ORFs of other adenoviruses) that might have suggested functions for these predicted proteins. For subgroup C adenoviruses (Ad2 and Ad5) in which the *E4* region is best characterized, mutation or deletion of these particular E4 ORFs has little or no effect on the ability of the virus to replicate in cell culture (*40–42*). Furthermore, besides the fact that ORF3 can partially complement viruses with mutations in E4 ORF6 (*40, 42*), no functions have been attributed to any of these three viral proteins.

From the study of subgroup C adenoviruses, it is known that the *E4* region influences such aspects of the virus life cycle as transcription of the viral E2 region, replication of viral DNA, and accumulation and transport of late viral mRNAs (*41, 43–48*). Two proteins encoded within this region have been studied in detail. The E4 ORF6 protein forms a complex (*49*) with the E1B 55-kD protein and regulates cytoplasmic accumulation of both viral and cellular mRNAs (*47, 50*). The E4 ORF6/7 protein, on the other hand, forms a stable complex with the cellular transcription factor E2F in an infected cell (*51–55*). The formation of the complex between E2F and ORF6/7 is needed for efficient transcription from the adenovirus E2 promoter (*43, 44, 46, 52, 54*) and for cooperative binding of E2F to a pair of cognate DNA binding sites (*55, 56*). In order for E2F to bind with E4 ORF6/7, the adenovirus E1A oncoprotein must first disrupt heterocomplexes that normally exist between E2F and the retinoblastoma tumor susceptibility protein (*57, 58*). Like the better studied E4 proteins, the Ad9 E4 ORF1, ORF2, or ORF3 product may also bind to an important cellular regulatory protein, alter its function, and contribute to the specific mammary gland oncogenicity of Ad9.

REFERENCES AND NOTES

1. F. L. Graham, in *The Adenoviruses*, H. S. Ginsberg, Ed. (Plenum, New York, 1984), pp. 339–398.
2. A. Houweling, P. J. Van Den Elsen, A. J. Van Der Eb, *Virology* **105**, 537 (1980).
3. B. Stillman, *Cancer Surv.* **5**, 389 (1986).
4. P. Whyte *et al.*, *Nature* **334**, 124 (1988).
5. M. E. Ewen, Y. Xing, J. B. Lawrence, D. M. Livingston, *Cell* **66**, 1155 (1991).
6. J. Pines and T. Hunter, *Nature* **346**, 760 (1990).
7. L.-H. Tsai, E. Harlow, M. Meyerson, *ibid.* **353**, 174 (1991).
8. P. Sarnow, Y. S. Ho, J. Williams, A. J. Levine, *Cell* **28**, 387 (1982).
9. J. Ankerst, N. Jonsson, L. Kjellen, E. Norrby, H. O. Sjogren, *Int. J. Cancer* **13**, 286 (1974).
10. R. Javier, K. Raska, Jr., G. J. Macdonald, T. Shenk, *J. Virol.* **65**, 3192 (1991).
11. J. Ankerst and N. Jonsson, *J. Natl. Cancer Inst.* **81**, 294 (1989).
12. J. J. Trentin, Y. Yabe, G. Taylor, *Science* **137**, 835 (1962).
13. C. Shellabarger, D. Smith, W. Topp, personal communication.
14. M. Green, J. K. Mackey, W. S. M. Wold, P. Rigden, *Virology* **93**, 481 (1979).
15. G. Chinnadurai, S. Chinnadurai, J. Brusca, *J. Virol.* **32**, 623 (1979).
16. The four recombinant viruses were produced as follows: First, two recombinant left-end clones {Ad9 [0 to 7.5 map units (mu)] to Ad26 (7.5 to 90 mu) and Ad26 (0 to 7.5 mu) to Ad9 (7.5 to 95 mu)} were constructed with the use of the two leftmost Eco RI DNA fragments of Ad9 and Ad26. Next, two right-end viral DNA fragments [Ad9 Xba IA (65 to 100 mu) and Ad26 Xba IA (65 to 100 mu)] were cloned that overlapped with the recombinant left-end fragments. Recombinant viruses were produced with these cloned viral DNAs by the overlap recombination method (*15*) as follows: Each cloned DNA was linearized by restriction enzyme digestion, and one left-end DNA fragment and one overlapping right-end DNA fragment were cotransfected onto A549 cells; homologous recombination resulted in the production of infectious virus. With the use of the two left- and two right-end cloned DNA fragments described above, four different recombinant viruses (AB-9, AB-26, AC-9, and AC-26) were produced. Restriction enzyme analysis was utilized to confirm the genomic structure of each virus (data not shown).
17. Ad9 and Ad26 viral genomes were cloned into a modified pUC19 vector by ligating Spe I linkers onto the termini of virion DNA (*59*) and introducing this modified viral DNA into an Spe I site previously created at the Sma I site of pUC19. Because Ad9 and Ad26 genomes do not encode Spe I sites, intact viral genomes could be released from these plasmids by Spe I digestion. Virus was subsequently generated by calcium phosphate precipitation of these linearized DNAs onto A549 cells. The resulting viruses retained the correct tumorigenic phenotype and served as control viruses for the recombinant virus studies.
18. The six recombinant viruses were constructed according to the methods described previously (*16*). For each recombinant virus, we used the same left-end clone [Ad26 (0 to 90 mu)] and the following different overlapping right-end clones: virus 9HA, Ad9 (70 to 100 mu); virus 26XHB, Ad9 (70 to 87 mu) to Ad26 (87 to 100 mu); virus 9HB, Ad9 (70 to 94 mu) to Ad26 (94 to 100 mu); virus 9SS, Ad26 (70 to 94 mu) to Ad9 (94 to 100 mu); virus 26ITR, Ad26 (70 to 94 mu) to Ad9 (94 to 99.2 mu) to Ad26 (99.2 to 100 mu); and virus 9SM, Ad26 (70 to 94 mu) to Ad9 (94 to 96 mu) to Ad26 (96 to 100 mu). The genomic structures of these six recombinant viruses were confirmed by restriction enzyme or sequence analysis (data not shown).
19. R. Javier and T. Shenk, unpublished results.
20. P. B. Fisher, L. E. Babiss, I. B. Weinstein, H. S. Ginsberg, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3527 (1982).
21. R. Jannun and G. Chinnadurai, *Virus Res.* **7**, 33 (1987).
22. R. Javier and T. Shenk, unpublished results.
23. K. Shiroki *et al.*, *J. Virol.* **50**, 854 (1984).
24. R. Javier and T. Shenk, unpublished results.
25. R. Bernards, A. Houweling, P. I. Schrier, J. L. Bos, A. J. Van Der Eb, *Virology* **120**, 422 (1982).
26. Y. Sawada, K. Raska, Jr., T. Shenk, *ibid.* **166**, 281 (1988).
27. R. Bernards, M. G. de Leeuw, M. J. Vaessen, A. Houweling, A. J. Van Der Eb, *J. Virol.* **50**, 847 (1984).
28. R. Bernards *et al.*, *Nature* **305**, 776 (1983).
29. P. I. Schrier, R. Bernards, R. T. Vaessen, A. Houweling, A. J. Van Der Eb, *ibid.*, p. 771.
30. G. H. Mellow, B. Fohring, J. Dougherty, P. H. Gallimore, K. Raska, Jr., *Virology* **134**, 460 (1984).
31. Y. Sawada, B. Fohring, T. Shenk, K. Raska, Jr., *ibid.* **147**, 413 (1985).
32. J. L. Cook, D. L. May, A. M. J. Lewis, T. A. Walker, *J. Virol.* **61**, 3510 (1987).
33. B. A. Werness, A. J. Levine, P. M. Howley, *Science* **248**, 76 (1990).
34. K. Munger, B. A. Werness, N. Dyson, W. C. Phelps, P. M. Howley, *EMBO J.* **8**, 4099 (1989).
35. K. Munger *et al.*, *J. Virol.* **65**, 3943 (1991).
36. R. Javier and T. Shenk, unpublished results.
37. J. Herisse, M. Rigolet, S. D. de Dinechin, F. Galibert, *Nucleic Acids Res.* **9** 4023 (1981).
38. T. Hogenkamp and H. Esche, *ibid.* **18**, 3065 (1990).
39. R. Javier and T. Shenk, unpublished results.
40. E. Bridge and G. Ketner, *J. Virol.* **63**, 631 (1989).
41. D. N. Halbert J. R. Cutt, T. Shenk, *ibid.* **56**, 250 (1985).
42. M. M. Huang and P. Hearing, *ibid.* **63**, 2605 (1989).
43. L. E. Babiss, *ibid.*, p. 2709.
44. S. Hardy, D. A. Engel, T. Shenk, *Genes Dev.* **3**, 1062 (1989).
45. C. Hemstrom, K. Nordqvist, U. Pettersson, A. Virtanen, *J. Virol.* **62**, 3258 (1988).
46. R. Reichel *et al.*, *ibid.* **63**, 3643 (1989).
47. A. B. Sandler and G. Ketner, *ibid.*, p. 624.
48. S. S. Yoder and S. M. Berget, *ibid.* **60**, 779 (1986).
49. P. Sarnow *et al.*, *ibid.* **49**, 692 (1984).
50. S. Pilder, M. Moore, J. Logan, T. Shenk, *Mol. Cell. Biol.* **6**, 470 (1986).
51. M. M. Huang and P. Hearing, *Genes Dev.* **3**, 1699 (1989).
52. M. J. Marton, S. B. Baim, D. A. Ornelles, T. Shenk, *J. Virol.* **64**, 2345 (1990).
53. S. D. Neill and J. R. Nevins, *ibid.* **65**, 5364 (1991).
54. S. D. Neill, C. Hemstrom, A. Virtanen, J. R. Nevins, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2008 (1990).
55. P. Raychaudhuri, S. Bagchi, S. D. Neill, J. R. Nevins, *J. Virol.* **64**, 2702 (1990).
56. S. Hardy and T. Shenk, *Mol. Cell. Biol.* **9**, 4495 (1989).
57. S. Bagchi, P. Raychaudhuri, J. R. Nevins, *Cell* **62**, 659 (1990).
58. S. P. Chellappan, S. W. Hiebert, M. Mudryj, J. W. Horowitz, J. R. Nevins, *ibid.* **65**, 1053 (1991).
59. D. Hanahan and Y. Gluzman, *Mol. Cell. Biol.* **4**, 302 (1984).
60. L. E. Babiss, P. B. Fisher, H. S. Ginsberg, *J. Virol.* **49**, 731 (1984).

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SV2, a Brain Synaptic Vesicle Protein Homologous to Bacterial Transporters

Sandra M. Bajjalieh, Karen Peterson, Rajesh Shinghal, Richard H. Scheller*

Synaptic vesicle protein 2 (SV2) is a membrane glycoprotein specifically localized to secretory vesicles in neurons and endocrine cells. As a first step toward understanding the function of SV2 in neural secretion, a rat brain complementary DNA (cDNA) that encodes SV2 was isolated and characterized. Analyses of this cDNA predict that SV2 contains 12 transmembrane domains. The NH_2-terminal half of the protein shows significant amino acid sequence identity to a family of bacterial proteins that transport sugars, citrate, and drugs. Expression of the SV2 cDNA in COS cells yielded a high level of SV2-like immunoreactivity distributed in a reticular and punctate pattern, which suggests localization to intracellular membranes. Its localization to vesicles, predicted membrane topology, and sequence identity to known transporters suggest that SV2 is a synaptic vesicle–specific transporter.

Neurotransmitters are concentrated and stored in small clear vesicles localized at the synapse. Neuronal communication is mediated by the release of neurotransmitters from these vesicles by means of vesicle fusion with the plasma membrane. Molecular characterization of synaptic vesicle components has identified proteins that contribute to several aspects of vesicle functioning, including vesicle interaction with cytoskeletal elements and docking at active zones along the plasma membrane (*1–3*). However, little is known about the molecules that regulate the contents of synaptic vesicles or effect the release of transmitters during exocytosis.

Synaptic vesicle protein 2 (SV2) was identified with a monoclonal antibody prepared against cholinergic vesicles from the electric organ of the marine ray *Discopyge ommata* (*4*). Immunolocalization by electron microscopy revealed that this antibody recognizes an epitope on the cytoplasmic face of synaptic vesicles. Biochemical evidence suggests that SV2 is a membrane-associated glycoprotein of ~80 kD. The SV2 epitope is present in fish, amphibians, and mammals and is specifically localized to neural and endocrine cells. Immunohistochemical studies demonstrate that the SV2 epitope is not limited to neurons that contain a specific neurotransmitter but rather is detected in all neuronal and endocrine cells surveyed (*4, 5*). In the endocrine cell lines AtT-20 and PC12, SV2 immunoreactivity localizes to the Golgi apparatus and to the tips of processes, where it is relatively concentrated. Because of its cellular localization and occurrence in a broad array of species, the antibody to SV2 is widely used as a marker for synaptic vesicles.

An important step toward understanding the role of SV2 in synaptic transmission is the determination of its amino acid sequence. To isolate a cDNA that encodes the SV2 protein, we purified an immunoreactive peptide fragment from rat brain synaptic vesicles (Fig. 1). Amino acid microsequencing of this fragment yielded a 40-residue sequence (Fig. 3A, boldface). Rat brain cDNA was amplified by the polymerase chain reaction (PCR) with primers based on the SV2 peptide sequence, which resulted in a 96-nucleotide fragment that encoded the first 31 amino acids of the sequence (*6*). The cloned PCR product was then used as a template in another PCR reaction to generate a radioactive nucleotide probe of high specific activity. This probe was used to screen a rat brain Lambda Zap II library (Stratagene). Approximately 400,000 plaques were screened, yielding 12 positive clones. One of these clones, containing a 3.8-kb insert, was used in subsequent studies.

To confirm that the isolated clone encodes SV2 and to explore the cellular localization of the protein product, we transiently expressed the cDNA in COS cells, a transformed exocrine cell line. The SV2 cDNA was inserted in both the forward (coding) and reverse (noncoding) directions in the mammalian expression vector pCMV. COS cells transfected with constructs that contained the cDNA in the forward direction expressed high concentrations of SV2, as assayed by fluorescent immunohistochemistry (Fig. 2).

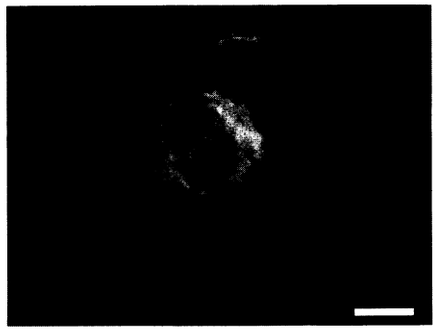

Fig. 2. COS cell transfected with the construct containing SV2 cDNA in the forward direction, showing localization of SV2-like immunoreactivity. COS cells were transfected with SV2 cDNA that had been subcloned into the pCMV expression vector and were fixed, permeabilized, and incubated with the antibody to SV2 followed by rhodamine-conjugated goat antibodies to mouse IgG. Cells transfected with the construct that contained SV2 cDNA in the reverse (noncoding) direction were not immunoreactive. Bar = 25 mm.

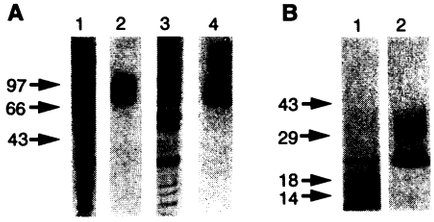

Fig. 1. Generation of a peptide fragment with the SV2 epitope for amino acid sequencing. (**A**) Synaptic vesicles (*22*) before (lanes 1 and 2) and after (lanes 3 and 4) removal of extrinsically associated proteins by incubation with 1 M KCl followed by 10 mM $NaCO_3$ (pH 11). Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred to PVDF paper (Immobilon; Millipore). Proteins in lanes 1 and 3 were stained with Coomassie blue, whereas those in lanes 2 and 4 were incubated sequentially with the monoclonal antibody to SV2 and ^{125}I-labeled antibodies to mouse immunoglobulin G (IgG) and then subjected to autoradiography. The samples in lanes 3 and 4 contain an identical proportion of the total vesicle preparation as the samples in lanes 1 and 2. The samples shown represent less than 1% of the total material processed in preparative gels. (**B**) The region of the PVDF membrane that contained SV2 immunoreactivity [lanes 3 and 4 in (A)] was removed and incubated with cyanogen bromide (60 mg/ml in 70% formic acid). The resulting peptide fragments were separated by SDS-PAGE, transferred to PVDF paper, and either stained with Coomassie blue (lane 1) or processed for anti-SV2 immunoreactivity as in (A) (lane 2). The sample shown represents ~5% of the total used to obtain the amino acid sequence. Positions of the molecular mass marker proteins are shown to the left of each panel in kilodaltons.

Howard Hughes Medical Institute and Department of Molecular and Cellular Physiology, Stanford University, Stanford, CA 94305.

*To whom correspondence should be addressed.