P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(7):1504-1507
World Journal of Gastroenterology
 ISSN 1007-9327

Inhibition of HBV targeted ribonuclease enhanced by introduction of linker

Wei-Dong Gong, Jun Liu, Jin Ding, Ya Zhao, Ying-Hui Li, Cai-Fang Xue

Wei-Dong Gong, Jun Liu, Jin Ding, Ya Zhao, Ying-Hui Li, Cai-Fang Xue, Department of Pathogenic Organisms, Fourth Military Medical University, Xi' an 710033, Shaanxi Province, China
Supported by National Natural Scientific Foundation of China, No. 30100157 and Innovation Project of FMMU, No.CX99005
Correspondence to: Professor Cai-Fang Xue or Dr. Jun Liu, Department of Pathogenic Organisms, Fourth Military Medical University, Xi' an 710033, Shaanxi Province, China. etiology@fmmu.edu.cn
Received: 2003-03-05 **Accepted:** 2003-04-01

Abstract

AIM: To construct human eosinophil-derived neurotoxin (hEDN) and HBV core protein (HBVc) eukaryotic fusion expression vector with a linker $(Gly_4Ser)_3$ between them to optimize the molecule folding, which will be used to inhibit HBV replication *in vitro*.

METHODS: Previously constructed pcDNA3.1(-)/TR was used as a template. Linker sequence was synthesized and annealed to form dslinker, and cloned into pcDNA3.1(-)/TR to produce plasmid pcDNA3.1(-)/HBc-linker. Then the hEDN fragment was PCR amplified and inserted into pcDNA3.1(-)/HBc-linker to form pcDNA3.1(-)/TNL in which the effector molecule and the target molecule were separated by a linker sequence. pcDNA3.1(-)/TNL expression was identified by indirect immunofluorescence staining. Radioimmunoassay was used to analyse anti-HBV activity of pcDNA3.1(-)/TNL. Meanwhile, metabolism of cells was evaluated by MTT colorimetry.

RESULTS: hEDN and HBVc eukaryotic fusion expression vector with a linker $(Gly_4Ser)_3$ between them was successfully constructed. pcDNA3.1(-)/TNL was expressed in HepG2.2.15 cells efficiently. A significant decrease of HBsAg concentration from pcDNA3.1(-)/TNL transfectant was observed compared to pcDNA3.1(-)/TR (P=0.036, P<0.05). MTT assay suggested that there were no significant differences between groups (P=0.08, P>0.05).

CONCLUSION: Linker introduction enhances the inhibitory effect of HBV targeted ribonuclease significantly.

Gong WD, Liu J, Ding J, Zhao Y, Li YH, Xue CF. Inhibition of HBV targeted ribonuclease enhanced by introduction of linker. *World J Gastroenterol* 2003; 9(7): 1504-1507
http://www.wjgnet.com/1007-9327/9/1504.asp

INTRODUCTION

Hepatitis B virus (HBV) remains a major public health problem worldwide[1-6] and causes transient and chronic infection of liver[7-9]. Transient infection may produce serious illness, and chronic infection may also have serious consequences: nearly 25 % of chronic HBV infected patients terminate in untreatable liver cancer of 350 million chronic HBV infected patients. The available treatments are of limited efficacy, such as interferon-α (INF-α)[10-12], nucleoside analogues[13-15] and gene therapy strategy[16,17]. Alternative approaches to inhibit HBV replication are urged. Capsid-targeted viral inactivation (CTVI, also called virion-targeted viral inactivation) was established by Natsoulis and Boeke in 1991, in which a viral capsid protein or other virion associated protein as a carrier guides a degradative enzyme into virus particles specifically to inhibit virus replication or kill it[18]. CTVI has been thoroughly investigated in experimental treatment for retrovirus, such as Moloney murine leukemia virus (MMLV) and HIV, showing a promising prospect as an antiviral treatment[19,20]. Previously we fused HBV core protein (HBVc) to human eosinophil-derived neurotoxin (hEDN), and after transfection of the fusion protein encoding plasmid into HepG2.2.15 cells HBV replication was inhibited, due to the fact that HBV pregenome RNA (pgRNA) was degraded by the effector molecule, hEDN, *via* the guiding of the target molecule, HBVc[21,22]. Here we reported the further enhancement of the degradative effect by introduction of a linker sequence $(Gly_4Ser)_3$ to separate the effector molecule and the target one.

MATERIALS AND METHODS

Reagents and equipments

pcDNA3.1(-)/TR was constructed in our laboratory[21]. HepG2.2.15 cells were kindly provided by Dr. Hao (Tangdu Hospital, Fourth Military Medical University). Restriction enzymes, alkaline dephosphoratase, *TaKaRa Ex Taq*™, DNA marker-DL2000 and T_4 DNA ligase (*TaKaRa* Biotechnology Co., Ltd., Dalian), Plasmid Miniprep Kit, Agarose Gel Extraction kit (Watson Biotechnology, Shanghai), LipofectinMine2000 (GIBCO), mouse anti-HA and rabbit anti-mouse IgG labeled with FITC (Sino-American Biotechnology Company), Radioimmunoassay Kit (Beijing Bei Mian Dongya Biotech Institute), GeneAmp PCR System 9600 (Perkin Elmer).

Linker and primers

All the oligomers were synthesized by Sangon (Shanghai).
LinkerF 5' -gcg cgg atc cgg tgg cgg tgg ctc ggg cgg tgg tgg gtc ggg tgg cgg cgg atc tga tga gct cgc gc- 3' (*Bam*HI, *Sac*I)
LinkerR 5' -gcg cga gctc aga tcc gcc gcc acc cga ccc acc acc gcc cga gcc acc gcc acc gga tcc gcg c- 3' (*Sac*I, *Bam*HI)
P1: 5' - gcg ggatcc acc atg aaa cct cca cag tt- 3' (*Bam*HI)
P2: 5' - gcg agatct gat gat tct atc cag gtg aa- 3' (*Bgl*II)

Cell culture

HepG2.2.15 cells integrated full-length HBV genome were cultured in DMEM medium containing 150 mL/L fetal bovine serum at 37 ℃, in 50 mL/L CO_2. G418 was added to screen cells at the final concentration of 100 g/L. The media were freshed once every two days and the cells were passaged every six days.

Introduction of linker

To prepare double stranded linker, linkerF and linkerR were annealed by heating at 100 ℃ for 5 min and then slowly cooling to room temperature, and dissolved at a concentration of 0.1 g/L.

Dslinker bearing *Bam*HI and *Sac*I restriction sites was digested and cloned into pcDNA3.1(-)/TR digested by the same restrictions, to produce plasmid pcDNA3.1(-)/HBc-linker.

hEDN fragment acquired from pcDNA3.1(-)/TR was PCR amplified by using primers and taking pcDNA3.1(-)/TR as a template. The PCR products digested by *Bam*HI/*Bgl*II were inserted into pcDNA3.1(-)/HBc-linker which was restricted by *Bam*HI and dephosphorated, and the direction of hEDN was identified by enzyme restriction. The constructed plasmid was called pcDNA3.1(-)/TNL in which the effector molecule and the target molecule were separated by a linker sequence. The linker sequence and the PCR products were confirmed by sequencing by gencore (Shanghai).

Transfection and indirect immunofluorescence

Transfections were performed as described by the provider of LipofectinMine2000 and the transfecting condition had already been optimized by our laboratory[21]. The cell density of 4×10^8 /L was added by 500 μL/well in 24-well plate in which the cover glasses were put in advance. Transfecting work was performed when cells were adhered, usually after 24 hours[18]. The experiment was divided into 3 groups: test group in which pcDNA3.1(-)/TNL was used, and two control groups in which blank vector pcDNA3.1(-) was used in the second group and the third was taken for mock transfection. Tri-wells were contained in each group. 48 hours post-transfection cells were quickly washed with 1mL sterile PBS (pH8.0) for 5 minutes, fixed in 1 g/L TritonX-100 diluted with 20 g/L paraformaldehyde and put on ice for 30 minutes. The fixed cells were washed three times with cold PBS, incubated with mouse anti-HA (1:100) for 15 minutes at 4 ℃, and then washed three times in cold PBS, followed by incubation in rabbit anti-mouse IgG labeled with FITC (1:100) for 10 minutes at 4 ℃. After rinsed with PBS for 1 hour, the slides were mounted with cover ships by using 500 g/L glycerol/PBS. The results were observed by fluoroscopy and pictures were taken.

Analysis of anti-HBV activity for pcDNA3.1(-)/TNL

24 hours before transfection, HepG2.2.15 cells were plated into a 96 well-plate with of 4×10^4 cells per well. Transfections were performed as described above. To determine HBsAg concentration, transfection experiment was divided into 7 groups, they were pcDNA3.1(-)/TNL, pcDNA3.1(-)/TR, pcDNA3.1(-)/hEDN$_{mut}$-HBVc, pcDNA3.1(-)/HBVc, pcDNA3.1(-)/hEDN, pcDNA3.1(-) and mock transfection, named as A to G respectively. Each transfection was performed in triplicate. After 48 hours of transfection the cell suspension was taken and HBsAg concentration was determined by RIA kit (completed by Nuclear Medicine Department of Xijing Hospital). Meanwhile the transfected cells were used to analyze the metabolic activity in order to analyze the effect of expressing protein on host cells. The data obtained were analyzed by SPSS software.

MTT assay

Metabolism of cells was evaluated by MTT colorimetry. 48 hours following transfections, 20 μL of MTT solution (5 g/L) was added into each well and incubated at 37 ℃ for another 4 h. 150 μL DMSO was added and surged for 10 min to dissolve the crystal completely. Absorbance values were identified at 490 nm wavelength by ELISA reader.

RESULTS

Linker introduction

To separate HBc and hEDN, a linker was introduced. Both the linker sequence cloned into plasmid pcDNA3.1(-)/HBc-linker and hEDN fragment from PCR products, were subsequently inserted into pcDNA3.1(-)/TNL, and confirmed by sequencing. plasmid pcDNA3.1(-)/TNL was identified by restrictions of *BamH*I/*Sac*I and *Sac*I/*Hind*III, the results suggested the construction was successful (Figure 1).

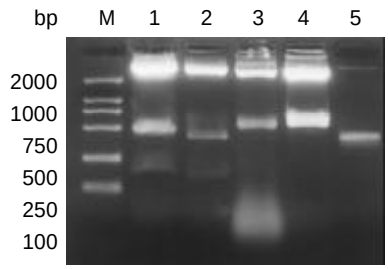

Figure 1 Identifications of plasmids in construction of pcDNA3.1(-)/TNL. 1: pcDNA3.1(-)/TNL digested by *Sac*I/*Hind*III; 2: pcDNA3.1(-)/TR by *Bam*HI/*Sac*I; 3: pcDNA3.1(-)/TR by *Sac*I/*Hind*III; 4: pcDNA3.1(-)/TNL by *Bam*HI/*Sac*I; 5: PCR products of hEDN.

Indirect immunofluorescence

To observe the expression of linker-separated fusion protein of pcDNA3.1(-)/TNL, indirect immunofluorescence was performed after 48 h of transfection (Figure 2).

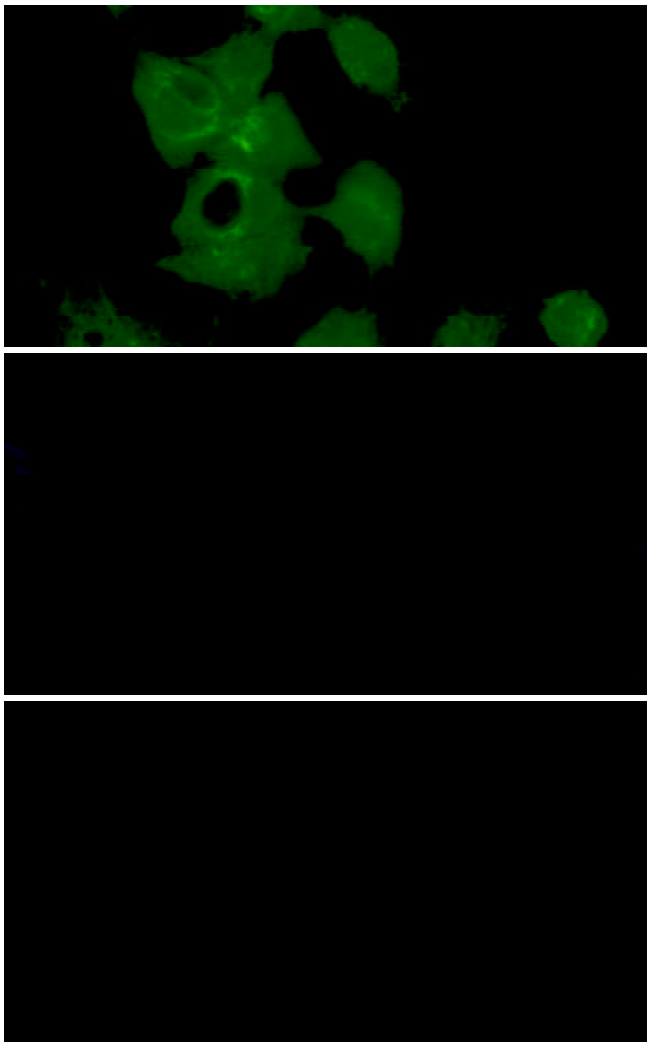

Figure 2 Detection of fusion protein by indirect immunofluorescence. 1: Green fluorescence in cells transfected by pcDNA3.1(-)/TNL; 2, 3: No fluorescence was observed in pcDNA3.1(-) transfectant and mock transfection.

Linker effect

To analyze the digestion effect of hEDN separated by a linker with HBVc. HBsAg concentration was determined by RIA after transfection. The significant decrease of HBsAg concentration in pcDNA3.1(-)/TNL transfectant, compared to pcDNA3.1(-)/TR(P=0.036, P<0.05, Figure 3), suggested that linker introduction enhanced hEDN digestion effect, which may be due to optimization of the folding of both hEDN and HBVc molecules. Also there were significant differences between groups A, B and groups C, D, E, F, G (P=0.0054, P<0.01), and no significant difference was found between groups C, D, E, F and group G (P=0.085, P>0.05).

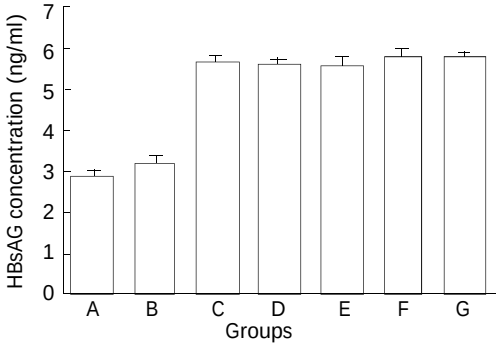

Figure 3 Comparision of HBsAg concentration in transfection groups.

Cell toxicity effect

48 hours post-transfection cell growth was observed and cell toxic effect of linker-separated fusion protein on host hepatocytes was detected by MTT assay. The A_{490} value of HepG2.2.15 cells transfected with pcDNA3.1(-)/TNL, pcDNA3.1(-)/TR, pcDNA3.1(-)/hEDN$_{mut}$-HBVc, pcDNA3.1(-)/HBVc, pcDNA3.1(-)/hEDN, pcDNA3.1(-) and mock transfection was 0.62±0.16, 0.69±0.10, 0.70±0.05, 0.64±0.04, 0.75±0.08, 0.78±0.04, 0.54±0.16, respectively ($\bar{x}$±s, n=3). The results suggested that there were no significant differences between groups (P=0.08, P>0.05).

DISCUSSION

HBV infection is an important health problem worldwide. Int-erferons and nucleosides analogues are effective drugs for chronic HBV infection, but only 20-30 % of treated patients maintain a long-lasting response to anti-viral drugs[23]. The expense of prolonged treatment makes these therapies poorly suitable for people in developing countries, where the prevalence of chronic HBV infection is often high. Therefore, new therapy strategy for HBV infection is urgent.

A new strategy called CTVI was established to guide effector molecule to target molecule and inhibit virus replication[18]. Previously we fused HBVc to hEDN, and after transfection of the fusion protein encoding plasmid into HepG2.2.15 cells, HBV replication was inhibited due to the fact that HBV pregenome RNA (pgRNA) was degraded by the effector molecule, hEDN, by guiding the target molecule, HBVc[21,22]. To further enhance the degradative effect of targeted ribonuclease, the classic linker $(Gly_4Ser)_3$ was introduced to separate the effector molecule and the target one. In our investigation, HBsAg concentration decreased significantly in pcDNA3.1(-)/TNL transfectant compared to pcDNA3.1(-)/TR transfectant due to linker introduction which may augment the digestion effect of hEDN significantly (P=0.036, P<0.05) via optimizing the protein folding.

Linker introduction, one of the gene fusion techniques, has become an increasingly useful tool in a variety of biomedical researches[24]. Many naturally occurring enzymes are composed of two or more distinct modules that are joined into a single macromole by stretches of amino acids referred to as linkers[25]. In structural biology, construction of recombinant fusion proteins has been used as a means to increase the expression of soluble proteins and to facilitate protein purification[26-28]. In recent years, a wide range of applications of gene fusion techniques has been reported in the fields of biotechnology. These applications include selection and prodution of antibodies and proteins with specialized functions, such as proteins that target specific genes[29-31]. Recent studies have provided examples of linkers that are important in establishing the structural and functional assembly of multi-domain proteins[32]. These interdomain linkers are relatively long and probably flexible in order to allow the two modules to perform independent functions. Recombinant production of chimeric enzymes requires stable linkers to join fusion partners without interfering with their function. The use of long linkers may result in low yield of active fusion protein since unprotected and flexible regions are often susceptible to proteolytic cleavage during recombinant protein production. A shorter linker might overcome problems associated with protease degradation. On the other hand, there is a risk that a shorter linker brings the modules too close to each other, resulting in a loss of function[25]. In many studies functional single-chain antibodies (scFvs) were engineered by linking immunoglobulin heavy and light chain variable domains (V_H and V_L) *via* $(G1y_4Ser)_3$, which satisfies the needs above. Further enhancement of hEDN digestion and inhibitory effect may be completed by further linker design and screening, and the work involved in this field is in progress in our lab.

REFERENCES

1 **Fleming J**. Current treatments for hepatitis. *J Infus Nurs* 2002; **25**: 379-382
2 **elSaadany S**, Tepper M, Mao Y, Semenciw R, Giulivi A. An epidemiologic study of hepatocellular carcinoma in Canada. *Can J Public Health* 2002; **93**: 443-446
3 **Hassan MM**, Hwang LY, Hatten CJ, Swaim M, Li D, Abbruzzese JL, Beasley P, Patt YZ. Risk factors for hepatocellular carcinoma: synergism of alcohol with viral hepatitis and diabetes mellitus. *Hepatology* 2002; **36**: 1206-1213
4 **Torbenson M**, Thomas DL. Occult hepatitis B. *Lancet Infect Dis* 2002; **2**: 479-486
5 **Lok AS**. Hepatitis B infection: Pathogenesis and management. *J Hepatol* 2000; **32**(Suppl 1): 89-97
6 **Mazumdar TN**. Management of chronic hepatitis B infection: an update. *J Indian Med Assoc* 2001; **99**: 306-308, 310
7 **Chen N**, Zhu C, Hu D, Zeng F. The clinical significance of negative serological markers of hepatitis B infection in hepatitis B virus carriers with chronic hepatic disease. *Zhonghua Neike Zazhi* 2002; **41**: 653-655
8 **Hino O**, Kajino K, Umeda T, Arakawa Y. Understanding the hypercarcinogenic state in chronic hepatitis: a clue to the prevention of human hepatocellular carcinoma. *J Gastroenterol* 2002; **37**: 883-887
9 **Esteban R**. Management of chronic hepatitis B: an overview. *Semin Liver Dis* 2002; **22**(Suppl 1): 1-6
10 **Wai CT**, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology* 2002; **36**: 1425-1430
11 **Manns MP**. Current state of interferon therapy in the treatment of chronic hepatitis B. *Semin Liver Dis* 2002; **22**(Suppl 1): 7-14
12 **Shindo M**, Hamada K, Koya S, Sokawa Y, Okuno T. The clinical significance of core promoter and precore mutations during the natural course and interferon therapy in patients with chronic hepatitis B. *Am J Gastroenterol* 1999; **94**: 2237-2245
13 **Liaw YF**. Therapy of chronic hepatitis B: current challenges and opportunities. *J Viral Hepat* 2002; **9**: 393-399
14 **Wolters LM**, Hansen BE, Niesters HG, de Man RA.Viral dynamics in chronic hepatitis B patients treated with lamivudine,

lamivudine-famciclovir or lamivudine-ganciclovir. *Eur J Gastroenterol Hepatol* 2002; **14**: 1007-1011
15 **Santantonio T**, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long-term follow-up of patients with anti-HBe / HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J Hepatol* 2000; **32**: 300-306
16 **Song YH**, Lin JS, Liu NZ, Kong XJ, Xie N, Wang NX, Jin YX, Liang KH. Anti-HBV hairpin ribozyme-mediated cleavage of target RNA *in vitro*. *World J Gastroenterol* 2002; **8**: 91-94
17 **Chiou HC**, Lucas MA, Coffin CC, Banaszczyk MG, Ill CR, Lollo CP. Gene therapy strategies for the treatment of chronic viral hepatitis. *Expert Opin Biol Ther* 2001; **1**: 629-639
18 **Natsoulis G**, Boeke JD. New antiviral strategy using capsid-nuclease fusion proteins. *Nature* 1991; **352**: 632-635
19 **VanBrocklin M**, Federspiel MJ. Capsid-targeted viral inactivation can eliminate the production of infectious murine leukemia virus *in vitro*. *Virology* 2000; **267**: 111-123
20 **Wu X**, Liu H, Xiao H, Kim J, Seshaiah P, Natsoulis G, Boeke JD, Hahn BH, Kappes JC. Tageting foreign proteins to human immunodeficiency virus particles via fution with Vpr and Vpx. *J Virol* 1995; **69**: 3389-3398
21 **Li YH**, Liu J, Xue CF. Construction of HBV targeted ribonuclease and its expression in 2.2.15 cell line. *Xibao yu Fenzi Mianyixue Zazhi* 2002; **18**: 217
22 **Ding J**, Liu J, Xue CF, Li YH, Gong WD. Construction and expression of prokaryotic expression vector for pTAT-HBV targeted ribonuclease. fusion protein. *Xibao yu Fenzi Mianyixue Zazhi* 2003; **19**: 49-51
23 **Raj V**. Treatment of hepatitis B. *Clin Cornerstone* 2001; **3**: 24-36
24 **Arai R**, Ueda H, Kitayama A, Kamiya N, Nagamune T. Design of linkers which effectively separate domains of a bifunctional fusion protein. *Protein Eng* 2001; **14**: 529-532
25 **Gustavsson M**, Lehtio J, Denman S, Teeri TT, Hult K, Martinelle M. Stable linker peptides for a cellulose-binding domain-lipase fusion protein expressed in *Pichia pastoris*. *Protein Eng* 2001; **14**: 711-715
26 **Stein A**, Dalal Y, Fleury TJ. Circle ligation of in vitro assembled chromatin indicates a highly flexible structure. *Nucleic Acids Res* 2002; **30**: 5103-5109
27 **Kiczak L**, Kasztura M, Koscielska-Kasprzak K, Dadlez M, Otlewski J. Selection of potent chymotrypsin and elastase inhibitors from M13 phage library of basic pancreatic trypsin inhibitor (BPTI). *Biochim Biophys Acta* 2001; **1550**: 153-163
28 **Reiter Y**, Schuck P, Boyd LF, Plaksin D. An antibody single-domain phage display library of a native heavy chain variable region: isolation of functional single-domain VH molecules with a unique interface. *J Mol Biol* 1999; **290**: 685-698
29 **Rojas G**, Almagro JC, Acevedo B, Gavilondo JV. Phage antibody fragments library combining a single human light chain variable region with immune mouse heavy chain variable regions. *J Biotechnol* 2002; **94**: 287-298
30 **Klug A**. Zinc finger peptides for the regulation of gene expression. *J Mol Biol* 1999; **293**: 215-218
31 **Tang L**, Li J, Katz DS, Feng JA. Determining the DNA bending angle induced by non-specific high mobility group-1 (HMG-1) proteins: a novel method. *Biochemistry* 2000; **39**: 3052-3060
32 **Gokhale RS**, Khosla C. Role of linkers in communication between protein modules. *Curr Opin Chem Biol* 2000; **4**: 22-27

Edited by Ren SY and Wang XL