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A mammalian two-hybrid screening system for inhibitors of interaction between HIV Nef and the cellular tyrosine kinase Hck

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Abstract

In the scope of the search for new anti-HIV agents interacting with a new target, we developed a high-throughput screening system to detect the interactions between Nef and Hck. Nef is an accessory protein of HIV, which is involved in the pathogenicity of the acquired immunodeficiency syndrome (AIDS). Nef is also a signaling molecule because it binds to many host molecules. It has especially high affinity to Hck, a member of src family tyrosine kinase. Using a mammalian two-hybrid system, the interaction between Nef and the SH3 domain of Hck induced luciferase activity with high sensitivity and a Nef–PXXP peptide inhibited this interaction; and so did the anticancer drug adriamycin. We also developed another assay system by coexpression of full-length Hck and Nef, and found that Hck kinase was activated depending on the dose of Nef plasmid. Using the second system, we found that adriamycin interfered with the Nef–Hck interaction by reducing the amount of the Hck protein. The mammalian two-hybrid system may show utility in screening inhibitors of Nef–Hck interaction.

Keywords: HIV; Nef; Hck; SH3; Mammalian two-hybrid system

1. Introduction

Many chemotherapeutic agents against HIV/AIDS have been developed, and several drugs targeting reverse transcriptase and protease have been of considerable benefit to AIDS patients. An

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inevitable problem, however, has been the rapid emergence of drug-resistant viruses. Overcoming this problem requires new drugs interacting with new molecular targets. Since resistance is the result of continuous mutation and selection by the pressure of antiviral drugs, drugs targeting virus pathogenicity independent of viral proliferation may be less prone to resistance development. We chose Nef and its effects on cellular signaling pathways as a possible target for HIV/AIDS.

Nef is an accessory protein and HIV is thought to be able to replicate without Nef, although the titer is lower than with Nef (Kirchhoff et al., 1995). However, Nef is known to have many important roles (Collette, 1997; Harris, 1996). It induces MHC class I down-regulation, which enables HIV to escape death by CTL (Schwartz et al., 1996) and seems to enhance the cellular response to T-cell receptor stimulation (Wu and Marsh, 2001). Nef is also thought to be responsible for the pathogenicity of HIV (Mariani et al., 1996; Hanna et al., 1998) and SIV (Kestler et al., 1991; Novembre et al., 1996). Research on transgenic mice harboring selected HIV genes has suggested that the pathogenicity exerted by the nef gene may be independent of viral replication (Hanna et al., 1998; Larsen et al., 1998). Research also revealed multiple interactions between Nef and cellular signaling molecules, such as the src family of protein kinases, p21-associated kinase, PKC and Vav (Renkema and Saksela, 2000). Especially, Nef binds the SH3 region of a member of the src family kinase, Hck, through its highly conservative proline-rich motif [PXXP](P = proline, X = any amino acid) with unusually high affinity in vitro (Moarefi et al., 1997) and in cells (Briggs et al., 1997). A recent report suggested that the PXXP domain of Nef is critical to pathogenicity and that Hck is involved (Hanna et al., 2001). In addition, a dominant negative mutant Hck has recently been shown to inhibit MHC class I down-regulation (Chang et al., 2001), which provides evidence of the role of Hck.

These findings led us to develop a screening system based on the interaction between Nef and the cellular tyrosine kinase Hck. To work out this strategy, we adopted a mammalian two-hybrid system. Although mammalian two-hybrid systems have been used less than yeast two-hybrid systems, they have some advantages over in vitro screening, the permeability and toxicity to mammalian cells of the drugs detected by these systems are to some extent already known. Some inhibitors may act on the interaction indirectly by binding other proteins that act on Nef or Hck or change their conformation. This technique is also superior to a yeast screening system because yeast has rigid cell walls that drugs have difficulty permeating.

In this study, we established a mammalian two-hybrid system as the primary screening method, and also developed another cell-based system to detect activation of Hck kinase by Nef for further evaluation. Using these two assay methods, we found that adriamycin inhibited Nef–Hck interaction by reducing the amount of Hck protein.

2. Materials and methods

2.1. Cells

Transformed human embryonic kidney cells, 293T, were kindly provided by Bruce J. Mayer (University of Connecticut) and maintained in DMEM supplemented with 10% fetal calf serum. NIH 3T3 cells were purchased from the American Type Culture Collection and cultured in DMEM supplemented with 5% calf serum.

2.2. Construction of plasmids

For the mammalian two-hybrid system, plasmid pM (encoding the yeast GAL4 DNA binding domain) and pVP16 (encoding the herpes simplex virus VP16 transactivating domain) were purchased from Clontech Co. (Mammalian MATCHMAKER Two-hybrid System, Palo Alto, CA). The reporter plasmid encoding luciferase (pG5-Luc) was constructed using pG5CAT (Clontech Co.) by replacing chloramphenicol acetylating enzyme (CAT) gene with luciferase gene from plasmid pTRE-Luc (Clontech Co.). To generate an in frame fusion protein of Nef and the GAL4 DNA binding domain, nef DNA (0.6 kb) was amplified by PCR from plasmid pcD-SRα

nef460 encoding HIV-1 Nef (from NL432) (Tsunetsugu-Yokota et al. 1992), and subcloned into pBluescript (pBluescript-nef) to allow DNA sequencing. To construct pM-nef coding GAL4 DNA binding domain fused with Nef, Nef gene was isolated from the pBluescript-nef coding Nef and inserted into pM to fuse with the GAL4 DNA binding domain; this is referred to as pM-nef. The partial DNA fragments coding Hck aa 1–112 (from N-terminus to the SH3 region, 0.3 kb) and whole Hck (aa 1–505, 1.9 kb) were created using PCR from cDNA of U937 cells. RNA of these cells was extracted by the guanidinium isothiocyanate method and the cDNAs were generated by first strand cDNA synthesis using random primers of the SuperScript Preamplification System (GIBCO BRL, Rockville, MD USA) following directions of the Manufacturer. The DNA fragments were subcloned into pUC19 for sequencing (pUC19 hckSH3 and pUC19-hck-wt). DNAs with correct sequences were digested and inserted into pVP16 to fuse with the VP16 transcription activating domain, and were termed pVP16-hckSH3 and pVP16-hck-wt.

For Hck kinase activation assay, pCAGGS-nef was constructed with pCAGGS vector (kindly provided by Dr J. Miyazaki) and PCR amplified nef gene (from NL432) with Myc-tag at the C-terminus. Whole hck gene was digested from pUC19-hck-wt and inserted into pIRES2-EGFP vector (Clontech Co.), and called pIRES2-hck-EGFP. pIRES2-EGFP and pEGFPC1 were purchased from Clontech Co.

2.3. Mammalian two-hybrid assays

293T cells and NIH 3T3 cells were seeded in a collagen-coated 24-well dish (4×10^5 cells per ml) and 24 h after cell seeding, pM-nef, pVP16-hckSH3 (or pVP16-hck-wt) and reporter pG5-Luc were cotransfected by lipofection using FuGENE6 (Roche Molecular Biochemicals, Hague Road, IN) and lipofectamine (GIBCO BRL), respectively, following the Manufacturers' procedures. Luciferase activity was measured at 2 days after transfection. For screening of inhibitors, transfected 293T cells in collagen-coated 60 mm dishes were trypsinized and re-seeded into 96-well plates for luciferase assay (Packard Instruments Co. Research Parkway, CT) at 24 h after transfection, and samples were added. Luciferase activity was measured at 24 h after addition of the samples. The SEAP (secreted alkaline phosphatase) expressing vector (Clontech Co.) was cotransfected and its activity (absorbance A_{405}) measured with p-nitrophenyl-phosphate (Berger et al. 1988) to standardize the efficiency of the transfection. Myristylated Nef–PXXP peptide (Myr-VTPQV-PLRP) was purchased from Sawady Tech. (Tokyo, Japan).

2.4. Luciferase assays

Luciferase activity was measured with a luminometer (Futaba Medical Co., Tokyo, Japan) using a PicaGene kit (Toyo Inki Co., Tokyo) or with a Packard liquid scintillation counter using a Luclite kit (Packard Instruments Co.) for 96-well assays. Transfected cells were lysed with lysis buffer of the PicaGene kit after removing the medium, and a portion of the lysate solution was mixed with a substrate of luciferase. Luciferase activity was then immediately measured with the luminometer. Alternatively, cells in 96-well plates were directly added with Luclite, and the activity was measured with the liquid scintillation counter.

2.5. Hck kinase activation assays

293T cells were seeded in a collagen-coated 24 well plate at 2×10^5 cells per well and transfected with pIRES2-hck-EGFP and either pCAGGS-nef or pEGFPC1 using FuGENE6, and harvested at 2 days after transfection. To evaluate inhibitors, we added test samples at 24 h after transfection, and harvested the cells 24 h later. Cell pellets were lysed with HNTG buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 100 KIU per ml aprotinin, 100 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml of each of antipain, leupeptin and pepstatin) on ice for 30 min, centrifuged, and the amount of protein in the supernatant was then measured. Cell lysate containing 10 μg of protein boiled with sample buffer was applied to SDS-

PAGE, transferred to a PVDF membrane and probed using anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY), anti-Hck antibody (sc-72, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-Nef antibody (Tsunetsugu-Yokota et al., 1992). The signal was detected with a horseradish peroxidase-linked second antibody (anti-rabbit IgG or anti-mouse IgG) and Enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Amersham Place, England).

3. Results and discussion

3.1. Primary screening by mammalian two-hybrid system

Adopting a mammalian two-hybrid system as the primary screening system, we constructed three plasmids, pM-nef coding GAL4 DNA binding domain fused with Nef, pVP16-hck encoding HSV VP16 transcription activating domain fused with Hck, and the reporter plasmid expressing luciferase. The three plasmids were transiently cotransfected into 293T cells. For Hck, we used the hck gene coding N-terminal 112 amino acids of the SH3 region (pVP16-hck-SH3). First, we inserted full-length hck gene in pVP16 (pVP16-hck-wt), but cotransfection with pM-nef and the pG5-Luc reporter failed to induce any luciferase activity. Some conformational change of Hck induced by fused VP16 protein might affect the interaction between Nef–PXXP and SH3 domain of Hck or the C-terminus domain of Hck might interrupt binding Gal4 DNA binding domain to its promoter. The cotransfection of pVP16-hck SH3 with pM-nef and the reporter pG5-Luc in 293T cells resulted in a 12-fold increase in luciferase activity over that seen upon transfection with the pG5-Luc reporter plasmid alone. As a control, we used supplementary plasmids, pM-p53 (tumor suppressor gene) and pVP16-T (SV40 large T antigen), a 31-fold increase in the activity

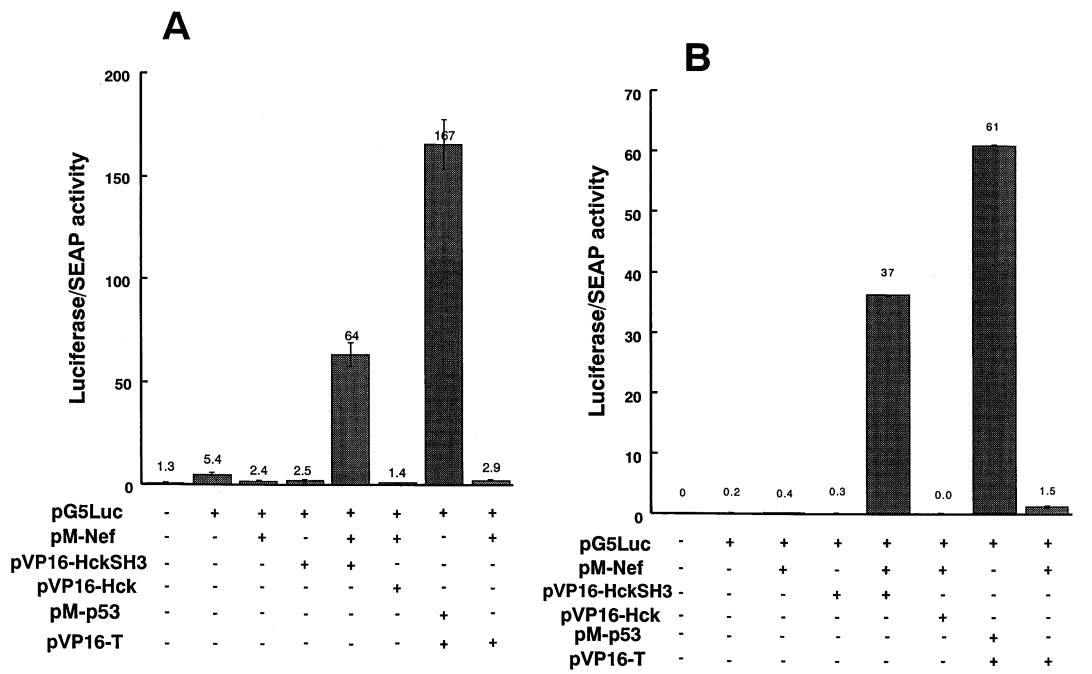

Fig. 1. Expression of luciferase by Nef–Hck interaction in 293T and NIH 3T3 cells. We transfected transiently the two plasmids together with the luciferase reporter plasmid into 293T cells (A) and NIH 3T3 cells (B). Only in the presence of the three plasmids was luciferase activity detected. The values represent luciferase arbitrary units standardized with SEAP activities for transfection efficiency.

was observed (Fig. 1A). Similar results were seen when these plasmids were transiently transfected into NIH3T3 cells (Fig. 1B). The results indicated that Nef and the SH3 region of Hck are interacting in these mammalian cells.

As we observed activity high enough for practical and sensitive high-throughput screening, we transfected pM-nef and pVP16-hckSH3 with the reporter pG5-Luc into 293T cells in 60-mm dishes. Then, 24 h later the transfected cells were reseeded into 96-well plates and test samples were added. The transfected cells had expressed luciferase activity at this time. As a control, we used pM-p53 and pVP16-large T. We expected that parallel addition of test samples to Nef–Hck and p53-large T expressing cells would reveal specific inhibition of the Nef–Hck interaction, because drugs with nonspecific toxicity would inhibit the luciferase activity to the same extent in both cells. The luciferase activity without samples was set as 100%, and the relative activity was compared with that of control, p53-large T expressing cells. We first tested a myristylated-peptide of 9 amino acids (Myr-VTPQVPLRP) containing Nef proline-rich domain termed Nef–PXXP peptide. Myristylation of peptides is thought to increase permeability to the cell membrane. The peptide inhibited the luciferase activity in a concentration-dependent manner; the activity by Nef–Hck with 100 μg/ml (82 μM) of Nef–PXXP peptide was about half that of p53-large T (Fig. 2). This suggests that the screening system represented Nef–Hck interaction. Next, we tried 500 samples from our library of synthetic and natural substances and obtained some positive substances which showed apparently selective inhibition of the Nef–Hck interaction. The four most intensive samples showing over 50% inhibition compared with control were adriamycin derivatives. Adriamycin showed specific inhibition at 0.1 μg/ml (0.18 μM; Fig. 3).

3.2. Another system of evaluating inhibitors by western blot with anti-phosphotyrosine antibody

To confirm the positive inhibition of samples, we also used another system as indicative for the Nef–Hck interaction. It is reported that Nef interacts with the SH3 region of Hck to give rise to intramolecular structural domain displacement and to activate its kinase activity (Moarefi et al.,

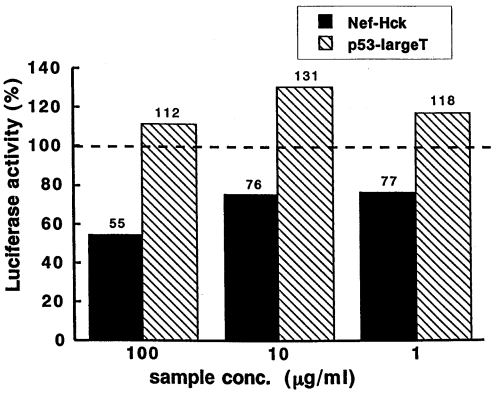

Fig. 2. Specific inhibition of luciferase activity by Nef–Hck with Nef–PXXP peptide. The effect of Nef–PXXP peptide (Myr-VTPQVPLRP) on the luciferase activity by Nef–Hck interaction compared with that by p53-large T. The values represent the percentage of each activity with solvent only. The toxicity determined by MTT assay of the peptide during the assay period was 0%.

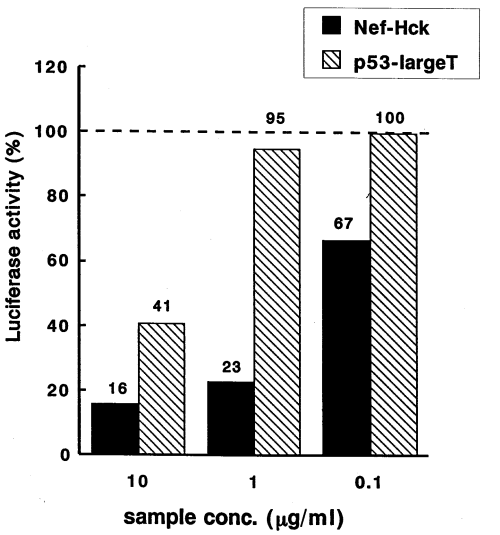

Fig. 3. Specific inhibition of luciferase activity by Nef–Hck with adriamycin. The effect of adriamycin on the luciferase activity by Nef Hck interaction compared with that by p53-large T. Values represent the percentage of each activity with solvent only. The toxicity determined by MTT assay of adriamycin was 5, 30 and 40% at 0.18 (0.1), 1.8 (1), and 18 μM, (10 μg/ml) respectively.

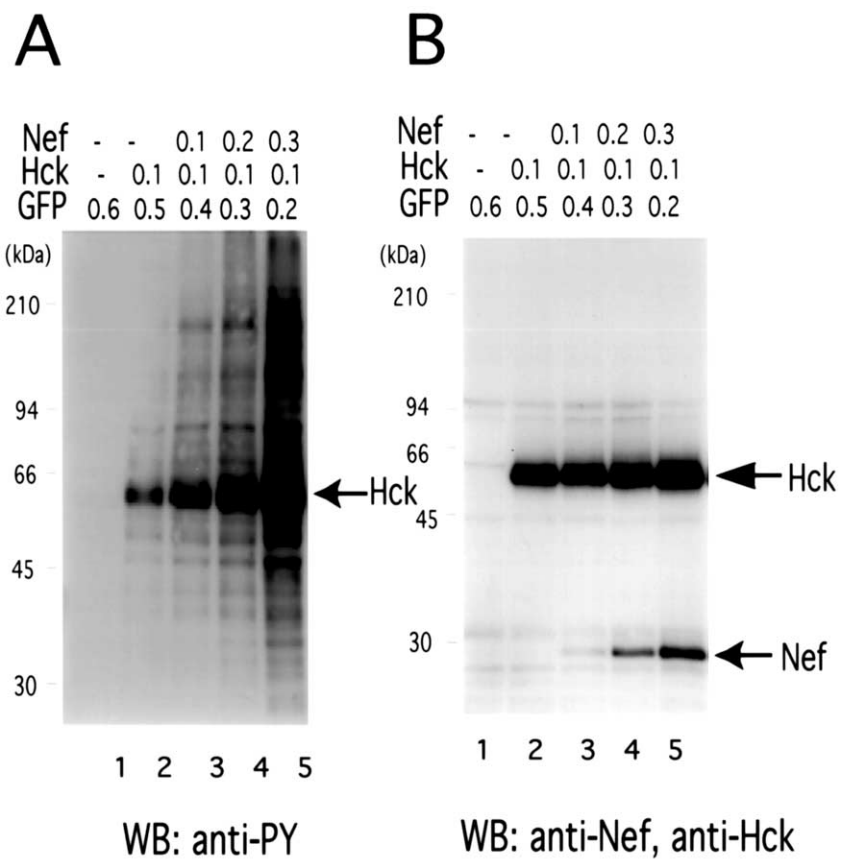

Fig. 4. Activation of Hck autophosphorylation by Nef. We cotransfected transiently into 293T cells, 0.6 μg of pEGFPC1 only (lane 1), 0.1 μg of pIRES2-hck-EGFP and 0.5 μg of pEGFPC1 (lane 2), 0.1 μg of pIRES2-hck-EGFP, 0.1 μg of pCAGGS-nef and 0.4 μg of pEGFPC1 (lane 3), 0.1 μg of pIRES2-hck-EGFP, 0.2 μg of pCAGGS-nef and 0.3 μg of pEGFPC1 (lane 4) and 0.1 μg of pIRES2-hck-EGFP, 0.3 μg of pCAGGS-nef and 0.2 μg of pEGFPC1 (lane 5). After 2 days, cells were lysed and applied to western blotting with anti-phosphotyrosine antibody (A) or anti-Hck antibody and anti-Nef antibody (B), and were detected by ECL.

1997). We constructed another plasmid expressing the whole Hck kinase domain, pIRES2-hck-EGFP, and cotransfected it with Nef-expressing plasmid pCAGGS-nef in 293T cells. Two days after the transfection, cells were lysed and cell lysates were applied to SDS-PAGE and western blotting with anti-phosphotyrosine antibody. The intensity of tyrosine phosphorylated Hck proteins (56 and 59 kDa) increased in a dose-dependent manner based on the DNA amount of plasmids expressing Nef (Fig. 4), suggesting that the autophosphorylation of Hck kinase was enhanced by Nef in the cells. We then examined and found that adriamycin inhibited autophosphorylation of Hck in the presence of Nef expression, but also in the absence of Nef (Fig. 5A). We then examined the protein amount of Hck by western blot using anti-Hck antibody, and found that adriamycin reduced the amount of Hck in a concentration dependent manner (Fig. 5B). Adriamycin did not reduce the amount of Nef protein (Fig. 5B) or endogenous kinases (Erk1, JNK, Raf-1) of 293T cells (data not shown). It also depleted VP16-Hck-SH3 fusion protein expressed in 293T cells (data not shown). These results suggest that adriamycin inhibits the Nef–Hck interaction by reducing the amount of Hck protein.

Endogenous Hck of U937 cells was also depleted by adriamycin treatment (data not shown). Adriamycin is known to be a DNA intercalator to inhibit RNA synthesis and is used as an anti-cancer drug (Arcamone et al. 1969), therefore, its effect on Hck cannot be considered as entirely specific. Although the mechanism of the specific depletion of Hck by adriamycin was not examined, it is possible that this depletion was the result of inhibition of RNA synthesis if the half-life of Hck is shorter than that of other proteins.

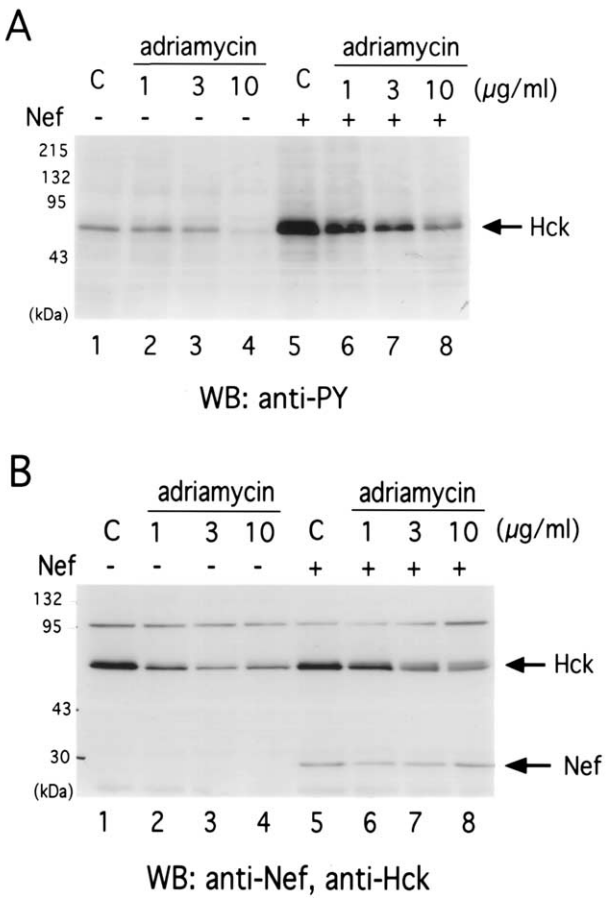

Fig. 5. Effect of adriamycin on autophosphorylation of Hck (A) and amount of protein of Hck and Nef (B); 0.1 μg of pIRES2-hck-EGFP and 0.3 μg of pEGFPC1 (lanes 1–4) or 0.1 μg of pIRES2-hck-EGFP and 0.3 μg of pCAGGS-nef (lanes 5–8) were cotransfected into 293T cells. At 24 h after transfection, 0.1% DMSO (solvent) (lanes 1 and 5) or adriamycin (lanes 2 and 6: 1.8 μM (1 μg/ml), lanes 3 and 7: 5.4 μM (3 μg/ml), lanes 4 and 8: 18 μM (10 μg/ml)) was added and incubated for another 20 h. The cells were lysed and applied to western blotting with anti-phosphotyrosine antibody (A) or anti-Hck antibody and anti-Nef antibody (B), and were detected by ECL.

We have elaborated a convenient, sensitive system for studying inhibitors of the interaction between Nef and Hck in intact mammalian cells; this system may be suitable for high-throughput screening. We found using this system that adriamycin inhibits interaction between Nef and Hck by reducing Hck protein. The system may also be applicable to assess interactions with other signal transducing molecules and inhibitors thereof.

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