*Rapid Communication*

*J. Biochem.* **122**, 490-493 (1997)

# Effects of Fibronectin Fragments on DNA Transfection into Mammalian Cells by Electroporation

**Kimikazu Hashino,[1] Hideyuki Matsushita, and Ikunoshin Kato**

*Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., Otsu, Shiga 520-21*

Received for publication, May 14, 1997

**Two kinds of cells, human epidermoid carcinoma A431 cells and simian kidney COS-7 cells, were transfected with the chloramphenicol acetyl transferase (CAT) gene by electroporation, and then cultivated on culture dishes coated with two different forms of recombinant fibronectin fragments consisting of cell-binding domain (C-274) or heparin-binding domain and CS1 region (H-296). In the case of A431 cells, H-296-coated dishes significantly increased the amount of expressed CAT and the adhesion of electroporated cells in comparison with non-coated dishes. C-274 was effective for COS-7 cells. Overall, these fibronectin fragments increased the recovery of the transfectants on A-431 cells and COS-7 cells, respectively.**

**Key words: cell-adhesion, electroporation, fibronectin, gene transfer, mammalian cell.**

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

A number of methods have been developed for the transient or stable transfection of mammalian cells with exogenous DNA. The methods frequently used include the use of calcium phosphate (*1, 2*), DEAE-dextran (*3*), liposomes (*4*), electroporation (*5*), and micro-injection (*6*). Electroporation is a method of gene transfer, which involves a physical approach and requires special equipment. However, the method has been widely applied to mammalian (*5, 7*), bacterial (*8, 9*), and plant (*10, 11*) cells. The method involves the exposure of cells to a pulsed electric field which presumably creates pores in the plasma membrane (*12*), and therefore the electric field pulse affects cell viability (*13*). Mammalian cells recover rapidly from electroporation (*14*). Cellular wounding has been studied in detail, together with the cytosis of materials by cells (*15-17*). On the other hand, fibronectin is a component of the extracellular matrix and plays important roles in wound healing of organs (*18*). We are interested in the effects of fibronectin on wound healing and thus studied the function of fibronectin in the repair of cell damage. Fibronectin is a multifunctional glycoprotein which is abundant in the extracellular matrix and plasma, and plays important roles in cell-matrix interactions, including cell-to-substrate adhesion (*19, 20*). At least two cell-adhesive sites have been mapped on fibronectin polypeptides. One site is located in the central cell-binding domain and contains an Arg-Gly-Asp-Ser (RGDS) sequence as a cell-adhesive core signal (*21, 22*). The other one is located within the type III-connecting segment (IIICS) and is represented by a Glu-Ile-Leu-Asp-Val (EILDV) or shorter Lue-Asp-Val (LDV) sequence as a minimal essential one (*23, 24*). We constructed several recombinant plasmids that expressed fragments of the cell-binding domain, the heparin-binding domain, or both, with or without the CS1 sequence, in *Escherichia coli*, and analyzed the cell-adhesive activity of these fragments (*25*). We attempted to determine the cell-adhesive activity of these fragments and tried to increase the viability of cells damaged by electroporation, which resulted in the development of an improved method giving high yields of transfectants.

The fibronectin fragments shown in Fig. 1 (C-274 and H-296) were prepared according to the method described previously (*25*). H-296 and C-274 possesses EILDV and RGDS sequences as a cell-adhesive signal, respectively. Furthermore, C-274 possesses a PHSRN sequence, which has been identified as a synergy site that enhances the cell-adhesive activity of the RGDS sequence of fibronectin (*26, 27*). Three kinds of cells (mouse myeloma B16-F10, human epidermoid carcinoma A-431, and simian kidney COS-7 cells) were, respectively, cultivated in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS).

The effects of these fibronectin fragments on the recovery of cells damaged by an electric pulse were examined by use of B16-F10 cells. Since the adhesion of the cells to the fibronectin fragments had previously been analyzed in detail (*25*), this cell line was suitable for our experiment. Twenty-four well dishes were coated with 1 $\mu$M fibronectin fragments (500 $\mu$l/well) at 4°C overnight. The treated dishes were washed with 1 ml/well of FCS-free DMEM before use. The detached B16-F10 cells were washed twice with phosphate-buffered saline (PBS). The washed cells were suspended in 1 ml of PBS, and then placed in a 0.4 cm electroporation cuvette (Gene Pulser cuvette; BioRad Laboratories, Richmond, CA). A pulse of 250 V and 960 $\mu$F was delivered using a BioRad Gene Pulser (BioRad Laboratories). After the pulse, the cell suspension was stood for 10 min on ice, and then added to 15 ml of DMEM containing 10% FCS. The cells were plated onto dishes coated with the fibronectin fragments (1 ml/well) and then cultivated under 5% $CO_2$ at 37°C for 5 h. Then, the adhered cells were fixed with 4% formaldehyde and photographed under a

[1] To whom correspondence should be addressed.

Abbreviations: CAT, chloramphenicol acetyl transferase; DMEM, Dulbecco's modified essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline.

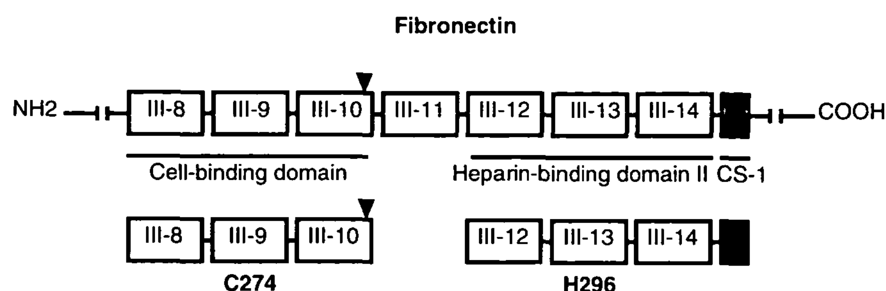


Fig. 1. **Domain structure of fibronectin and its relation to the recombinant fibronectin fragments.** The boxes represent type III repeats and the CS1 sequence of fibronectin. The type III repeats are numbered as described elsewhere (*34*). The location of the RGDS sequence is indicated by an arrowhead. The EILDV sequence is located in the CS-1 region (*23, 24*).

phase-contrast microscope. The cells adhered to the dishes were counted as surviving cells. The number of cells that adhered on H-296-coated dishes or C-274-coated dishes was more than that on non-coated dishes (Exp. 1 in Table I). These fragments were effective for increasing the cell viability. Interestingly, the numbers of adhered cells were in the order of H-296>C-274.

Cell viability decreased with increasing field strength (*28*). This phenomenon would be due to the increase in cell-damage caused by the electrical pulse. On the other hand, B16-F10 cells adhere to both H-296 and C-274, and the cell adhesion activity of H-296 is greater than that of C-274 [ED50 value for cell adhesion on H-296=9.2 nM, and that on C-274=68 nM (*25*)]. Cell-recovery with H-296 was higher than that with C-274 (Exp. 1 in Table I). These results suggest that cell adhesion could be important for survival of damaged cells. Survival after plasma membrane disruption, *i.e.*, wound healing of cells, requires that the cells have a rapid resealing mechanism (*29*). Electric pulse-mediated cell damage, as shown in this study, would be greater than plasma membrane disruption. Such cells would not be able to survive when left unadhered for a long time. In other words, cell adhesion in the early period on cultivation after such damage is considered to be an important factor for highly efficient recovery of the cells. Cell adhesion in an environment similar to the extracellular matrix would accelerate the healing of such damage to the cells.

Considering the results described above, it was assumed that the fibronectin fragments were involved in the DNA transfer into the cells on electroporation. To test the hypothesis that efficient recovery of cells damaged by an electrical pulse is required for a high yield of DNA-transfected cells, we investigated the transfection efficiencies of two different cell lines with the fibronectin fragments. The chloramphenicol acetyl transferase (CAT) gene was used as a reporter gene in the present experiments. The CAT expression plasmid, *i.e.*, pCAT control vector (Promega, Madison, WI), was prepared by CsCl-EtBr centrifugation (*30*). Electroporation was performed in the same way except that the detached cells were suspended in 1 ml of PBS containing 15 $\mu$g/ml of pCAT. After electroporation, the cell suspension was stood for 10 min on ice, and then added to 15 ml of DMEM containing 10% FCS, the cells were plated onto a fibronectin fragment-coated dish (1 ml/well) and then cultivated under 5% $CO_2$ at 37°C overnight. Then the medium in the culture was exchanged with fresh medium, and the cells were further cultivated overnight for use for the CAT assay. Determination of CAT was performed by means of an enzyme immunoassay (CAT-ELISA; Boehringer Mannheim GmbH, Germany). As shown in Table I, the fragments induced an increase in the amount of CAT. A431 cells cultivated on H-296-coated dishes were found to produce a larger amount of CAT than on C-274-coated or non-coated dishes. H-296-coated dishes were about threefold as effective as non-coated dishes (Exp. 2 in Table I). Next, we investigated the adhesion of cells damaged by an electrical pulse to these fragments. As shown in Table I (Exp. 3), it was observed that H-296 more strongly promoted the adhesion of wounded cells than C-274.

TABLE I. **Effects of fibronectin fragments on cell adhesion and DNA transfection.**

| Experiment | Cell | Fragment | % Cell adhesion mean±SD | % DNA transfection mean±SD |
|---|---|---|---|---|
| Exp. 1 | B16F10 | C-274 | 164.0±15.2[¶] | |
| | | H-296 | 233.1±31.8[¶,‡] | |
| | | None | 100.0±15.6 | |
| Exp. 2 | A431 | C-274 | | 136.4±2.1[§] |
| | | H-296 | | 321.2±41.7[§] |
| | | None | | 99.8±10.9 |
| Exp. 3 | A431 | C-274 | 170.8±35.7[¶] | |
| | | H-296 | 280.8±45.5[¶,‡] | |
| | | None | 100.1±12.3 | |
| Exp. 4 | COS-7 | C-274 | 168.9±19.6[¶,#] | |
| | | H-296 | 141.7±22.0[§] | |
| | | None | 100.1±26.7 | |
| Exp. 5 | COS-7 | C-274 | | 168.9±29.0[§] |
| | | H-296 | | 132.6±13.1 |
| | | None | | 100.1±16.4 |

The number of cells adhered on non-coated wells and the amount of CAT expressed in cells on non-coated wells were set at 100%. Differences between two groups were examined by Student's *t*-test. [¶]Significantly different from "none" group; $p<0.01$. [§]Significantly different from "none" group; $0.01<p<0.05$. [‡]Significantly different from "C-274" group; $p<0.01$. [#] Significantly different from "H-296" group; $0.01<p<0.05$.

More interestingly, in the case of COS-7 cells, the efficiency of cell-adhesion on C-274-coated dishes was higher than that on H-296-coated or non-coated dishes (Exp. 4 in Table I). This might indicate that C-274 promotes a higher yield of gene-transferred COS-7 cells than H-296. C-274-coated dishes were found to produce a larger amount of CAT than H296-coated or non-coated dishes (Exp.5 in Table I). These results suggest that the difference in transfection efficiency between A431 and COS-7 cells is due to the difference in the number of adhered cells, *i.e.*, the viability of the cells. Although the profiles of cell-adhesion and CAT differ between the two kinds of cells, these results showed that the recovery of cells damaged by an electric pulse is related to the yield of DNA-transfected cells.

Gene transfer by electroporation involves the exposure of cells to a pulsed electric field, which presumably creates



Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

pores in the plasma membrane (*12*). Therefore, the transformation frequency increases with increasing strength of the electric field, but cell viability decreases with increasing strength (*28*). The reason is that the electric field pulse affects cell viability (*13*). Thus, in order to attain highly efficient transfection of cells by electroporation, it is rational to raise the cell viability of the electrically pulsed cells. On the other hand, it has been reported that cells damaged by electroporation recover rapidly (*14*). In the present study, it was observed that the profile of gene transfer efficiency was very similar to that of cell adhesion (Exp. 2 and 3 on A431 cells, and Exp. 4 and 5 on COS-7 cells in Table I). Cultivation of cells on a substrate precoated with the fibronectin fragments enhanced the wound healing of the damaged cells, causing the recovery of a part of the population of the cells transfected with DNA which can not survive on non-coated dishes due to the damage by electroporation. It is an important factor that the damaged cells efficiently recover in an early period on cultivation followed by electroporation, because the population of transfectants in this period would affect to transfection efficiency. The same methodology will be applicable to other cell-adhesive molecules, for example, collagen, laminin, vitronectin, *etc.*

Gene transfer by electroporation has been applied to medical science, *i.e.*, gene therapy (*31, 32*). Improvement of electroporation for gene transfer would contribute to the highly efficient transduction of an exogenous gene into target cells. On the other hand, Hanenberg *et al.* (*33*) showed that efficient retroviral-mediated gene transfer into primitive hematopoietic progenitor and stem cells can be achieved on recombinant fibronectin fragments containing type III12-14 repeats in combination with the binding site(s) for $\alpha_4\beta_1$-integrin and/or $\alpha_5\beta_1$-integrin without the need for co-cultivation on the producer cell line. In this study, we showed that fibronectin fragments should be useful materials for gene transfer into mammalian cells not only by means of a virus vector but also by electroporation.

The authors wish to thank Y. Uemori and H. Chono for the technical assistance in preparation of the fibronectin fragments.

REFERENCES


1. Chen, C. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752
2. Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. (1978) Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell* **14**, 725-731
3. Lopata, M.A., Cleveland, D.W., and Sollner-Webb, B. (1984) High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. *Nucleic Acids Res.* **12**, 5707-5717
4. Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M., and Danielsen, M. (1987) Lipofection: A highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. USA* **84**, 7413-7417
5. Potter, H., Weir, L., and Leder, P. (1984) Enhancer-dependent expression of human $\kappa$ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. *Proc. Natl. Acad. Sci. USA* **81**, 7161-7165
6. Capecchi, M.R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**, 479-488
7. Chu, G., Hayakawa, H., and Berg, P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic Acids Res.* **15**, 1311-1326
8. Hashimoto, H., Morikawa, H., Yamada, Y., and Kimura, A. (1985) A novel method for transformation of intact yeast cells by electroinjection of plasmid DNA. *Appl. Microbiol. Biotechnol.* **21**, 336-339
9. Dower, W.J., Miller, J.F., and Ragsdale, C.W. (1988) High efficiency transformation of *E. coli* by high voltage electroporation. *Nucleic Acids Res.* **16**, 6127-6145
10. Riggs, C.D. and Bates, G.W. (1986) Stable transformation of tobacco by electroporation: Evidence for plasmid concatenation. *Proc. Natl. Acad. Sci. USA* **83**, 5602-5606
11. Cocking, E.C. and Davey, M.R. (1987) Gene transfer in cereals. *Science* **236**, 1259-1262
12. Kinosita, K. and Tsong, T.Y. (1977) Voltage-induced pore formation and hemolysis of human erythrocytes. *Biochim. Biophys. Acta* **471**, 227-242
13. Knutson, J.C. and Yee, D. (1987) Electroporation: Parameters affecting transfer of DNA into mammalian cells. *Anal. Biochem.* **164**, 44-52
14. Knight, D.E. and Scrutton, M.C. (1986) Gaining access to the cytosol: The technique and some applications of electropermeabilization. *Biochem. J.* **234**, 497-506
15. McNeil, P.L., Muthukrishnan, L., Warder, E., and D'Amore, P.A. (1989) Growth factors are released by mechanically wounded endothelial cells. *J. Cell Biol.* **109**, 811-822
16. Miyake, K. and McNeil, P.L. (1995) Vesicle accumulation and exocytosis at sites of plasma membrane disruption. *J. Cell Biol.* **131**, 1737-1745
17. Bi, G.-Q., Alderton, J.M., and Steinhardt, R.A. (1995) Calcium-regulated exocytosis is required for cell membrane resealing. *J. Cell Biol.* **131**, 1747-1758
18. Colvin, R.B. (1989) Fibronectin in wound healing in *Fibronectin* (Mosher, D.F., ed.) pp. 213-254, Academic Press, San Diego, CA
19. Ruoslahti, E. and Pierschbacher, M.D. (1987) New perspectives in cell adhesion: RGD and integrins. *Science* **238**, 491-497
20. Yamada, K.M. (1989) Fibronectin domains and receptors in *Fibronectin* (Mosher, D.F., ed.) pp. 47-121, Academic Press, San Diego, CA
21. Pierschbacher, M.D. and Ruoslahti, E. (1984) Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33
22. Yamada, K.M. and Kennedy, D.W. (1984) Dualistic nature of adhesive protein function: fibronectin and its biologically active peptide fragments can autoinhibit fibronectin function. *J. Cell Biol.* **99**, 29-36
23. Humphries, M.J., Komoriya, A., Akiyama, S.K., Olden, K., and Yamada, K.M. (1987) Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.* **262**, 6886-6892
24. Komoriya, A., Green, L.J., Mervic, M., Yamada, S.S., Yamada, K.M., and Humphries, M.J. (1991) The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J. Biol. Chem.* **266**, 15075-15079
25. Kimizuka, F., Taguchi, Y., Ohdate, Y., Kawase, Y., Shimojo, T., Hashino, K., Kato, I., Sekiguchi, K., and Titani, K. (1991) Production and characterization of functional domains of human fibronectin expressed in *Escherichia coli*. *J. Biochem.* **110**, 284-291
26. Aota, S., Nomizu, M., and Yamada, K.M. (1994) The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. *J. Biol. Chem.* **269**, 24756-24761
27. Danen, E.H.J., Aota, S., van Kraats, A.A., Yamada, K.M., Ruiter, D.J., and van Muijen, G.N.P. (1995) Requirement for the synergy site for cell adhesion to fibronectin depends on the activation state of integrin $\alpha_5\beta_1$. *J. Biol. Chem.* **270**, 21612-21618
28. Patterson, M.K. (1979) Measurement of growth and viability of


Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012

cells in culture. *Methods Enzymol.* **58**, 141-152

29. Steinhardt, R.A., Bi, G., and Alderton, J.M. (1994) Cell membrane resealing by a vesicular mechanism similar to neurotransmitter release. *Science* **263**, 390-393
30. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
31. Nishi, T., Yoshizato, K., Yamashiro, S., Takashima, H., Sato, K., Hamada, K., Kitamura, I., Yoshimura, T., Saya, H., Kuratsu, J., and Ushio, Y. (1996) High-efficiency *in vivo* gene transfer using intraarterial plasmid DNA injection following *in vivo* electroporation. *Cancer Res.* **56**, 1050-1055
32. Zhang, L., Li, L., Hoffmann, G.A., and Hoffman, R.M. (1996) Depth-targeted efficient gene delivery and expression in the skin by pulsed electric fields: An approach to gene therapy of skin aging and other diseases. *Biochem. Biophys. Res. Commun.* **220**, 633-636
33. Hanenberg, H., Xiao, X.L., Dilloo, D., Hashino, K., Kato, I., and Williams, D.A. (1996) Colocalization of retrovirus and target cells on specific fibronectin fragments increases genetic transduction of mammalian cells. *Nature Med.* **2**, 876-882
34. Kornblihtt, A.R., Umezawa, K., Vibe-Pedersen, K., and Baralle, F.E. (1985) Primary structure of human fibronectin: Differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J.* **4**, 1755-1759

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 2, 2012