Identification of subunits of the 650 kDa 12(S)-HETE binding complex in carcinoma cells

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Abstract Cytosol and nuclei of Lewis lung carcinoma (LLC) cells contain high affinity binding sites specific for the arachidonic acid metabolite 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE). In this report we present evidence that the cytosolic 12(S)-HETE binding complex also occurs in human erythroleukemia (HEL) and promonocytic leukemia (U937) cells as well as in murine 3T3-L1 preadipocytes but not in intestinal epithelial cells (Int407). The cytosolic 650 kDa 12(S)-HETE-binding complex was found to consist of subunits; raising the ATP concentration in cytosol led to conversion of the 650 kDa complex to a 50 kDa binding component, presumably the actual 12(S)-HETE binding polypeptide. Lowering of the cytosolic concentration of ATP had the opposite effect, i.e., the amount of the 650 kDa complex increased. Another subunit of the 650 kDa complex was identified as heat shock protein 70 (hsp70) by Western blot analyses and coimmunoprecipitation. Hsp70 was present in substoichiometric amounts, in an approximate 1:6 ratio. The multimeric nature of the binding complex and the identification of hsp70 as a subunit suggest that there are similarities between the 12(S)-HETE binding protein and receptors of the steroid/thyroid hormone superfamily.—**Herbertsson, H., T. Kühme, U. Evertsson, J. Wigren, and S. Hammarström.** Identification of subunits of the 650 kDa 12(S)-HETE binding complex in carcinoma cells. *J. Lipid Res.* 1998. **39:** 237–244.

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Lipoxygenases (1) catalyze stereospecific insertion of molecular oxygen into polyunsaturated fatty acids at different positions of their 1,4-*cis*-pentadiene systems to form hydroperoxyeicosatetraenoic acids (HPETEs). HPETEs are either reduced to hydroxyeicosatetraenoic acids (HETEs) or converted to leukotrienes, lipoxins, or hepoxilins. Leukotrienes, formed by 5-lipoxygenase in conjunction with other enzymes, are important mediators in the pathogenesis of allergic and inflammatory diseases (2). The biological function of 5-, 12-, and 15-H(P)ETEs is less well understood. Two distinct types of 12-lipoxygenase with different substrate specificity have been demonstrated (1). 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) is formed in several cells and tissues, e.g., platelets (3), epidermis (4), and nervous system (5). It has been proposed to play a role in platelet activation (6), in the pathogenesis of psoriasis (4, 7), in postsynaptic responses to histamine (5), and in modulating the metastatic potential of melanoma and carcinoma cells (8). It has also been reported to be a mediator of the essential regulation of cell survival and apoptosis by influencing the expression of bcl-2 protein (9).

Lewis lung carcinoma (LLC) cells contain specific high affinity binding sites for 12(S)-HETE (10). The subcellular distribution of these sites is mainly cytosolic and nuclear (11). Size determination by gel permeation chromatography and sedimentation analysis indicated a molecular size of 650 kDa. This report describes the identification of two subunits of the complex and its occurrence in some other cell lines.

MATERIALS AND METHODS

Materials

12(S)-hydroxy-[5,6,8,9,11,12,14,15(n)-^{3}H]eicosatetraenoic acid (specific activity 219 Ci/mmol) and unla-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FPLC, fast protein liquid chromatography; GRP, glucose regulated protein; HEL, human erythroleukemia; 12(S)-HETE, 12(S)-hydroxy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid; hsp70, heat shock protein 70; IBMX, 3-isobutyl-1-methylxanthine; Int407, a human intestinal epithelial cell line (intestine 407); LLC, Lewis lung carcinoma; LTD_4, leukotriene D_4; PBS, phosphate-buffered saline; SE, standard error; TNF, tumor necrosis factor; TPA, 12-O-tetradecanoylphorbol 13-acetate.

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beled 12(S)-HETE were bought from New England Nuclear (Boston, MA) and Cayman Chemicals (Ann Arbor, MI), respectively. Cell medium, antibiotics, and fetal calf serum were purchased from GibcoBRL (Paisley, Scotland). Insulin was obtained from Novo Nordisk (Lyngby, Denmark). Dexamethasone, IBMX (3-isobutyl-1-methylxanthine), TPA (12-O-tetradecanoylphorbol 13-acetate), apyrase, phosphokinase, and creatine phosphate were all Sigma products (St. Louis, MO). Monoclonal hsp70 antibody (MA3-007) was bought from Affinity Bioreagents (Golden, CO) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG from Affiniti (Nottingham, England). ECL Western blotting detection reagents and Hyperfilm were obtained from Amersham (Little Chalfont, England). Protein A Sepharose CL-4B was from Pharmacia Biotech AB (Uppsala, Sweden).

Cell culture

LLC cells (LL/2; CRL 1642) were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown at 37°C in a humidified atmosphere containing 8% (v/v) CO_2 using Dulbecco's modified Eagle's medium supplemented with 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) heat-inactivated (30 min at 56°C) fetal calf serum. Cultures were passaged twice a week using 0.54 mm EDTA in isotonically buffered saline. Cells were not used beyond 20 consecutive passages.

Human erythroleukemia (HEL) cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in RPMI 1640 medium with the same supplements as LLC cells. The culture was kept in a humidified atmosphere containing 5% (v/v) CO_2 and maintained by the addition of fresh medium or replacement of medium every 2–3 days. Cells were kept at a density of 2×10^5–10^6/ml and differentiated by addition of 0.62 ng/ml TPA for 4 days.

U937 promonocytic leukemia cells were obtained from American Type Culture Collection (Rockville, MD). They were grown using RPMI 1640 medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mm l-glutamine, and 10% fetal calf serum. The culture was kept in a humidified atmosphere containing 5% (v/v) CO_2. Medium was replaced every 4–5 days and the cell density was kept at 5×10^5–1.5×10^6/ml. U937 cells were differentiated by addition of 20 ng/ml TPA for 4–5 days.

Human intestinal epithelial cells (Int407) were originally obtained from Flow Laboratories. The cells were grown using Eagle's basal medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mm l-glutamine, and 15% newborn calf serum (atmosphere = 5% (v/v) CO_2). Cells were passaged by use of 0.54 mm EDTA and used for experiments the fifth day after passage.

3T3-L1 cells were originally obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% (v/v) newborn calf serum at 37°C in a humidified atmosphere containing 5% (v/v) CO_2. The cells were passaged every fifth day by use of 0.5 g trypsin and 0.2 g EDTA per liter of Modified Puck's Saline A. Quiescent cells were differentiated by addition of 5 μg/ml insulin (dissolved in 1% BSA-PBS, pH 3), 0.25 μm dexamethasone (in ethanol), and 0.1 mm IBMX (in ethanol) to the medium mentioned above supplemented with 10% fetal calf serum (day 0). On day 2 this medium was changed to the medium containing 5 μg/ml insulin but no dexamethasone or IBMX. On day 4 insulin was also excluded from the medium and thereafter the medium was changed every second day until the experiments were run on days 12–13.

Whole cell binding assay

Adherent cells were washed twice with PBS before incubation with 0.1 nm 12(S)-[^{3}H]HETE with or without nm 12(S)HETE in serum-free medium buffered with 10 mm HEPES. Cells growing in suspension were centrifuged at 200 *g* during washes. After incubating for 2 h at 4°C with gentle shaking, the incubation medium was removed and the adherent cells were washed twice with ice-cold medium. Cells in suspension were centrifuged twice. Adherent cells were removed with a rubber policeman, cell numbers were determined in a hemocytometer, and cell-bound radioactivity was measured in an 1214 Rackbeta liquid scintillation counter. Specific binding was calculated as the difference between the total binding of 12(S)-[^{3}H]HETE and the non-specific binding (determined in the presence of 100 nm unlabeled 12(S)-HETE).

Preparation of cytosol

Cells, preincubated with 12(S)-[^{3}H]HETE for 2 h at 4°C or non-incubated control cells, were washed twice. Adherent cells were removed with a rubber policeman and sedimented at 200 *g* for 5 min. The pellet was suspended in a hypotonic solution (1 mm $NaHCO_3$, 2 mm $CaCl_2$, and 5 mm $MgCl_2$). After swelling for 2 min the cells were homogenized in a Dounce homogenizer using the loose-fitting pestle. Sucrose (0.25 m), Tris-HCl (50 mm) pH 7.5, KCl (25 mm) and $MgCl_2$ (5 mm, final concentrations) were added. The homogenate was centrifuged at 100,000 *g* for 1 h in a Beckman TLA 100.2 rotor and the supernatant was used.

Gel permeation chromatography

A Superdex™200 FPLC™ HR10/30 column (Pharmacia) was eluted at room temperature with 1 ml/min of 25 mm Tris-HCl, 0.25 m NaCl, pH 8.0 (buffer A) or 15 mm Tris-HCl, pH 8.0 (buffer B). The total volume, V_t, was 24 ml, and the void volume, V_0, was determined as described previously (11). Fractions of 1 ml were collected and those containing the binding complex were detected by measuring the amount of tritium using an LKB Wallac 1214 Rackbeta liquid scintillation counter.

Cell-free binding assay

Cytosol from non-incubated LLC cells was fractionated by gel permeation chromatography as described above. Appropriate fractions (in buffer B) were incubated with 1 nm 12(S)-[^{3}H]-HETE in the presence or absence of 1 μm unlabeled 12(S)-HETE for 1 h at 4°C with gentle shaking and then rechromatographed on Superdex™ 200. In other experiments different concentrations (0–10 μm) of unlabeled 12(S)-HETE were used to displace 12(S)-[^{3}H]HETE.

Depletion and regeneration of ATP

In these experiments the following mixture of protease inhibitors was added to the cytosol: 4-(2-aminoethyl)bensenesulfonylfluoride, HCl (60 μg/ml), pepstatin A (0.7 μg/ml), and leupeptin (0.5 μg/ml). ATP was depleted by incubating with apyrase (50 units/ml) for 15 min at room temperature. In some experiments, ATP was regenerated to a final concentration of about 5 mm (12) using creatine phosphokinase (50 mg/ml)/creatine phosphate (12.5 mm) and incubating at room temperature for 15 min.

Monoclonal hsp70 antibody used for Western blot analyses and immunoprecipitation

A monoclonal mouse IgG hsp70 antibody from Affinity Bioreagents was used for Western blot analyses and immunoprecipitation experiments. The supplier has determined by Western blot that it reacts with hsp70, hsc70 (the constitutively expressed counterpart of hsp70), GRP78 (a glucose regulated 78 kDa protein), and hsp72 (induced by heat-shock in HeLa cells). This antibody is suitable for immunofluorescence, Western blot, and immunoprecipitation procedures to detect hsp70 family members.

Polyacrylamide gel electrophoresis and Western blotting

SDS-PAGE was performed using a Miniprotean II electrophoresis cell (Bio-Rad) with 7.5% (w/v) polyacrylamide gel. Samples were diluted 1:2 in sample buffer (125 mm Tris-HCl, pH 6.8, 20% (v/v) glycerol, 2% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue) and heated to 95°C for 4 min. PAGE-separated proteins were electrophoretically (360 mA for 3 h) transferred to nitrocellulose membranes in 50 mm Tris, 380 mm glycine buffer containing 20% (v/v) methanol and 0.01% (w/v) SDS. The blot was blocked overnight at 4°C with 5% (w/v) BSA in PBS with nutation and then incubated 24 h at 4°C with hsp70 antibodies in PBS containing 1% (w/v) BSA. Bound hsp70 antibodies were detected with HRP-conjugated anti-mouse IgG, using the ECL protocol from Amersham. The density of the resulting bands was measured by an LKB Ultroscan XL densitometer.

Coimmunoprecipitation of the 12(S)-HETE binding complex with hsp70 antibody

Cytosol depleted of ATP (as described above) and supplemented with protease inhibitors (60 μg/ml phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin A) was incubated for 1 h at 4°C with monoclonal hsp70 antibody. Protein A Sepharose CL-4B (20 mg, preequilibrated with water) was added and the mixture was nutated for 1 h at 4°C. The sample was centrifuged (12,500 *g*, 1 min) in a Beckman Microfuge E and the supernatant was removed. The protein A Sepharose pellet was washed three times with PBS, resuspended in 0.5 m NaCl in PBS, and incubated for 1 h at 4°C (13). After removing the protein A Sepharose by centrifugation, both the first supernatant (hsp70-depleted cytosol) and the second supernatant (from the resuspended immunopellet) were incubated with 1 nm 12(S)-[^{3}H]HETE for 1 h at 4°C. Samples were analyzed by gel permeation chromatography using elution buffer B. The remaining protein A Sepharose pellet as well as an aliquot of the first supernatant were boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE.

RESULTS

Cell-free 12(S)-HETE binding

To remove proteases from the 650 kDa 12(S)-HETE binding component, cytosol was fractionated by gel permeation chromatography (11). The fraction containing the 650 kDa complex was incubated with tritium-labeled 12(S)-HETE in the presence or absence of unlabeled 12(S)HETE. As shown in **Fig. 1** specific binding was observed. Non-specific binding was considerably higher than when whole cells were incubated with 12(S)-HETE. To somewhat compensate for this, a higher concentration of 12(S)-HETE was used in the cell-free binding assay. Removal of proteases by gel permeation chromatography or using protease inhibitors,

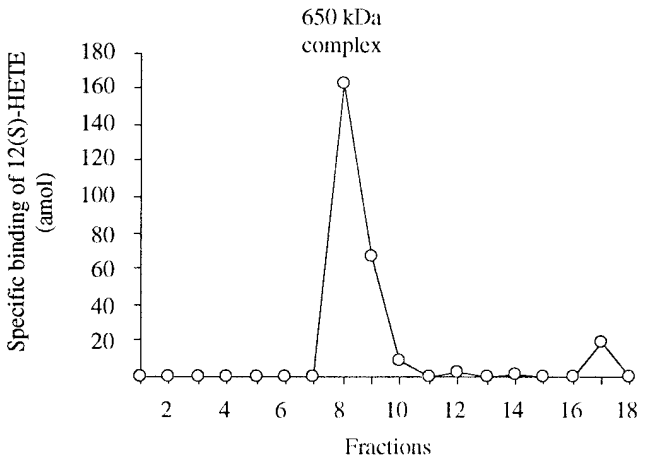

Fig. 1. Cell-free binding of 12(S)-HETE. Cytosol from 2×10^7 cells was fractionated on Superdex™200 and the fraction containing the 650 kDa complex was incubated for 1 h at 4°C with 1 nm 12(S)-[^{3}H]HETE in the absence or presence of 1 μm unlabeled 12(S)-HETE. The samples were then rechromatographed on Superdex™200. The results are shown as specific binding (total minus non-specific binding in corresponding chromatography fractions).

respectively, were about equally effective in allowing cell-free binding to occur. Competition with unlabeled 12(S)-HETE showed that the specific cell-free binding was reduced to 50% at about 0.65 nm unlabeled 12(S)-HETE (**Fig. 2**). This suggested that the ligand affinity was similar to that determined in whole cell experiments (K_d = 0.3 nm, ref. 11).

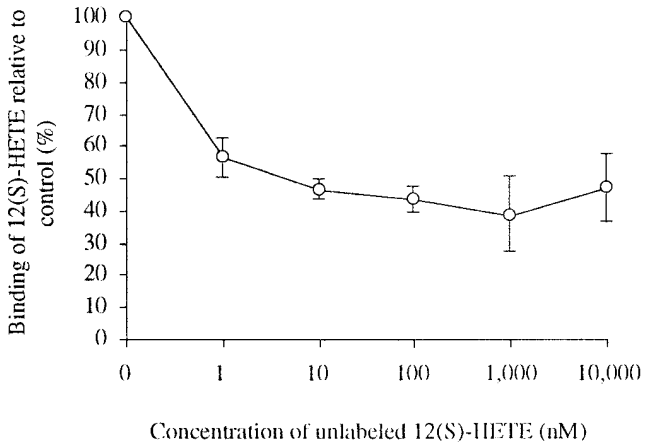

Fig. 2. Ligand affinity for cell-free 12(S)-HETE binding. The 650 kDa binding component was isolated as described for Fig. 1 and incubated for 1 h at 4°C with 1 nm 12(S)-[^{3}H]HETE in the presence of varying concentrations (0–10 μm) of unlabeled 12(S)-HETE. The samples were analyzed on Superdex™200. Data are presented as mean values ± SE from three experiments.

Detection of the 650 kDa 12(S)-HETE binding complex in other cell lines

Table 1 shows a comparison of the ability of five different cell lines to form a 650 kDa 12(S)-HETE binding complex. The cells were incubated with 0.1 nm 12(S)-[^{3}H]HETE in the presence or absence of unlabeled 12(S)-HETE. After 2 h at 4°C cytosol was prepared and analyzed by gel permeation chromatography. The amount of radioactivity in fractions containing the 650 kDa binding complex was compared. Lewis lung carcinoma cells specifically bound 0.22 fmol 12-HETE in the 650 kDa complex/10^6 cells. In contrast to LLC cells, HEL cells bound 45% 12(S)-HETE in the 650 kDa complex and 55% in a 50 kDa subunit fraction, cf. below. TPA-treatment did not affect the binding ability. Interestingly, U937 promonocytic leukemia cells bound about twice as much as Lewis lung carcinoma cells. When treated with TPA these cells differentiate to monocyte-like cells; concomitantly the binding capacity was doubled. 3T3-L1 preadipocytes bound approximately the same amount of 12(S)-HETE as Lewis lung carcinoma cells. When these cells had been differentiated to adipocytes, the binding capacity was reduced about six times. Intestine 407 cells have been used in our laboratory to study LTD_4 receptor signaling (14) and arachidonic acid metabolism (15). These cells were tried as a negative control as they do not produce 12(S)-HETE (15). The 650 kDa 12(S)-HETE binding complex was not detectable in intestine 407 cells.

Effects of ATP depletion and regeneration on the apparent size of the binding complex

Cytosol, prelabeled with 12(S)-[^{3}H]HETE, was incubated with apyrase to deplete ATP (12). An increase

TABLE 1. Occurrence of a 650 kDa 12(S)-HETE binding complex in various cell lines

Cell Line	650 kDa-Bound 12(S)-HETE fmol/10^6 Cells	n
LLC (murine Lewis lung carcinoma)	0.22 ± 0.07	3
HEL (human erythroleukemia)	0.14 ± 0.04	3
TPA-differentiated (0.62 ng/ml, 4 days)	0.18 ± 0.07	3
U937 (human promonocytic leukemia)	0.41 ± 0.16	4
TPA-differentiated (20 ng/ml, 4–5 days)	0.84 ± 0.31	4
Int407 (human intestinal epithelium)	0.01 ± 0.004	3
3T3-L1 (murine preadipocytes)	0.19 ± 0.03	3
Insulin/IBMX/dexamethasone-differentiated (as described in Materials and Methods)	0.03 ± 0.01	3

Cells were incubated with 0.1 nm 12(S)-[^{3}H]HETE in the absence or presence of 0.1 μm unlabeled 12(S)-HETE for 2 h at 4°C. Cytosol was prepared and fractionated on Superdex™200. Specific binding was calculated as the difference between total binding of 12(S)-[^{3}H]HETE and non-specific binding.

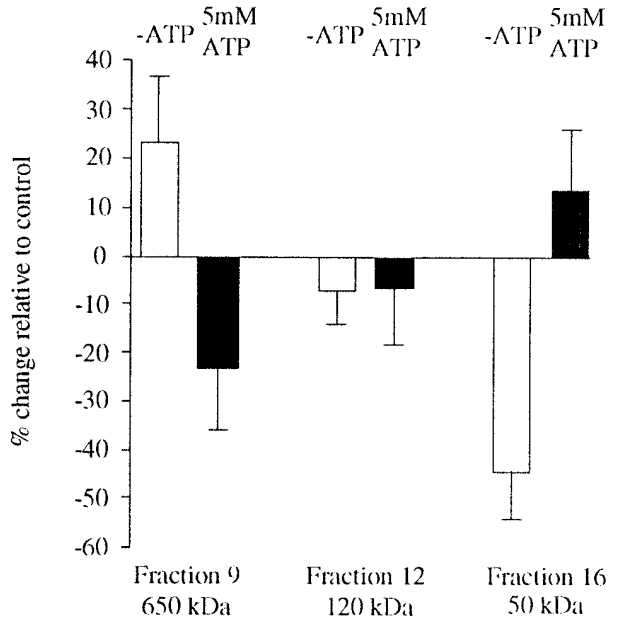

Fig. 3. ATP depletion enhances formation of the 650 kDa heterocomplex. LLC cells were incubated with 0.1 nm 12(S)-[^{3}H]HETE for 2 h at 4°C. Cytosol was prepared and divided into three parts; a) control, b) sample in which ATP was depleted, and c) sample in which ATP was first depleted and then regenerated. The three samples were analyzed on Superdex™200. Data are presented as mean values ± SE from n = 5.

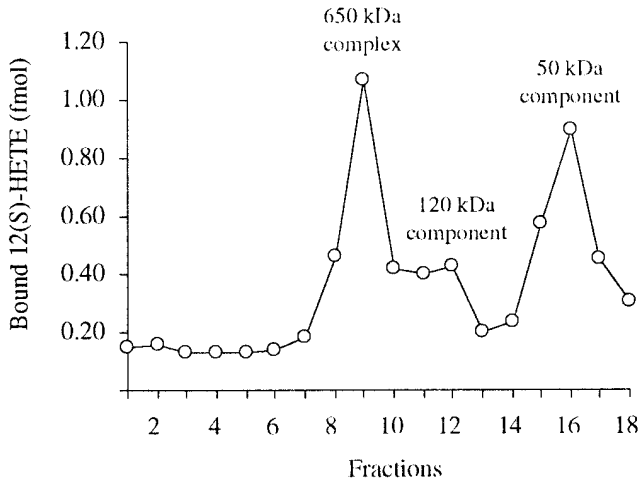

Fig. 4. ATP regeneration leads to dissociation of the 650 kDa binding complex to a 50 kDa 12(S)-HETE binding subunit. Apyrase-treated cytosol from cells preincubated with 12(S)-[^{3}H]HETE (0.1 nm, 2 h, 4°C) was incubated with an ATP-regenerating system. It was then analyzed on Superdex™200.

(24%, mean value; n = 5) in the amount of the 650 kDa complex was observed compared to a non-treated control (**Fig. 3**). When ATP was regenerated in apyrase-treated cytosol (12) there was a decrease (23%, mean value; n = 5) in the amount of the 650 kDa complex. Two additional radiolabeled components appeared (apart from the 650 kDa complex) of molecular weight 120 kDa and 50 kDa (**Fig. 4**). The relative amount of the 120 kDa binding component was not significantly influenced by the ATP concentration but the 50 kDa component was increased (14%, mean value; n = 5) when ATP was regenerated. A 50 kDa subunit was also observed in the control cytosol that had been incubated for 30 min at room temperature without apyrase or ATP regeneration. ATP depletion resulted in a 45% reduction (mean value; n = 5) of the 50 kDa component. These results suggest that ATP destabilizes the association of subunits in the 650 kDa 12(S)-HETE binding complex.

Identification of hsp70 as a 12(S)-HETE binding complex subunit

Cytosol was prepared from LLC cells that had been preincubated with unlabeled 12(S)-HETE (0.1 nm) or from non-incubated control cells. After fractionation on Superdex™200, the high molecular weight fractions (K_{av} = 0–0.39) were analyzed on 7.5% SDS-PAGE, electrotransferred to nitrocellulose, and incubated with a monoclonal hsp70 antibody followed by HRP-conjugated anti-mouse IgG for ECL detection (**Fig. 5**). The intensity of the hsp70 band was highest in fraction 9 which also exhibited the highest specific 12(S)-HETE binding (**Fig. 6**). In control cells a lower concentration of hsp70 was detected compared to cells incubated with 12(S)-HETE. Also, in this case, the intensity of the hsp70 band was highest in the fraction containing the 650 kDa complex. Finally, hsp70 was detected in the 650 kDa fraction after cell-free binding of 12(S)-HETE.

To immunoprecipitate the 650 kDa complex with hsp70 antibody, cytosol was first depleted of ATP by apyrase to stabilize the 12(S)-HETE binding complex

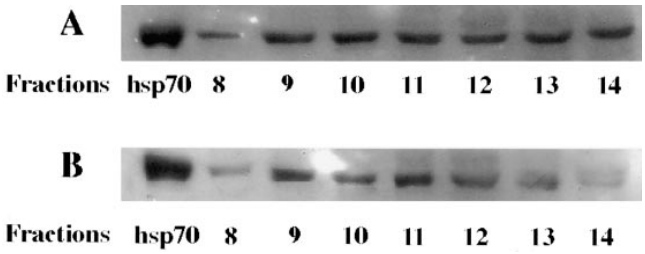

Fig. 5. Detection of hsp70 by Western blot analyses. Cytosol was prepared and fractionated on Superdex™200. Fractions 8–14 were analyzed by Western blot using a monoclonal hsp70 antibody. Lane 1: pure hsp70 control. Panel A: Cytosol fractions from nonincubated control cells. Panel B: Cytosol fractions from cells that were preincubated with 12(S)-HETE (0.1 nm, 2 h, 4°C).

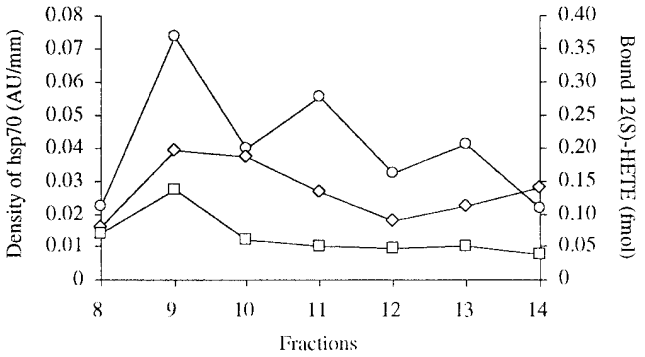

Fig. 6. Densitometric measurements of hsp70 immunoreactivity. Densitometry was performed on the blots shown in Fig. 5: ○, LLC cells preincubated with 0.1 nm 12(S)-HETE (2 h, 4°C); ◇ nonincubated control cells; □, specific 12(S)-HETE binding is also shown for comparison.

and then incubated with anti-hsp70. After sedimentation of immunocomplexes bound to protein A Sepharose, the immunoprecipitate was treated with 0.5 m NaCl (13) to release the 12(S)-HETE binding complex from hsp70. Western blot analyses showed that hsp70 was only present in the protein A Sepharose pellet but not in the remaining cytosol after immunoprecipitation. Gel permeation chromatography after incubation with 12(S)-[^{3}H]HETE showed that 16 ± 4% (mean value ± SE, n = 3) of the 650 kDa complex had coprecipitated with the immunocomplexes while 84% still resided in the hsp70-depleted cytosol. This indicated that hsp70 was associated with the 12(S)-HETE binding complex in substoichiometric amounts at an approximate 1:6 ratio.

DISCUSSION

We have previously demonstrated binding sites for 12(S)-HETE in LLC cells that exhibit high ligand affinity (K_d = 0.3 nm) and specificity (10, 11). These sites are predominantly cytosolic with an apparent molecular weight of 650 kDa (11). Cell-free binding has been more difficult to demonstrate than binding to whole cells but was achieved after including a mixture of protease inhibitors in the incubation medium (11). As an alternative approach we have now demonstrated specific binding after removing endogenous proteases from the 12(S)-HETE binding protein complex by gel permeation chromatography. Using either method, cell-free binding was not as efficient as when whole cells were incubated. Competition experiments with unlabeled 12(S)-HETE demonstrated that cell-free binding had a ligand affinity comparable to that observed in whole cell experiments.

12(S)-HETE binding to a 650 kDa cytosolic component was also demonstrated in human promonocytic leukemia (U937), erythroleukemia (HEL) cells, and murine 3T3-L1 preadipocytes. The relative amount of the complex was doubled in TPA-differentiated compared to control U937 cells. In contrast, differentiated compared to non-differentiated 3T3-L1 cells contained less of the binding complex.

Phorbol esters are known to induce expression of the proto-oncogene c-fos during differentiation of U937 cells to macrophages (16). Under similar conditions we observed an increase in the amount of 12(S)-HETE binding complex. In contrast, TNF-induced c-fos expression in preadipocytes, which appears to be dependent on formation of HETEs, prevented differentiation to adipocytes (17–20). The observed decrease in the amount of 12(S)-HETE binding complex in differentiated 3T3-L1 cells thus parallels the reported changes in c-fos expression. This could suggest a role for the 12(S)-HETE binding complex in the c-fos signal pathway. Human intestinal epithelial cells (Int 407 (15)) had no detectable binding complex.

The present investigation also addressed the question of whether the cytosolic binding complex is multimeric. Part of the 650 kDa complex dissociated into 120 kDa and 50 kDa 12(S)-HETE binding subunits when incubated at room temperature. The dissociation was potentiated by increasing the ATP concentration. The 120 kDa 12(S)-HETE binding complex that was observed is probably an intermediate in the transformation of the 650 kDa complex to the 50 kDa binding subunit. Other protein complexes are also known to be influenced by nucleoside trisphosphate concentrations for the association and dissociation of subunits (21, 22).

The observation that the 12(S)-HETE binding complex coeluted with hsp70 immunoreactivity and that ligand binding increased the hsp70 immunoreactivity of the complex suggested that hsp70 is associated with the 12(S)-HETE binding complex. The results of immunoprecipitation experiments provided further evidence for this and indicated that hsp70 binds substoichiometrically to the HETE binding complex in a ratio of 1:6. This is similar to what has been reported for the glucocorticoid hormone receptor (the corresponding ratio was 1:5(23)). The monoclonal hsp70-antibody used for Western blot and coimmunoprecipitation experiments cross-reacts to some extent with hsc70, GRP78, and following heat-shock hsp72. However, our cells were not subjected to heat-shock; pure hsp70 was used as a control and the electrophoretic mobility indicated a size of 70 kDa. Based on these considerations it

is likely that the observed immunoreactivity represents hsp70.

Hsp70 prevents misfolding and aggregation at an early stage during folding of newly synthesized proteins either when the polypeptide is still ribosome-bound or in a state of transit across a membrane and thus not yet available for folding (22). In addition, hsp70 has intrinsic ATPase activity and ATP binding and hydrolysis result in peptide release.

In summary, the present and previous investigations from our laboratory (11) have revealed several similarities between a 12(S)-HETE binding protein complex in carcinoma cells and ligand-dependent transcription factors of the steroid/thyroid hormone receptor superfamily (24). The similarities include cytosolic/nuclear localization, subnanomolar K_d, (for example the K_d for the interaction between estradiol and its receptor is 0.3 nm (25)), and substoichiometric occurrence of hsp70 in a receptor containing heterocomplex. Recent data showing that the eicosanoids 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 and leukotriene B_4 are ligands for orphan receptors belonging to the same gene superfamily (26–28) has also contributed to the likelihood of such a relationship.

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