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Nuclear Vitamin K_2 Binding Protein in Human Osteoblasts

HOMOLOGUE TO GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

Kazuko Hoshi, Kohji Nomura,† Yoshihisa Sano‡ and Yasuko Koshihara*§*

DEPARTMENTS OF *NUTRITION, †PROTEIN BIOCHEMISTRY, TOKYO METROPOLITAN INSTITUTE OF GERONTOLOGY, TOKYO 173-0015; AND ‡TSUKUBA RESEARCH LABORATORY, THE EISAI CO., TSUKUBA, IBARAKI 300-26, JAPAN

ABSTRACT. The importance of vitamin K in bone metabolism has been suggested previously. The binding protein of vitamin K_2 (menatetrenone, 2-methyl-3-*all-trans*-tetraphenyl-1,4-naphthoquinone, menaquinone-4), found in nuclear extract of human osteoblasts, binds to vitamin K_1 and K_2, but not K_3. Since the binding protein does not bind to other steroids or vitamins, such as hydrocortisone, vitamin A, 1,25$(OH)_2$vitamin D_3, trolox (a derivative of vitamin E), and warfarin, a specific binding protein to vitamin K_1 and vitamin K_2 in osteoblasts was suggested. The size of the specific binding protein was revealed to be 6S by sucrose density gradient and about 40,000 daltons by SDS–PAGE. Twenty amino acid residues from the N-terminal were the same as human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), but the 21st residue, alanine, was replaced with serine. The binding protein was precipitated with anti-human GAPDH antibody, and authentic human GAPDH could bind vitamin K_2. We propose that the nuclear binding protein for vitamin K_2 exists in nuclei similarly to other vitamin receptors and that the molecular structure is very close to human GAPDH. BIOCHEM PHARMACOL **58**;10:1631–1638, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. vitamin K; osteoblasts; binding protein; GAPDH; menatetrenone; human

The role of vitamin K in bone metabolism *in vivo* and *in vitro* has received much attention recently. MK-4[‖], one form of vitamin K_2 with four isoprene units at the 3 position of the quinone structure, has a significant therapeutic effect on involutional osteoporosis [1]. One of the important functions of vitamin K in bone metabolism is the post-translational modification of osteocalcin, that is, vitamin K serves as a cofactor of γ-carboxylase, which converts glutamic acid residue (Glu) into γ-carboxyglutamic acid residue (Gla) in osteocalcin molecules [2]. However, other functions without post-translational modification have been found: (a) inhibition of osteoclast formation and resorption activity [3, 4], (b) inhibition of osteoclast bone resorption by inducing osteoclast apoptosis [5], (c) inhibition of 1,25$(OH)_2$vitamin D_3-induced osteocalcin mRNA level [6], and (d) inhibition of prostaglandin synthetase activity through inducing new protein biosynthesis [7]. These functions suggest the existence of a specific binding protein in osteoblasts or osteoclasts as well as other hormones or vitamins. Until now, there has been no direct evidence that vitamin K_2 binds a specific protein. Since vitamin E and vitamin K, fat-soluble vitamins, are scarcely soluble in water, the binding proteins have not been investigated. However, vitamin E (α-tocopherol) transfer protein, which exhibits a structural homology with cellular retinaldehyde-binding protein, was found in rat liver cytosol [8].

In this study, we revealed a specific binding protein for vitamin K_2 in human osteoblasts derived from ulnar periosteum, whose cells had osteoblastic characteristics such as production of both type I collagen and osteocalcin [9, 10], an increase in intracellular cyclic AMP induced by parathyroid hormone treatment, alkaline phosphatase activity of over 160 IU/10^5 cells, and accumulation of hydroxyapatite. The vitamin K_2 binding protein was different from α-tocopherol transfer protein and was 95.2% identical to human GAPDH, which is a glycolytic protein. However, GAPDH has been shown recently to have many new functions in human cells, as reviewed by Sirover [11], such as uracil DNA glycosylase activity that mediates DNA repair [12] as well as binding to transfer RNA [13] and DNA [14, 15]; tubulin binding [16]; and participation in neuronal and non-neuronal cell death after nuclear translocation [17]. These authors propose that GAPDH is a general mediator of cell death and uses nuclear translocation as a signaling mechanism. GAPDH is often regarded as a static marker protein in contrast to the dynamic alterations of other proteins.

In osteoblasts, vitamin K_2 binding protein, having high homology to GAPDH, may play roles in bone metabolism

§ Corresponding author: Yasuko Koshihara, Ph.D., Department of Nutrition, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan. Tel. (81) 3-3964-3241, Ext. 3095; FAX (81) 3-3579-4776; E-mail: ykoshi@center.tmig.or.jp

‖ *Abbreviations:* MK-4, menaquinone-4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PDL, population doubling levels; NSF, nuclear soluble fraction; and PBS, Ca^{2+} and Mg^{2+}-free phosphate-buffered saline.

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except in the post-translational region. In this study, the specificity of vitamin K binding protein to other vitamins or hormones was investigated.

MATERIALS AND METHODS

Materials

Vitamin K_2 (menatetrenone, 2-methyl-3-*all-trans*-tetraprenyl-1,4-naphthoquinone, MK-4) was synthesized chemically by the Eisai Co. Purity and quality were confirmed by HPLC and NMR; the purity was 99.8%. Vitamin K_2 labeled with ^3H on the side chain (sp. act. 4140 MBq/mg), almost 100% pure by HPLC analysis, was supplied by the Eisai Co. Fetal bovine serum (FBS) and α-minimum essential medium (α-MEM) were purchased from Irvine Scientific. Human GAPDH from human erythrocytes (EC 1.2.1.12), pullulanase (EC 3.2.1.41), and activated charcoal were obtained from the Sigma Chemical Co. Dextran T-70 was from Pharmacia LKB. Anti-human GAPDH polyclonal antibody against the synthetic peptide was from Trevigen Inc. Purified IgG was from Organon Teknika Co., protein A-agarose was from Oncogene Research Products (Calbiochem).

Cell Culture

Human osteoblasts were isolated from the periosteum of a patient who gave his informed consent prior to the procedure. The periosteum specimen from the ulna was obtained from a 20-year-old patient who underwent surgery for treatment of a traumatic fracture. The details of osteoblast isolation from the periosteum have been described previously [9, 18]. In brief, cell populations migrated from an explant were obtained by screening for alkaline phosphatase activity above 160 IU/well. These cells (SaM-1) were cultured in α-MEM supplemented with 10% FBS at 37° in 5% CO_2/95% air. Confluent cells were dispersed with 0.05% trypsin containing 0.05% EDTA and then transferred to new plastic dishes in a split ratio of 1:2 or 1:4. The culture medium was replaced three times each week. The cells were identified as osteoblasts according to the characteristic features displayed. Since every clonal cell from the SaM-1 cells produced osteocalcin [19], the possibility of other cell contamination was very low. The cells were differentiated by the addition of 1,25$(OH)_2$ vitamin D_3 after they reached confluence, and the cells began to produce osteocalcin. The cells were diploid cells having mortality with 34 PDL.

Uptake of ^3H-Labeled Vitamin K_2

When osteoblasts at 17–18 PDL in 12-well multi-well plates (4-cm^2/well) reached confluence, they were incubated with 20 nM (2 μCi) [^3H]-vitamin K_2 for 12 hr. [^3H]-Vitamin K_2 was dissolved in DMSO. The final concentration of DMSO was less than 0.1%. After incubation, the cell layer was washed two times with PBS to remove unincorporated [^3H]-vitamin K_2. The cells on the plate were dissolved in 0.2 N NaOH and then added with scintillation fluid (Aquazol, New England Nuclear Dupont). The radioactivities were counted in a liquid scintillation counter.

Preparation of Cytoplasm and Nuclear Fraction

Human osteoblast-like cells (SaM-1 cells) on a 9-cm dish at 21–23 PDL were washed two times with 10 mL PBS and once with homogenization buffer that consisted of 0.25 M sucrose, 0.1 mM EDTA, and 20 mM Tris–HCl, pH 7.4. Cells on a 10-cm dish were scraped into 0.5 mL of the homogenization buffer and homogenized by a teflon–glass homogenizer to disrupt the cells. The cell homogenate from 10–20 dishes was centrifuged at 1500 *g* at 4° for 10 min. The supernatant was centrifuged further at 7500 *g* for 10 min and 105,000 *g* for 20 min, sequentially, to prepare the cytoplasmic fraction. On the other hand, the precipitate was washed once with the homogenization buffer and sonicated to disrupt the nuclei in an extraction buffer that consisted of 0.08 M NaCl, 1 mM EDTA, and 20 mM Tris–HCl, pH 7.4, and then centrifuged at 7500 *g* for 10 min. The resulting supernatant was centrifuged further at 105,000 *g* for 20 min. The final supernatant was designated as the NSF to use for binding with [^3H]-Vitamin K_2.

Binding Assay with [^3H]-Vitamin K_2

One hundred microliters of NSF or cytoplasmic fraction prepared from enucleated fraction by centrifugation at 105,000 *g* for 20 min was incubated at 4° for 3 hr with 20 nM [^3H]-vitamin K_2 in the presence of a 500-fold molar excess of unlabeled vitamin K_2. After that, the fraction was

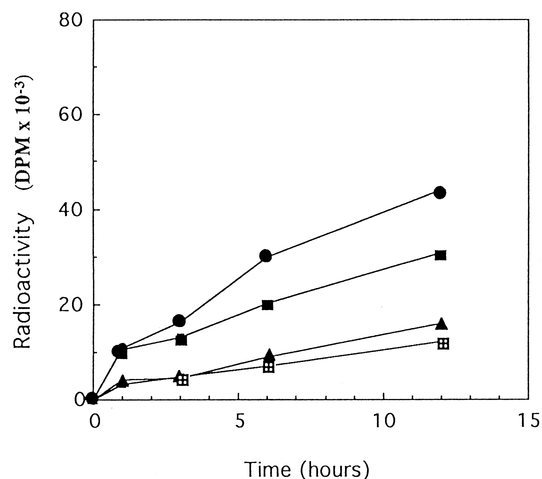

FIG. 1. Uptake of [^3H]-vitamin K_2 into human osteoblasts. Human osteoblasts (SaM-1 cells) incubated with 20 nM [^3H]-vitamin K_2 for 1, 3, 6, or 12 hr were separated into four subcellular fractions [nuclei (●), microsomes (■), cytoplasm (▲), and mitochondria (▦) by sequential centrifugation. The radioactivity of each fraction was counted. Similar results were obtained in three separate experiments.

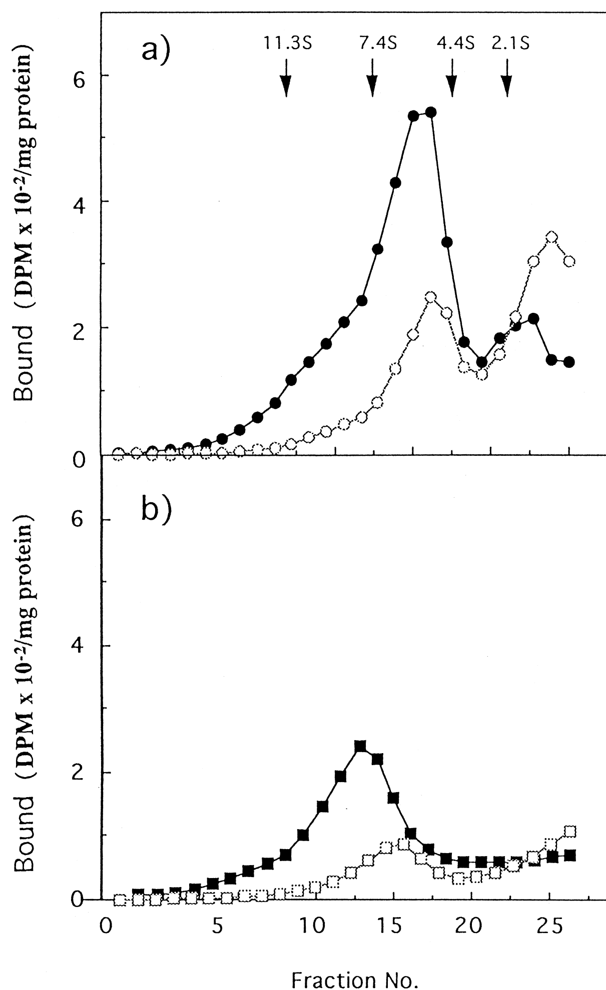

FIG. 2. Vitamin K_2 binding protein by sucrose density gradient. NSF (a) and cytoplasmic fraction (b) prepared from human osteoblasts were incubated with 20 nM [^{3}H]-vitamin K_2 in the presence (○, □) or absence (●, ■) of a 500-fold molar excess of unlabeled vitamin K_2. After removing unbound [^{3}H]-vitamin K_2 with dextran-coated charcoal, the mixture was layered on a 2–25% sucrose density gradient in the presence of 0.3 M KCl and centrifuged at 200,000 g for 18 hr. Similar results were obtained in four separate experiments.

incubated with dextran-coated charcoal, which was prepared from 200 μL of a mixed solution of 1% charcoal and 1% dextran in PBS and centrifuged to remove unbound [^{3}H]-vitamin K_2. The supernatant was layered on a 2–25% (w/v) sucrose density gradient containing 0.05 M Tris–HCl, pH 7.4, 1.5 mM EDTA, 5 mM dithiothreitol, and 0.3 M KCl, and centrifuged at 200,000 *g* for 18 hr. After fractionation, radioactivity of each fraction was counted in a liquid scintillation counter.

SDS–PAGE

Each fraction after sucrose density gradient centrifugation was heat-denatured in the presence of 10 mM β-mercaptoethanol and 0.1% SDS and then analyzed by 10% SDS–PAGE in the presence of 0.1% SDS. Analyzed protein bands were stained with Coomassie brilliant blue. For analysis of the amino acid sequence, western-blot samples were analyzed with a protein sequencer (model 476A, Applied Biosystems, Inc.).

Immunoprecipitation of [^{3}H]-Vitamin K_2

Nuclear fraction (300 μL) was incubated with 20 nM [^{3}H]-vitamin K_2 at 4° for 2 hr, and unbound [^{3}H]-vitamin K_2, separated by charcoal-coated dextran as described above, was incubated with 3 μL of anti-rabbit IgG (1.8 mg/mL) at 4° for 1 hr to avoid non-specific immunoprecipitation. The IgG-adsorbed protein complex was precipitated by the addition of 7.5 μL of protein A-agarose solution (50% in PBS), and centrifuged at 600 *g* for 20 min. The supernatant (200 μL) was incubated with anti-human GAPDH antibody (50.8 mg/mL) or anti-rabbit IgG in various concentrations for 16 hr at 4°. At that time, heat-denatured anti-human GAPDH antibody was used to investigate the specificity of the antibody. After immunoprecipitation by the addition of protein A-agarose, the resulting supernatant fraction was layered on sucrose density gradient and then separated from the remaining binding protein as described previously.

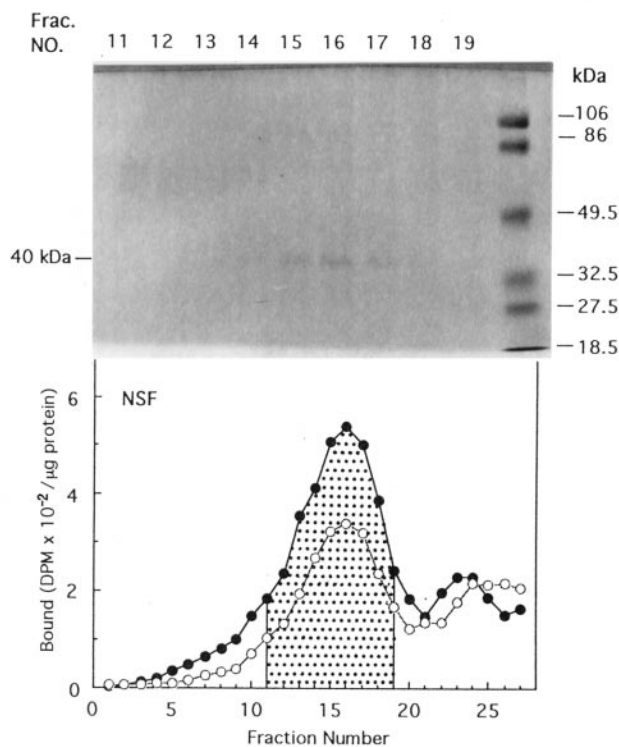

FIG. 3. SDS–PAGE of vitamin K_2 binding protein of NSF. The upper panel shows the band stained with Coomassie brilliant blue after analysis by SDS–PAGE of each fraction separated by sucrose density gradient centrifugation. The fraction numbers coincides with fractions shown in the lower panel, [^{3}H]-vitamin K_2 binding protein separated by sucrose density gradient centrifugation in the presence (○) or absence (●) of a 500-fold molar excess of unlabeled vitamin K_2. Similar results were obtained in three separate experiments.

TABLE 1. Amino acid sequence of vitamin K_2 binding protein

Protein	Sequences	Identity (%)
Vitamin K_2 binding protein	GKVKVGVNGFGRIGRLVTRA S	
Human GAPDH	GKVKVGVNGFGRIGRLVTRA A	95.2
Mouse GAPDH	VKVGVNGFGRIGRLVTRA AIC	94.7
Bovine GAPDH	VKVGVNGFGRIGRLVTRA AFN	94.7

N-Terminal amino acid sequences were analyzed using a protein sequencer model 476A (Applied Biosystems, Inc.).

RESULTS

Uptake of Vitamin K_2 into Human Osteoblasts

First, we investigated the uptake of [^{3}H]-vitamin K_2 into osteoblasts and the localization of radioactivity in subcellular organelles. [^{3}H]-vitamin K_2 was taken up in a time-dependent manner up to 12 hr. In the subcellular fractions, the radioactivity in the nuclear fraction was the highest of the four fractions separated by sequential centrifugation, into nuclei, mitochondria, microsomes, and cytoplasm (Fig. 1). The radioactivity in the cytoplasmic fraction was much lower than that in the microsomal and nuclear fractions. As expected, fairly high radioactivity was detected in the microsomal fraction where the enzyme converting vitamin K to vitamin K hydroquinone and vitamin K epoxide exists. Since it is difficult for [^{3}H]-vitamin K_2 to be transferred into nuclei without so-called carrier protein(s), we next tried to identify the nuclear binding protein to [^{3}H]-vitamin K_2.

Identification of [^{3}H]-vitamin K_2 Binding Protein by Sucrose Density Gradient Centrifugation

Cytoplasm or NSF was incubated with [^{3}H]-vitamin K_2 in the presence or absence of unlabeled vitamin K_2, which removed unbound vitamin K_2 by agitation with charcoal–dextran. Then the fraction was separated by sucrose density gradient centrifugation in the presence of 0.3 M KCl. As shown in Fig. 2, a sharp peak observed around 6S in NSF was diminished significantly by the addition of unlabeled [^{3}H]-vitamin K_2. However, in the cytoplasm, the peak was very small and was seen around 7.4S. In the presence of unlabeled vitamin K_2, the peak was diminished and was observed around 6S, the same as in the nuclear fraction. This pattern implied the existence of a specific binding protein for vitamin K_2 that was located markedly more in nuclei than cytoplasm.

Identification of [^{3}H]-Vitamin K_2 Binding Protein in NSF

After separation by sucrose density gradient centrifugation, each fraction from fraction 11 to fraction 19 was subjected to analysis using SDS–PAGE. As shown in Fig. 3, fraction 11 or 12 showed faint and diffuse bands around 70 kDa. One band around 40 kDa appeared to accompany the radioactivity peak in fractions 15 through 17. The intensity of the band correlated with the peak height of radioactivity. This band was probably a candidate for the binding protein. After western blot analysis of the PVF membrane, the N-terminal amino acid sequence in the band was analyzed by a protein sequencer. As shown in Table 1, vitamin K_2-binding protein was 95.2, 94.7 and 94.7% identical to human, mouse, and bovine GAPDH, respectively. The difference between human GAPDH and binding protein was the 21st amino acid from the N-terminal, which was replaced with alanine instead of serine. The first 2 amino acids and the last 3 amino acids in mouse and bovine GAPDH are different from vitamin K_2-binding protein.

To further identify vitamin K_2 binding protein, the binding activity of authentic human GAPDH to [^{3}H]-vitamin K_2 was investigated. Moreover, other proteins such as albumin, one of the ubiquitous proteins, and pullulanase, a glycolytic protein of the same size (debranching enzyme), were investigated for their specific binding activity to vitamin K_2. Authentic GAPDH specifically bound to [^{3}H]-vitamin K_2, because the radioactivity around 6S on the sucrose density gradient diminished to 20% by the addition of unlabeled vitamin K_2 as well as that seen in NSF. On the other hand, human albumin and pullulanase did not show any radioactive peaks around 6S (Fig. 4). A peak near the top of the gradient did not bind with [^{3}H]-vitamin K_2.

Furthermore, it was confirmed by using a specific antibody that GAPDH is a vitamin K_2 binding protein. As shown in Fig. 5, the radioactive peak around 6S was decreased by the addition of antiserum of GAPDH. The diminution of the peak depended upon the dilution rate of the antiserum. However, a high concentration of heat-denatured antiserum did not decrease the radioactive peak. These data suggest that the vitamin K_2-binding protein is homologous to GAPDH.

Specificity of Vitamin K_2 Binding Protein

NSF of osteoblasts was incubated with [^{3}H]-vitamin K_2 in the presence or absence of a 500-fold molar excess of unlabeled vitamin K analogues or warfarin and separated by sucrose density gradient centrifugation. By the addition of unlabeled vitamin K_1, the radioactive peak clearly decreased, but the potency was lower than that caused by the addition of unlabeled vitamin K_2. Warfarin and

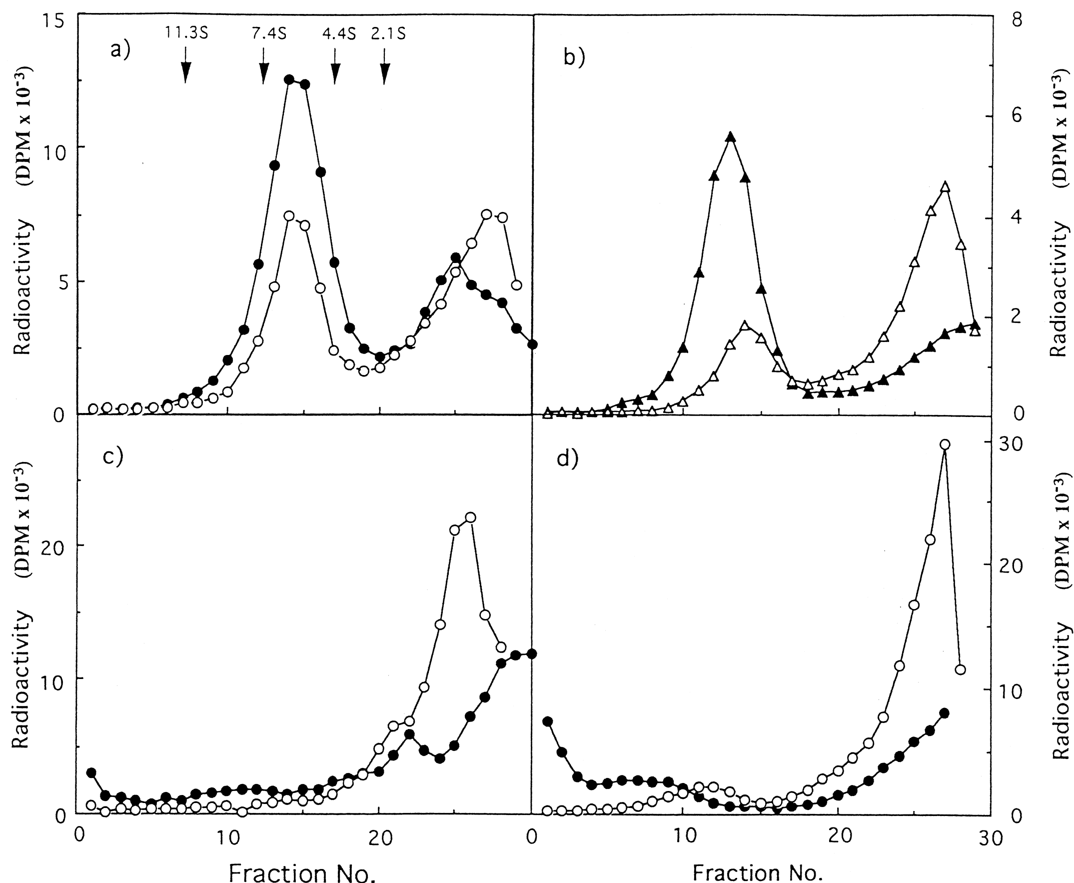

FIG. 4. Specific binding of [^{3}H]-vitamin K_2 to osteoblast NSF or other authentic proteins. NSF (a), authentic human GAPDH (b), human albumin (c), and pullulanase (d) were bound to 20 nM [^{3}H]-vitamin K_2 in the presence (○, △) or absence (●, ▲) of a 500-fold molar excess of unlabeled vitamin K_2. Similar results were obtained in two separate experiments.

vitamin K_3 did not decrease the radioactivity, that is, these two compounds did not compete with [^{3}H]-vitamin K_2 (Fig. 6).

Next, a competition assay was carried out by the addition of 10- to 1000-fold molar excess of unlabeled vitamin K or other compounds, when NSF was incubated with [^{3}H]-vitamin K_2. Unlabeled excess vitamin K_2 inhibited [^{3}H]-vitamin K_2 binding to NSF in a concentration-dependent manner but the addition of hydrocortisone, vitamin A, and trolox did not inhibit the binding (Fig. 7). However, binding was inhibited slightly by $1,25(OH)_2$ vitamin D_3, especially by the addition of a 1000-fold molar excess of $1,25(OH)_2$vitamin D_3 (25% inhibition). These results suggest that osteoblasts have a specific binding protein for vitamin K_2.

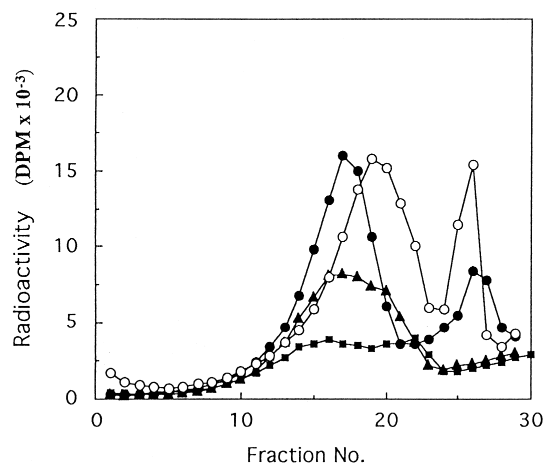

FIG. 5. Inhibition of [^{3}H]-vitamin K_2 binding to osteoblasts NSF by GAPDH antibody. After binding [^{3}H]-vitamin K_2 to NSF and removing unbound [^{3}H]-vitamin K_2 by charcoal, the reaction mixture was incubated with non-immune rabbit serum (●), anti-human GAPDH antibody [50 μg (▲) or 150 μg (■)], and heat-denatured anti-GAPDH antibody [150 μg (○)] as described in Materials and Methods. After immunoprecipitation by the addition of protein A-agarose, the resulting supernatant fraction was layered on a sucrose density gradient and then separated from the remaining binding protein by centrifugation as described in Materials and Methods. Similar results were obtained in three separate experiments.

DISCUSSION

In the 50 years since its discovery, vitamin K has attracted far less research attention than other fat-soluble vitamins. However, the clinical importance and the therapeutic use of vitamin K for the synthesis of four plasma clotting proteins and inhibition of decreased bone mineral density have been investigated. The mechanism of vitamin K

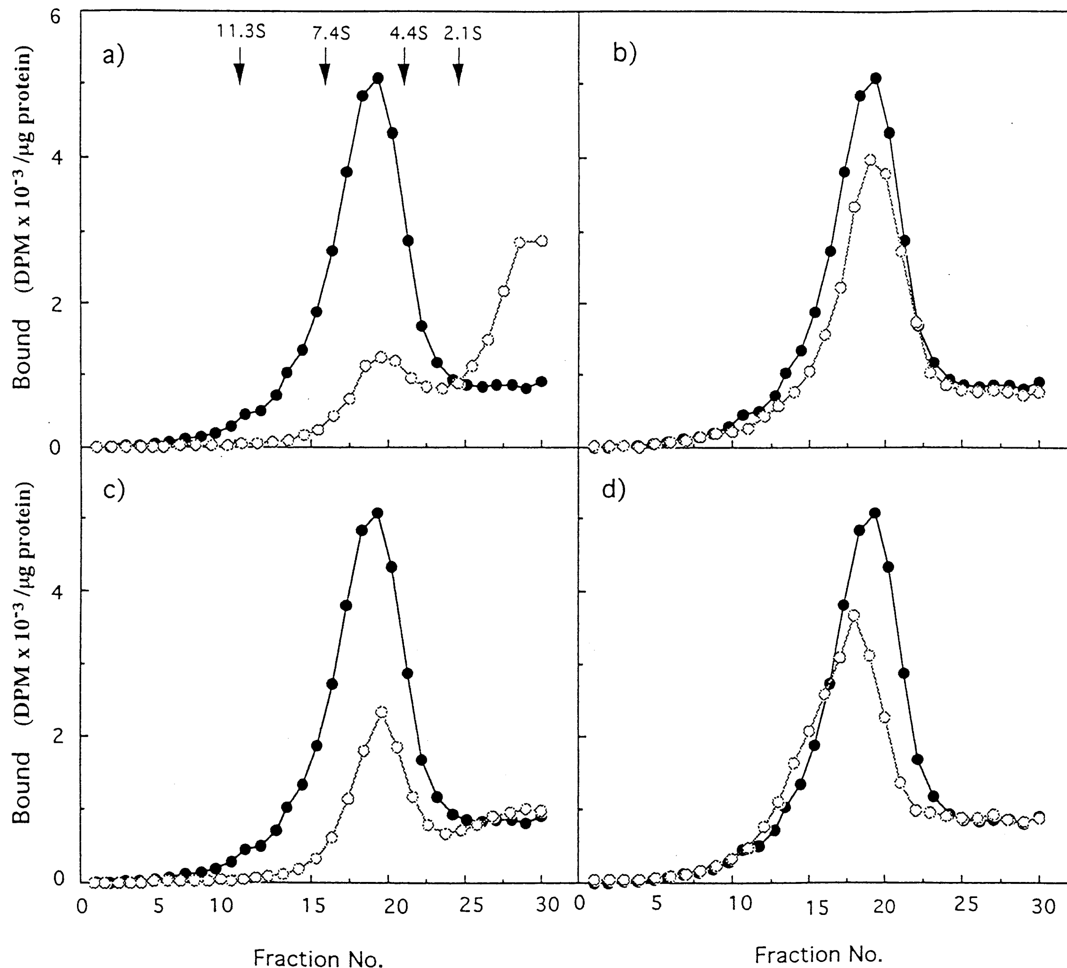

FIG. 6. Inhibition of [^{3}H]-vitamin K_2 binding to NSF of osteoblast-like cells by vitamin K analogues and warfarin. NSF was incubated with 20 nM [^{3}H]-vitamin K_2 in the presence (○) or absence (●) of a 500-fold molar excess of unlabeled vitamin K analogues. Vitamin K_2 (MK-4) (a), warfarin (b) vitamin K_1 (c), or vitamin K_3, (d) was applied on a sucrose density gradient. Similar results were obtained in three separate experiments.

function is thought to be in a role of post-translational regulation by vitamin K-dependent γ-carboxylation reaction of the glutamic acid residue. However, vitamin K has been reported to perform other functions, such as modulation of osteocalcin mRNA expression induced by 1,25(OH)$_2$vitamin D_3 [6], induction of osteoclastic apoptosis [5], and inhibition of osteoblastic proliferation [20]. Those functions indicate that vitamin K directly or indirectly acts on genomic function. The nuclear receptor of farnesol, which is a vitamin K_2 side chain, has been identified by Forman *et al.* [21]. The farnesol receptor is a mammalian orphan receptor that forms a heterodimeric complex with the retinoid X receptor. Farnesol metabolites are generated intracellularly and are required for the synthesis of cholesterol, bile acids, steroids, retinoids, and farnesylated proteins. However, farnesol does not have any effect on the inhibition of osteoclast formation and resorption [4]. On the other hand, geranylgeraniol, the other side chain of vitamin K_2, affects osteoclastic function. Geranylgeraniol also induces the differentiation of human leukemia cells (HL60 cells) to macrophages [22] by penetrating into nuclei and binding to DNA. These reports support the existence of vitamin K_2 binding protein or receptors that are different from geranylgeraniol binding protein. In this study, we presented a specific binding protein of vitamin K_2 in the nuclei of human osteoblasts. It has homology closely related to GAPDH, which is a well-known classical glycolytic protein localized in cytoplasm. However, immunocytochemical analysis demonstrated that the subcellular localization of the human GAPDH protein displayed a defined pattern as a function of cell growth [16]. In non-growing normal human cells, GAPDH was detected only in the cytoplasm. In actively growing human cells, GAPDH was localized in the nucleus or perinuclear regions. Moreover, it was determined recently that GAPDH plays a role not only in glycolysis, but also in apoptosis, as when GAPDH antisense oligonucleotides were found to protect against cytosine arabinonucleotide-induced apoptosis in cultured rat cerebellar neurons [17]. Ishitani and Chuang [23] revealed that the protein was the same as rat

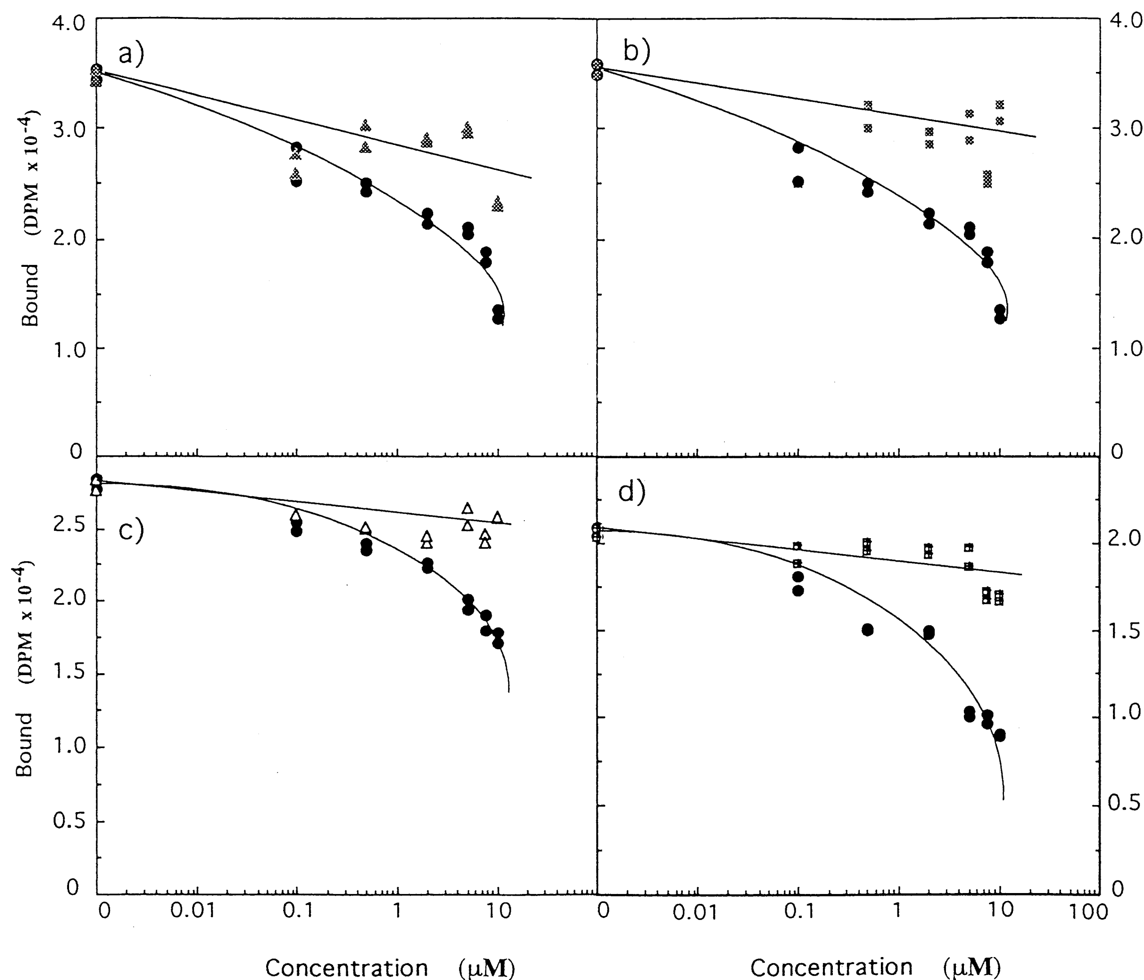

FIG. 7. Inhibition of [^{3}H]-vitamin K_2 binding to osteoblast-like cells by various compounds. NSF of osteoblast-like cells was incubated with 20 nM [^{3}H]-vitamin K_2 in the presence or absence of 10- to 1000-fold molar excess of unlabeled vitamin K_2 (●) or other compounds (▲, ▒, △, ▩): 1,25(OH)$_2$ vitamin D_3 (a), hydrocortisone (b), vitamin A (c), and trolox (water-soluble vitamin E) (d). Similar results were obtained in three separate experiments.

GAPDH except for the 22nd amino acid from the N-terminal to the 23th amino acid. The vitamin K_2 binding protein in this study had homology to GAPDH except for the 21st amino acid from the N-terminal. When GAPDH induced apoptosis, the protein was translocated into the nuclei. Interestingly, vitamin K_2 also induces apoptosis of osteoclasts [5] and inhibits apoptosis of osteoblasts [24]. Although the mechanism is not clear, vitamin K_2 binding protein may be translocated into the nuclei to play a role in apoptosis after adherence to DNA. GAPDH is a highly conserved protein, yet differences in amino acid sequences are known. Many of the new functions of the GAPDH protein were identified in human cells. It is unknown whether all or some of these new activities are restricted to humans. It is not clear whether vitamin K_2 binding protein acts with itself or with a heterodimer or other binding proteins, such as vitamin A receptor and vitamin D_3 receptor [25]. However, the functional vitamin K_2 is probably the polymeric form of the GAPDH (36 kDa) homologue, because of the size of vitamin K_2 binding protein (120 kDa) estimated by sucrose gradient centrifugation. A vitamin K_2-binding factor secreted from *Bacillus subtillis* upon the synthesis of vitamin K_2 was found in 1990 [26]. However, it was a different function from the general meaning of binding protein in nuclei or cytoplasm. We found vitamin K_2 binding protein in osteoblasts.

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