Detection of High Levels of 2 Specific Isoforms of 14-3-3 Proteins in Synovial Fluid from Patients with Joint Inflammation

RUHANGIZ T. KILANI, WALTER P. MAKSYMOWYCH, ALASTAIR AITKEN, GILLES BOIRE, YVES ST-PIERRE, YUNYUAN LI, and AZIZ GHAHARY

ABSTRACT. ***Objective.*** To investigate whether 14-3-3 proteins were detectable in synovial fluid (SF) of patients with inflamed joints, and if so, what isoform(s); and to examine whether there was a correlation between the levels of these proteins and those of MMP-1 and MMP-3 in the same samples.

Methods. In general, 2 sets of synovial and serum samples were analyzed. The first set of 17 SF samples from patients with inflamed joints were analyzed for 14-3-3 η isoform by Western blot. The second set of 12 matching serum and SF samples were analyzed for 14-3-3 η, γ, MMP-1, and MMP-3 by the same procedure. The MMP-1 stimulatory effect of various concentrations of 14-3-3 η in cultured fibroblasts was then evaluated.

Results. We found that of the seven 14-3-3 isoforms tested (ß, γ, ε, η, σ, θ, and ζ), the levels of only 2 isoforms, η and γ, were easily detectable in SF samples from patients with inflammatory joint diseases. The levels of these proteins were significantly higher in inflammatory SF and serum samples relative to controls. The values of these proteins correlated strongly with the levels of MMP-1 and MMP-3, 2 biomarkers for rheumatoid arthritis, detected in sera. Further, the level of 14-3-3 η was significantly higher in a pool of 12 serum samples from patients with inflammatory joint disease than those from healthy individuals.

Conclusion. Detection of only 2 (14-3-3 η and γ) out of 7 different isoforms in SF suggests they are specific to the site of inflammation, and that distinguishes them from barely detectable levels of these isoforms found in normal serum. The MMP-1 stimulatory effect of the η isoform explains its correlation with MMP-1 levels seen in these samples. (First Release July 1 2007; J Rheumatol 2007;34:1650–7)

Key Indexing Terms:
PROTEINS 14-3-3 ARTHRITIS SYNOVIAL FLUID MATRIX METALLOPROTEINASES

Inflammatory joint diseases that frequently lead to pain, joint damage, and eventually disfiguration and disability affect around 1% of the population[1]. As the cause of these diseases including rheumatoid arthritis (RA) is not known, the current theory is a "multifactorial threshold model," in which many genetic and environmental influences must act on the same person for the disease to manifest[1]. Thus the first line of treatment is to primarily suppress the inflammatory response at the joint site[2]. In normal joints, a small amount of synovial fluid (SF) lubricates cartilage and the synovium, and acts as a reservoir for solutes and a few resting mononuclear and synovial cells[3]. During chronic inflammation, SF volume and the concentration of immune cells and soluble proteins increase[4]. A salient feature of RA and other forms of inflammatory arthritis is synovial hyperplasia characterized by fibroblast-like synoviocyte (FLS) proliferation and inflammatory cell infiltration into the subintima[5]. The FLS, which comprise about two-thirds of the synovium population, have a well defined secretory system[5] and secrete large amounts of destructive matrix metalloproteases (MMP)[6], specifically MMP-1, 3, 8, 9, 10, 11, and 13[7-9]. Numerous researchers have shown that MMP-1 and MMP-3 play important roles in joint damage and that the most abundant collagenase is MMP-1[6]. Both MMP-1 and MMP-3 are biomarkers that have been shown to have predictive validity for structural damage in arthritis[10,11]. Local expression of MMP in inflammatory arthritis, especially MMP-1, is particularly prominent in the joint pannus adjacent to the site of cartilage and bone destruction[12], and the FLS in the pannus are the major source of MMP. The collagenases,

From the Department of Surgery, University of British Columbia, Vancouver, British Columbia, Canada.

Supported by the Canadian Institute of Health Research (NSM 66119) and in part by the Canadian Arthritis Network. Prof. Maksymowych is a Senior Scholar of the Alberta Heritage Foundation for Medical Research.

R.T. Kilani, PhD; A. Ghahary, PhD, BC Professioonal Fire Fighters' Burn and Wound Healing Research Lab, Department of Surgery, University of British Columbia, Vancouver, British Columbia; W.P. Maksymowych, FRCP; Y. Li, MD, PhD, Department of Medicine, University of Alberta, Edmonton, Alberta; A. Aitken, PhD, School of Biological Sciences, the University of Edinburgh, Edinburgh, UK; G. Boire, MD, Department of Medicine, Division of Rheumatology, University of Sherbrooke, Sherbrooke, Quebec; Y. St-Pierre, PhD, INRS-Institut-Armand-Frappier, University of Quebec, Laval, Quebec.

Address reprint requests to A. Ghahary, 351-2660 Oak Street, Jack Bell Research Centre, Vancouver, BC V6H 3Z6, Canada. E-mail: aghahary@interchange.ubc.ca

Accepted for publication April 25, 2007.

particularly MMP-1, are the only enzymes capable of cleaving native collagen molecules at neutral pH, rendering them susceptible to further enzymatic degradation[13].

Known factors that activate FLS to produce MMP-1 are the classical proinflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α)[14]. These cytokines are capable of stimulating the production of other potent MMP, including stromelysins, in synovial fibroblasts and chondrocytes *in vitro*[15]. Further, direct cell contact of FLS with membrane-associated TNF-α and IL-1α from blood-derived activated T cells, or synovial CD4+ and CD8+ T cell clones, induces expression of MMP-1[16]. T cells can activate FLS to produce an array of inflammatory mediators[17]. Similarly, FLS contact with T cells leads to T cell activation, proliferation, and cytokine production[18]. Thus, FLS/T cell interaction is important in the pathogenesis of RA. Indeed, it has been suggested that the therapeutic anti-TNF-α antibodies act by blocking FLS/T cell interactions and activation through neutralization of T cell membrane-associated TNF-α[19].

We recently conducted experiments to test our working hypothesis that a keratinocyte-derived antifibrogenic factor might function as a wound-healing stop signal(s) by modulating the expression of key extracellular proteins such as collagenase (MMP-1) and/or possibly collagen type I and type III in fibroblasts[20]. To test this hypothesis, we established a model of keratinocyte/fibroblast coculture system and observed a significant increase in MMP-1 expression at the levels of mRNA and enzyme activity in fibroblasts cocultured with keratinocytes relative to that of a control. To identify any keratinocyte-conditioned medium (KCM) related MMP-1-stimulating factors for dermal fibroblasts, sequential chromatography of KCM followed by peptide purification, mapping, and amino acid sequencing on the active fraction of KCM were conducted. Stratifin, also known as 14-3-3 sigma (σ), was then identified to be the MMP-1-stimulating active fraction of KCM. Further experiments confirmed that recombinant stratifin mimics the MMP-1-stimulating effect of KCM in dermal fibroblasts[20].

The 14-3-3 proteins are an abundant family of acidic dimeric molecules that have a wide range of functions. There are 7 known mammalian isoforms, ß, γ, ε, π, σ, τ, and ζ[21]. Since the discovery of the first 14-3-3 protein in 1967[22], the members of the 14-3-3 protein family have been repeatedly rediscovered based on their new biological activities, primarily in signal transduction pathways. They have been identified as activators of tryptophan and tyrosine hydroxylase[23] and protein kinase C (PKC) inhibitors[24]. Subsequent studies identified the 14-3-3 proteins as molecules that interact with PKC, Raf family members, and now more than 200 other intracellular proteins with critical biological functions[25,26], including cellular response to DNA damage and cell cycle regulation[27,28]. Our finding regarding the MMP-1 stimulatory effect of 14-3-3 for fibroblasts is the first indication of a relevant extracellular biological function for this important family of proteins[20]. Since it has been shown that MMP-1 and MMP-3 are biomarkers that have predictive validity for joint structural damage, we asked 2 specific questions: (1) are 14-3-3 proteins detectable in SF of patients with inflamed joints, and if so, what isoform(s); and (2) is there a correlation between the levels of these proteins and those of MMP-1 and MMP-3.

MATERIALS AND METHODS

Synovial fluid and serum samples. In general, 2 sets of synovial and serum samples were analyzed in our study. Table 1 describes the demographic features and disease modifying treatment of the first set of 17 SF samples from patients attending a rheumatology outpatient clinic that were analyzed for 14-3-3 η. Table 2 shows a second set of 12 matching serum and SF samples from patients attending a rheumatology outpatient clinic that were analyzed for 14-3-3 η, γ, MMP-1, and MMP-3. To collect these samples, patients were evaluated by rheumatologists from the Rheumatology Division at either the University of Alberta (Table 1) or the Centre Hospitalier Universitaire de Sherbrooke (Table 2). Samples were almost entirely obtained from knee joints. The most common diagnosis was RA (48.3%), followed by spondyloarthritis (SpA; 20.7%), and osteoarthritis (17%). Most patients with RA had active disease [Disease Activity Score (DAS) > 3.2] and virtually all were receiving a standard disease modifying agent at the time of joint aspiration. Only one patient, who had SpA, was receiving anti-TNF-α therapy. Criteria for admission to the study were the clinical indication for a therapeutic and/or diagnostic arthrocentesis of one or several articulations and willingness to participate. After informed consent had been obtained, serum samples and SF were collected in excess of the 2 ml needed for clinical evaluation (i.e., bacterial culture and SF analysis). All sera were stored at –80˚C until used. The SF were clarified by centrifugation at 1500 g for 15 min and stored in aliquots at –80˚C for subsequent analysis.

Western blot analysis. For Western blot analysis, 2 µl of either a pooled sample or an individual patient's SF sample and serum was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% (wt/vol) acrylamide gel, and electrotransferred onto PVDF membranes (Millipore, Bedford, MA, USA). Nonspecific proteins on membranes were blocked in 5% skim milk powder in phosphate buffered saline (PBS) –0.05% Tween20 overnight. Immunoblotting was performed using 2 µg/ml of 7 isoform-specific rabbit anti-human 14-3-3 polyclonal antibodies, which had been raised against acetylated specific peptides for each isoform in the laboratory of one author (AA). The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO, USA) or anti-mouse IgG (Bio-Rad, Hercules, CA, USA) antibodies (1:2500 dilution). Immunoreactive proteins were then visualized using the enhanced chemical luminescence (ECL) plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, England). Keratinocyte cell lysate was used as a positive control. Using the same procedure, each individual patient's matched SF and serum sample were examined by immunoblotting of the samples with specific antibodies against 14-3-3 η, γ, MMP-1, and MMP-3. Levels of these proteins were then quantified by densitometry and used to calculate correlation coefficients between the proteins.

In another set of experiments, pooled samples of either 12 sera from healthy individuals or 12 patient sera (Table 2) were prepared and different volumes of these samples ranging from 0.1 to 2.0 µl/lane were subjected to Western blot analysis using specific antibodies for 14-3-3 η, γ, MMP-1, and MMP-3. For semiquantitative purposes, known quantities of recombinant 14-3-3 η isoform ranging from 0.01 to 2.0 µg/lane were subjected to Western blot in parallel with 2 µl of pooled samples of sera from healthy individuals or patients and a pooled sample of SF using the Western blot procedure described above.

Cloning, expression, and purification of recombinant 13-3-3 η isoform. To clone cDNA for the 14-3-3 η isoform, we used a procedure similar to a method established to clone 14-3-3 σ[20]. Briefly, total RNA was prepared from human keratinocytes by the acid-guanidium-phenol-chloroform method.

Table 1. Demographic features of 17 patients whose synovial samples were analyzed for 14-3-3 ∈ as shown in Figure 3. All samples were obtained from the knee.

Sample	Age, yrs	Sex	Disease	Disease Duration, yrs	DAS	DMARD Treatment
1	75	M	RA	3	6.3	Leflunomide
2	69	F	RA	1	4.7	Nil
3	39	F	JRA	31	NA	Methotrexate
4	29	M	SpA	3	NA	Infliximab, cyclosporine
5	37	M	SpA	14	NA	Nil
6	41	F	RA	1	4.4	Hydroxychloroquine
7	54	M	SpA	31	NA	Nil
8	84	F	RA	7	4.1	Methotrexate
9	51	F	OA	6	NA	Nil
10	67	M	Gout/RA	26	NA	Allopurinol/hydroxychloroquine
11	71	F	RA	2	NA	Methotrexate
12	42	F	RA	2	4.9	Methotrexate/hydroxychloroquine
13	56	F	Gout/RA	10	4.5	Methotrexate
14	62	F	RA	2	6.6	Methotrexate
15	47	F	JRA	39	NA	Penicillamine
16	33	F	RA	5	4.7	Gold
17	36	F	SpA	0.5	NA	Nil

DAS: Disease Activity Score, DMARD: disease modifying antirheumatic drug, JRA: juvenile rheumatoid arthritis, OA: osteoarthritis, SpA: spondyloarthritis, NA: not available.

Table 2. Demographic features of 12 patients whose matched synovial and serum samples were analyzed for 14-3-3 η, γ, MMP-1, and MMP-3 as shown in Figure 4.

Sample	Age, yrs	Sex	Disease	Disease Duration	DAS-CRP	DMARD Treatment	Joint
1	66	M	OA	18 mo	NA	None	Knee
2	55	F	RA/OA	13 yrs	3.50	Prednisone, methotrexate	Knee
3	83	F	RA	30 yrs	6.34	Prednisone, chloroquine	Knee
4	63	F	RA	22 yrs	5.39	Auranofin	Knee
5	31	F	SpA	12 yrs	NA	None	Knee
6	76	M	Inflammatory arthritis	7 days	NA	None	Knee
7	79	F	OA	20 yrs	NA	None	Knee
8	88	F	OA	5 yrs	2.07	Prednisone, azathioprine, hydroxychloroquine	Shoulder
9	74	M	Pseudogout	3 days	NA	None	Knee
10	60	M	RA	13 yrs	3.35	Prednisone, methotrexate, hydroxychloroquine, leflunomide	Olecranon Bursa
11	56	F	SpA	10 yrs	3.82	Leflunomide	Knee
12	66	M	Hemodialysis arthropathy	2 mo	NA	None	Shoulder

cDNA was then synthesized with oligo(dT) primer and MMLV reverse transcriptase (Gibco-BRL, Grand Island, NY, USA). Samples were then incubated at 42˚C for 60 min, and the reaction was terminated by heating at 70˚C for 15 min followed by rapid chilling on ice. Polymerase chain reaction (PCR) amplification was carried out using 14-3-3 ε primer [sense: 5'-GCG AAT TCC TGC AGC GGG CGC GGC TGG CCG A-3' (GAATTC is an EcoR1 restriction site); and antisense: 5'-GCT CGA GCC TGA AGG ATC TTC AGT TGC CTT C-3' (CTCGAG is an XhoI restriction site)]. PCR was carried out for 30 cycles and PCR product was separated by electrophoresis on 1% agarose gel. The separated DNA product was stained with ethidium bromide and visualized under UV light.

DNA in the agarose gel was purified with a QIAEX II gel extraction kit according to manufacturer's instructions (Qiagen, Mississauga, ON, Canada). Purified DNA was then digested with EcoRI/XhoI for 2 h at 37˚C. The digested products were separated by electrophoresis on a 1% agarose gel and the specific DNA band reflecting 14-3-3 η was purified using the QIAEX II gel extraction kit. Finally, the purified DNA was ligated into a pGEX-6P-1-expressing vector using GST fusion protein (Amersham/Pharmacia Biotech).

For bacterial transformation, the ligated products were transformed to competent XL0blue-1 cells with the regular heat shock transformation method. Positive clones were identified by the size of restriction enzyme digested products. DNA sequence was confirmed by fluorescence dNTP sequence analysis. The plasmid DNA containing 14-3-3 η was transformed into protein-expressing bacteria BL-21 (DE3) (Novagene, Madison, WI, USA).

For protein expression, a single positive clone was grown in 100 ml of LB medium containing 50 μg/ml ampicillin for 4–6 h at 29˚C until an OD_{600nm} of 0.4–0.6 was reached. Bacteria were then diluted to 1:10 with fresh LB medium grown in the presence of 0.1 mM of IPTG for 24 h. For protein purification, bacteria were collected by centrifugation and lysed with 50 mM Tris-

HCl (pH 7.4) containing 10 mM EDTA, 5 mM EGTA, Protease cocktail (Sigma), 1% Triton X-100, and 0.5% IGEPAL CA630. Cell lysate was passed through a glutathione Sepharose 4B affinity column. The column was then washed with PBS containing 0.1% Triton X-100 until an OD_{280nm} equal to 0 was reached. Free 14-3-3 η was eluted by PreScission protease digestion according to the manufacturer's instructions (Amersham/Pharmacia Biotech). Purified protein was dialyzed against PBS and concentrated by Centricon (Millipore) and used to treat dermal fibroblasts. To confirm the purity of 14-3-3 η, the expressed proteins before (GST-14-3-3 η) and after (14-3-3 η) digestion were run on SDS-PAGE along with a molecular weight marker.

RESULTS

To address the question of whether there are 14-3-3 proteins in inflammatory joint SF, and if so, what isoform(s), we first evaluated the levels of 7 different isoforms of 14-3-3 proteins (α/ß, γ, ε, η, τ, σ, and ζ) in pooled samples consisting of either serum or SF from 3 different patients (the first 3 patients shown in Table 2), by Western blot analysis using keratinocyte cell lysate as a positive control and size marker for the different isoforms of 14-3-3 proteins. Patients 1 and 2 had evidence of osteoarthritis, while Patients 2 and 3 had evidence of RA. Patient 2 had both disorders. The results revealed a high level of only η and γ isoforms in SF that was markedly higher than that detected in serum (Figure 1). Comparing the levels of these 2 isoforms, the level of 14-3-3 η was at least 3-fold higher than that of 14-3-3 γ detected in the same pooled samples.

Prior to evaluating more synovial samples, we asked whether the 14-3-3 η isoform possesses any MMP-1 stimulatory effect in fibroblasts. To address this question, we cloned 14-3-3 η cDNA into a pGEX-6P-1-expressing vector and as shown in Figure 2 (lane 4), the isolated 14-3-3 η was more than 95% pure. Fibroblast cells were then treated with various concentrations of recombinant (0, 0.5, 1, 2, and 4 μg/ml) 14-3-3 η protein for 24 h (Figure 2B). Western blot analysis showed that fibroblast MMP-1 expression increased in a dose-dependent manner when incubated with increasing doses of recombinant 14-3-3 η protein. As a positive control, the level of MMP-1 expressed by squamous carcinoma cells was also evaluated. As shown in Figures 2B and 2C there was a more than 2-fold increase in MMP-1 expression relative to untreated control cells at the highest dose used (lane 5 vs lane 1). The pattern of ß-actin (Figure 2B) showed that the MMP-1 stimulatory effect of 14-3-3 η is not due to variation of protein loading, as the level of this protein remained the same in treated and untreated samples. This finding suggests that a higher level of 14-3-3 η found in SF is likely to function as an MMP-1 stimulatory factor for mesenchymal cells.

We then evaluated the level of 14-3-3 η in SF samples from 17 patients with inflamed knee joints (Table 1) who had active synovitis despite standard disease modifying therapies (Figure 3). All patients with RA had a DAS score > 4.0. Each sample showed easily detectable, although variable, levels of 14-3-3 η on Western blots. Although numbers are small, this variability is not related to diagnostic category, DAS in RA patients, disease duration, or treatment, and larger studies are required (data not shown).

We then examined to what degree the levels of 14-3-3 γ and η isoforms correlated with those of MMP-1 and MMP-3 in another set of 12 SF and their matched patients' serum samples (Table 2). The results, shown in Figure 4, again revealed an easily detectable level of 14-3-3 η in all samples. Interestingly, the levels of this protein correlated significantly with those of MMP-1 and MMP-3 detected in the same SF and patient serum samples (Figure 4, Table 3). The same 12 different samples were also evaluated for the presence of the 14-3-3 γ isoform, and the result showed a pattern of detectable 14-3-3 γ protein similar to that found for 14-3-3 η in patient SF and serum samples (Figure 4, Table 3). Significant correlations were also noted between serum or SF levels of 14-3-3 η and γ and corresponding samples of both MMP-1 and MMP-3 (Table 3).

To determine the detection level of 14-3-3 η in serum, samples from the 12 patients (Table 2) and 12 age matched healthy individuals were pooled separately, and various volumes ranging from 0.1 to 2.0 μl were then subjected to Western blot. As shown in Figure 5A, the signals for 14-3-3 were easily detectable in quantities as small as 0.1 μl/sample

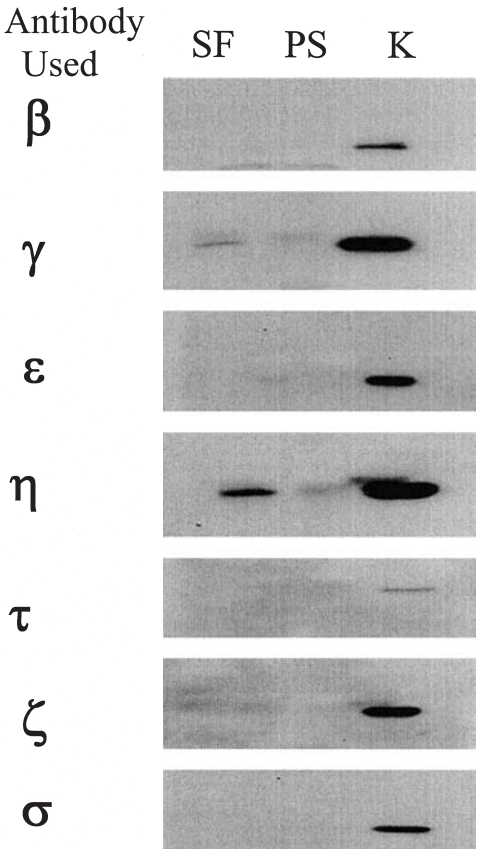

Figure 1. Detection of different isoforms of 14-3-3 in synovial fluid (SF) and serum of patients with inflammatory joints (PS). SF and serum samples related to the first 3 patients whose disease status is shown in Table 2 were pooled for analysis as described. Immunoblotting was performed using 2 μg/ml of 14-3-3 rabbit polyclonal antibodies raised against 7 human isoform-specific peptides. Keratinocyte cell lysate (K) was used as a positive control.

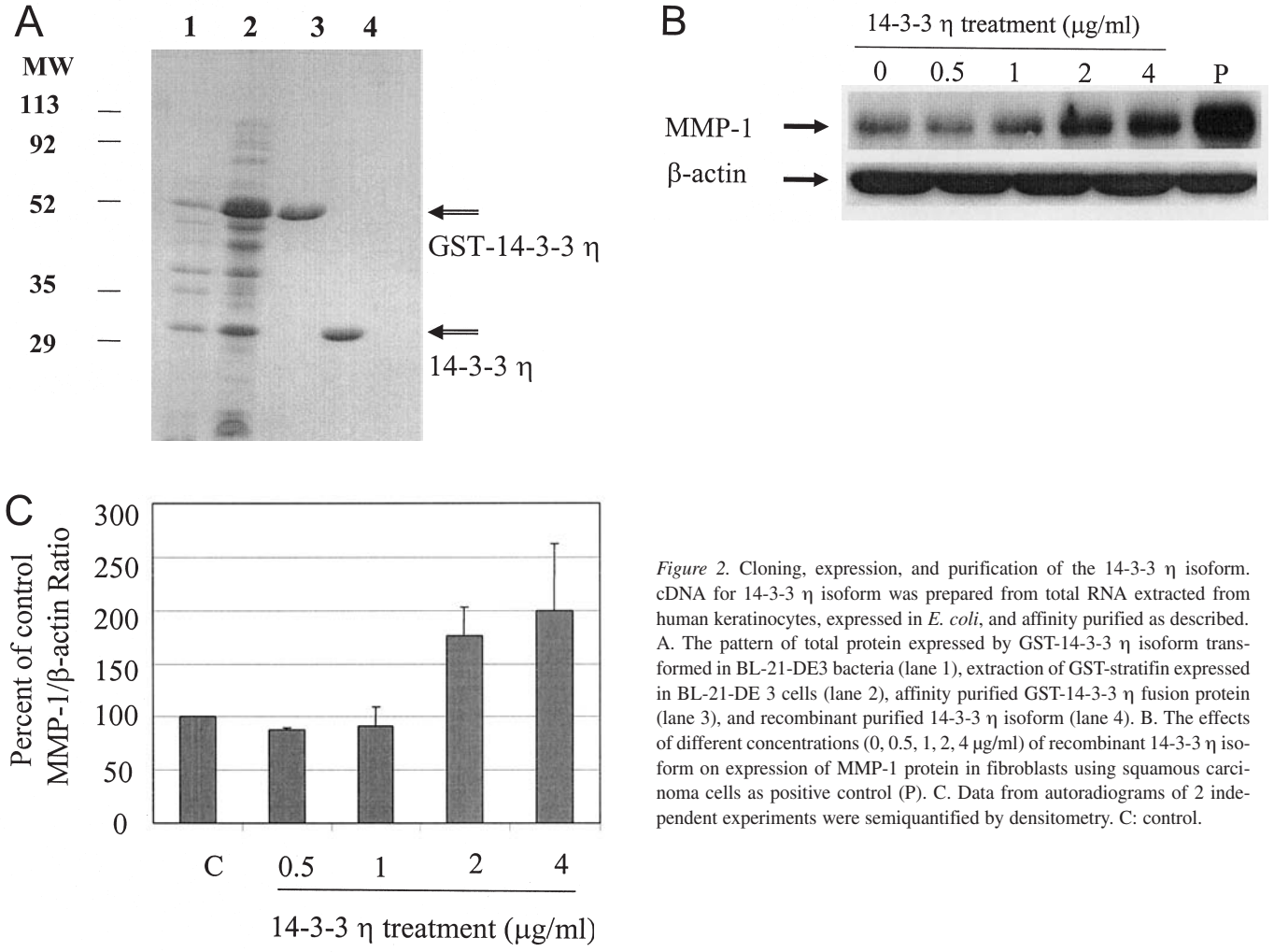

Figure 2. Cloning, expression, and purification of the 14-3-3 η isoform. cDNA for 14-3-3 η isoform was prepared from total RNA extracted from human keratinocytes, expressed in *E. coli*, and affinity purified as described. A. The pattern of total protein expressed by GST-14-3-3 η isoform transformed in BL-21-DE3 bacteria (lane 1), extraction of GST-stratifin expressed in BL-21-DE 3 cells (lane 2), affinity purified GST-14-3-3 η fusion protein (lane 3), and recombinant purified 14-3-3 η isoform (lane 4). B. The effects of different concentrations (0, 0.5, 1, 2, 4 μg/ml) of recombinant 14-3-3 η isoform on expression of MMP-1 protein in fibroblasts using squamous carcinoma cells as positive control (P). C. Data from autoradiograms of 2 independent experiments were semiquantified by densitometry. C: control.

of patient serum. However, the level of 14-3-3 η was barely detectable in normal sera at 0.1 μl. As shown in Figure 5, the level of 14-3-3 η was detectable in normal serum when larger volumes (≥ 1.0 μl) of serum were used. Using the same samples and experimental conditions, the quantities of 14-3-3 γ, MMP-1, and MMP-3 were also evaluated. As shown in Figure 5A, the levels of these proteins were markedly greater in any given volume of serum from patients relative to those of healthy controls. For a semiquantitative comparison, various concentrations of recombinant 14-3-3 η ranging from 0.05 to 2.0 μg/sample (Figure 5B) were run in parallel to an equal volume (2 μl/sample) of pooled samples consisting of sera from either 12 healthy individuals or 12 patients (described in Table 2) as well as 12 SF samples in a Western blot analysis (Figure 5C). The level of recombinant 14-3-3 η was detectable in quantities as small as 0.5 μg/sample and this level increased in proportion to the concentration of 14-3-3 loaded. When the autoradiograms reflecting the 14-3-3 η detected in pooled samples from normal and patient sera and SF were quantified by densitometry, the level of this protein was significantly greater (more than 5-fold) in patient sera relative to normal sera. Consistent with the data shown in Figure 1, the level of 14-3-3 η was even greater (more than 5-fold) in SF than that found in patient serum (Figure 5C). The results showed that for every 2 μl of serum from healthy individuals or patients and SF there is 0.3, 1.1, and 2.7 μg of 14-3-3 η isoform, respectively. This finding indicates that the level of 14-3-3 η is at least 3 times higher in patient sera relative to control sera, and at least 9 times higher in SF samples compared to sera from healthy individuals.

DISCUSSION

The rationale for this study was to address 2 questions: (1) Are 14-3-3 proteins detectable in the synovial fluid of patients with inflamed joints, and if so, what isoform(s) is detectable; and (2) Is there a correlation between the quantities of these proteins and those of MMP-1 and MMP-3? Our findings provide compelling evidence that only 2 specific 14-3-3 isoforms, η and γ, are readily detectable in SF from patients with inflamed joints. Comparing quantities of these 2 isoforms, the

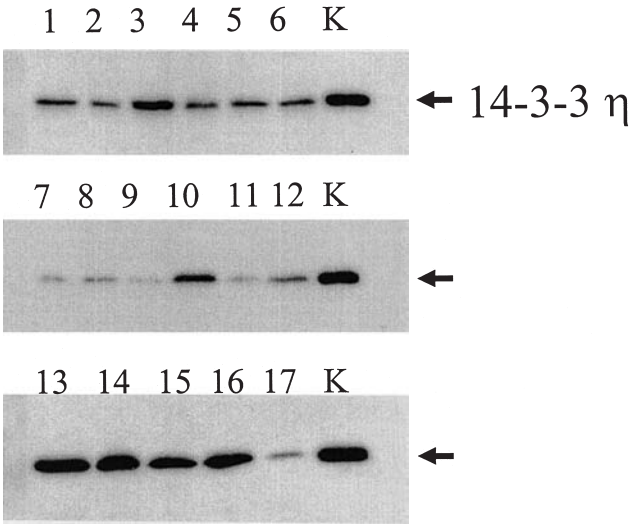

Figure 3. Detection of 14-3-3 η in SF from 17 patients with inflamed joints: 17 samples (2 μl/lane) were assessed for quantities of the most abundant 14-3-3 isoform, η, in synovial samples as shown in Figure 1. The signal for the 14-3-3 η protein was visualized by immunoblotting with anti-14-3-3 η antibody. The remainder of the Western blot analysis was the same as that in Figure 1. Lysate of keratinocytes (K) was used as positive control.

quantity of 14-3-3 η was at least 3-fold greater than that of 14-3-3 γ in the same pooled samples. Interestingly, when the levels of these proteins were compared with those of MMP-1 and MMP-3 in 12 matched serum and SF samples, a significant correlation was noted between 14-3-3 proteins and either MMP-1 or MMP-3. As 14-3-3 σ has a potent MMP-1 stimulatory effect in fibroblasts[18], we then asked whether the 14-3-3 η isoform can also stimulate MMP-1 production in fibroblasts. We observed that fibroblast MMP-1 expression increased in a dose-dependent fashion, suggesting that the high level of MMP-1 seen in inflamed joints might be due at least in part to the MMP-1 stimulatory effect of 14-3-3 η. It is not known if the regulatory effects of TNF-α and IL-1 on MMP expression[14,15] might be mediated through 14-3-3 proteins, and this deserves further study.

Although 14-3-3 proteins are primarily found intracellularly, certain 14-3-3 isoforms are present in the extracellular environment in some degenerative diseases, e.g., cerebrospinal fluid of patients with Creutzfeldt-Jakob disease[29]. Stratifin (14-3-3 σ) was also included in a catalog of proteins found to be secreted by epidermal keratinocytes[30], but no physiological function was assigned to these proteins. Although it is not clear why SF samples from inflamed joints contain such high concentrations of only 14-3-3 η and γ but not other isoforms, it is possible that this reflects differential expression of 14-3-3 isoforms by cells within the synovium. The mechanism by which these proteins are released into SF is also not known. We speculate that these 2 specific isoforms of 14-3-3 may be released from cells via exosomes: a proteomic analysis of proteins from small constitutively secreted membrane vesicles known as exosomes revealed that dendritic cells release three 14-3-3 isoforms[31]. Our recent observations showed that HaCaT cells release exosomes in which the levels of 14-3-3 ß, γ, and η are easily detectable (data not shown). The presence of these isoforms in SF may also be due to synovial cell-releasable exosomes containing 14-3-3 proteins.

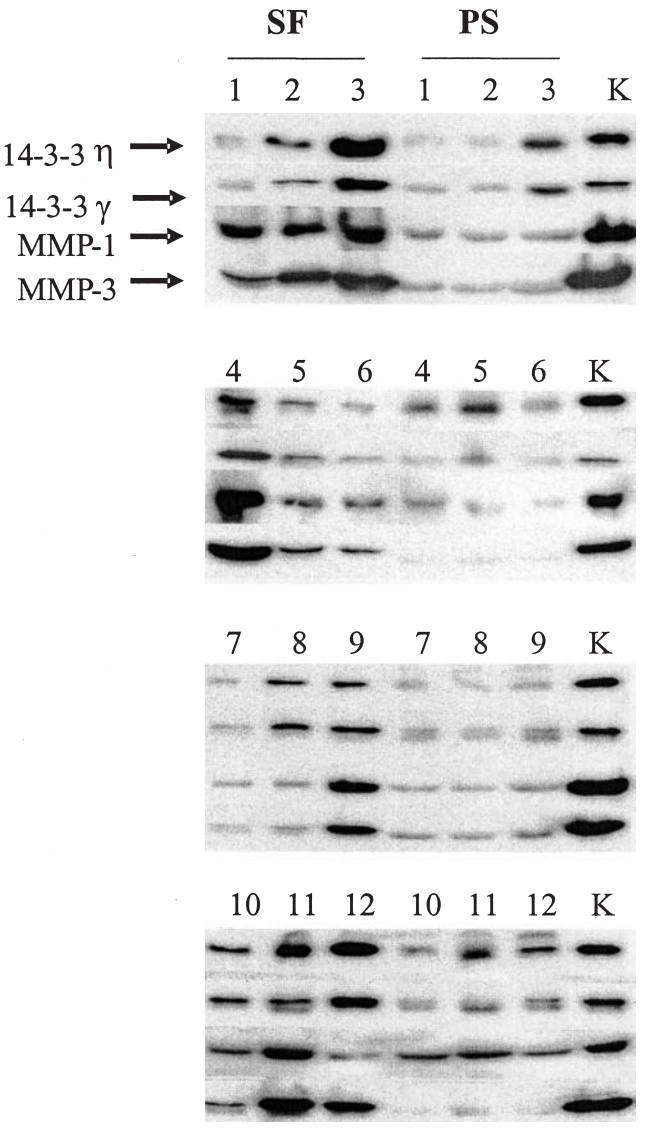

Figure 4. Detection of 14-3-3 η, 14-3-3 γ, MMP-1, and MMP-3 in patient serum samples (PS) and corresponding SF samples from 12 patients described in Table 2. SF or serum sample (2 μl/lane) was individually assessed by immunoblotting with specific antibodies against 14-3-3 η, 14-3-3 γ, MMP-1, or MMP-3. Keratinocyte cell lysate (K) was used as a positive control.

The presence of high levels of 14-3-3 η and γ isoforms in SF and serum samples from patients with inflamed joints provides the first indication of their possible involvement in arthritis. Thus, our results generate a new field of research through which questions of source, target, and biological activity of these proteins in arthritic joints may be addressed.

Table 3. Correlation coefficient (r) and p values of either MMP-1 or MMP-3 with corresponding serum and synovial levels of 14-3-3 η and γ in 12 patients (described in Table 2).

	Serum 14-3-3 η	Synovial 14-3-3 η	Serum 14-3-3 γ	Synovial 14-3-3 γ
MMP-1	r = 0.62, p = 0.02	r = 0.83, p = 0.003	r = 0.77, p = 0.02	r = 0.65, p = 0.03
MMP-3	r = 0.68, p = 0.01	r = 0.77, p = 0.003	r = 0.80, p = 0.03	r = 0.76, p = 0.04

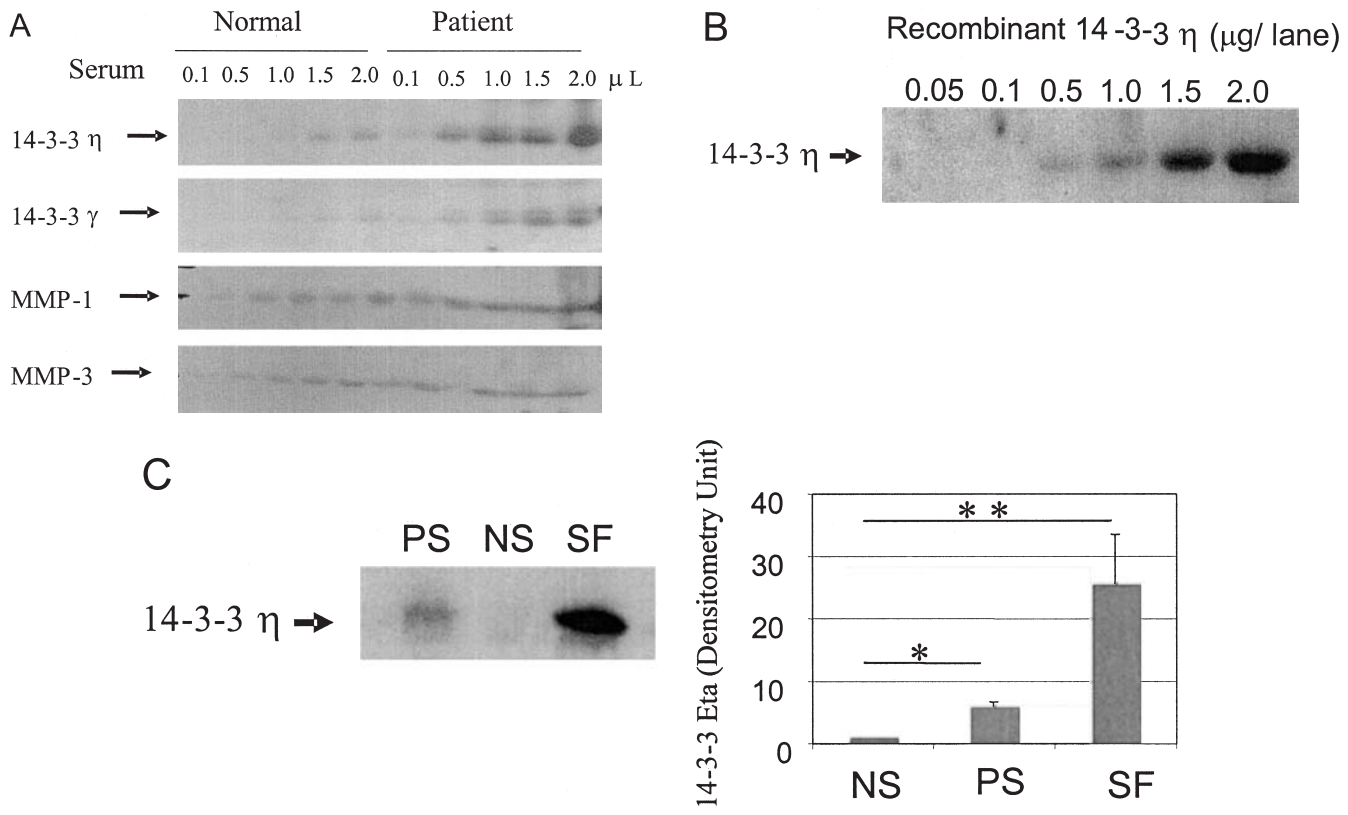

Figure 5. Detection of 14-3-3 η, γ, MMP-1, and MMP-3 in different volumes of normal and patient sera. A. Pooled samples of 12 sera from healthy individuals or those with arthritis (Table 2) ranging from 0.1 to 2.0 μl/lane were analyzed by Western blot using specific antibodies for 14-3-3 η, γ, MMP-1, and MMP-3. B and C. Known quantities of recombinant 14-3-3 η isoform ranging from 0.01 to 2.0 μg/lane (panel B) were analyzed by Western blot in parallel with 2 μl from pooled samples of normal serum (NS), patient serum (PS), and SF (panel C). The signals related to 3 independent autoradiograms similar to those shown in panel C were quantified by densitometry (graph). Significance of differences in 14-3-3 η levels between normal and patient samples, *p < 0.05 and **p < 0.01, respectively (Student t test).

We have used a microarray analysis and shown that 14-3-3 proteins stimulate other members of the MMP protein family such as stromelysin-1 (MMP-3), MMP-2, neutrophil collagenase (MMP-8), and membrane-type 5 MMP (MMP-24) in dermal fibroblasts[32]. Thus, because local expression of MMP in arthritis is particularly prominent at the site of cartilage and bone destruction[12], our observation of a direct correlation between MMP-1 and MMP-3 with 2 specific isoforms of 14-3-3, η and γ, suggests that either of these isoforms or both might be involved in the process that leads to structural damage. Due to the diagnostic heterogeneity of patients from whom samples were obtained, it would be difficult to draw any definitive conclusions as to the source of variability in levels of 14-3-3 η.

In summary, 14-3-3 η and γ are specific isoforms found in serum and SF from patients with inflamed joints; the level of 14-3-3 η isoform was higher in SF compared to serum; there was a significant correlation between the levels of 14-3-3 η and γ and those of MMP-1 and MMP-3 in both SF and patient serum samples; and sera from healthy individuals contain very low levels of these isoforms. Finally, this is the first report describing the presence of 2 specific 14-3-3 isoforms in SF of patients with inflamed joints; this suggests new directions in research focusing on the primary basis of joint damage in inflammatory diseases.

REFERENCES

1. Harris ED Jr. History and epidemiology of rheumatoid arthritis:

How long has it affected us, and who is at risk? In: Harris ED Jr. Rheumatoid arthritis. Philadelphia: W.B. Saunders; 1997:21-7.
2. Harris ED Jr. Introduction. In: Harris ED Jr. Rheumatoid arthritis. Philadelphia: W.B. Saunders; 1997:xix-xxiii.
3. Harris ED Jr. Rheumatoid synovium: Complex, and more than the sum of its parts. In: Harris ED Jr. Rheumatoid arthritis. Philadelphia: W.B. Saunders Company; 1997:127-49.
4. Raza K, Falciani F, Curnow J, et al. Early rheumatoid arthritis is characterized by a distinct and transient synovial fluid cytokine profile of T cell and stromal cell origin. Arthritis Res Ther 2005;7:R784-95.
5. Firestein GS. Rheumatoid synovitis and pannus. In: Klippel JH, Dieppe PA, editors. Rheumatology. London: Mosby; 1997: 5/13.1-5/13.5.
6. Pap T, Shigeyama Y, Kuchen S, et al. Differential expression pattern of membrane-type matrix metalloproteinases in rheumatoid arthritis. Arthritis Rheum 2000;43:1226-32.
7. Tolboom TC, Pieterman E, van der Laan WH, et al. Invasive properties of fibroblast-like synoviocytes: correlation with growth characteristics and expression of MMP-1, MMP-3, and MMP-10. Ann Rheum Dis 2002;61:975-80.
8. Lindy O, Konttinen YT, Sorsa T, et al. Matrix metalloproteinase 13 (collagenase 3) in human rheumatoid synovium. Arthritis Rheum 1997;40:1391-9.
9. Ahrens D, Koch AE, Pope RM, Stein-Picarella M, Niedbala MJ. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. Arthritis Rheum 1996;39:1576–87.
10. Tchetverikov I, Lard LR, DeGroot J, et al. Matrix metalloproteinases-3, -8, -9 as markers of disease activity and joint damage progression in early rheumatoid arthritis. Ann Rheum Dis 2003;62:1094-9.
11. Green MJ, Gough AK, Devlin J, et al. Serum MMP-3 and MMP-1 and progression of joint damage in early rheumatoid arthritis. Rheumatology Oxford 2003;42:83-8.
12. Konttinen YT, Ceponis A, Takagi M, et al. New collagenolytic enzymes/cascade identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above. Matrix Biol 1998;17:585-601.
13. Katrib A, McNeil HP, Youssef PP. What can we learn from the synovium in early rheumatoid arthritis? Inflamm Res 2002; 51:170-5.
14. Harris ED Jr. Cytokines, lymphokines, growth factors, and chemokines. In: Harris ED Jr. Rheumatoid arthritis. Philadelphia: W.B. Saunders; 1997:105-25.
15. Jasser MZ, Mitchell PG, Cheung HS. Induction of stromelysin-1 and collagenases synthesis in fibrochondrocytes by TNF-alpha. Matrix Biology 1994;14:241-9.
16. Burger D, Rezzonico R, Li JM, et al. Imbalance between interstitial collagenase and tissue inhibitor of metalloproteinases 1 in synoviocytes and fibroblasts upon direct contact with stimulated T lymphocytes: involvement of membrane-associated cytokines. Arthritis Rheum 1998;41:1748-59.
17. Yamamura Y, Gupta R, Morita Y, et al. Effector function of resting T cells: activation of synovial fibroblasts. J Immunol 2001;166:2270–5.
18. Miranda-Carus ME, Balsa A, Benito-Miguel M, Perez de Ayala C, Martin-Mola E. IL-15 and the initiation of cell contact-dependent synovial fibroblast-T lymphocyte cross-talk in rheumatoid arthritis: effect of methotrexate. J Immunol 2004;173:1463–76.
19. McInnes IB, Leung BP, Liew FY. Cell-cell interactions in synovitis. Interactions between T lymphocytes and synovial cells. Arthritis Res 2000;2:374-8.
20. Ghahary A, Karimi-Busheri F, Marcoux Y, et al. Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. J Invest Dermatol 2004;122:1188-97.
21. Fu H, Subramanian RR, Masters SC. 14-3-3 proteins: structure, function, and regulation. Annu Rev Pharmacol Toxicol 2000;40:617-47.
22. Moore B, Perez VJ. Specific acidic proteins of the nervous system. In: Carlson FD, editor. Physiological and biochemical aspects of nervous integration. Englewood Cliffs, NJ: Prentice Hall; 1967:343-59.
23. Ichimura T, Isobe T, Okuyama T, et al. Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. Proc Natl Acad Sci USA 1988;85:7084-8.
24. Toker A, Ellis CA, Sellers LA, Atiken A. Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. Eur J Biochem 1990;191:421-9.
25. Craparo A, Freund R, Gustafson T. 14-3-3 (epsilon) interacts with the insulin-like growth factor I receptor and insulin receptor substrate I in a phosphoserine-dependent manner. J Biol Chem 1997;272:11663-9.
26. Yaffe MB. How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. FEBS Letts 2002;513:53-7.
27. Chan TA, Hermeking H, Lengauer C, Kinzler KW, Volgelstein B. 14-3-3 Sigma is required to prevent mitotic catastrophe after DNA damage. Nature 1999;401:616-20.
28. Laronga C, Yang HY, Neal C, Lee MH. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. J Biol Chem 2000;275:23106-12.
29. Boston PF, Jackson P, Thompson RJ. Human 14-3-3 protein: radioimmunoassay, tissue distribution, and cerebrospinal fluid levels in patients with neurological disorders. J Neurochem 1982;38:1475-82.
30. Katz AB, Taichman LB. A partial catalog of proteins secreted by epidermal keratinocytes in culture. J Invest Dermatol 1999;112:818-21.
31. Thery C, Boussac M, Veron P, et al. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. J Immunol 2001;166:7309-18.
32. Ghaffari A, Li Y, Karami A, Ghaffari M, Tredget EE, Ghahary A. Fibroblast extracellular matrix gene expression in response to keratinocyte-releasable stratifin. J Cell Biochem 2006;98:383-93.