Differential subcellular distribution of estrogen receptor isoforms: Localization of ERα in the nucleoli and ERβ in the mitochondria of human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cell lines

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Abstract

The localization of estrogen receptors alpha (ERα) and beta (ERβ) in osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cells was studied by immunofluorescence labelling and confocal laser scanning microscopy, as well as by subcellular fractionation and immunoblotting of the proteins of the fractions with respective antibodies. In both cell types, ERα was localized mainly in the nucleus, particularly concentrated on nuclear structures, which on the basis of their staining with pyronin and with antibodies against the nucleoli-specific Ki67 antigen and C23-nucleolin, were characterized as nucleoli. A faint, diffuse ERα staining was also observed in the cytoplasm. ERβ was specifically enriched at the site of the mitochondria, visualized by labelling with the vital dye CMX and antibody against the mitochondrial-specific cytochrome oxidase subunit I. Immunoblotting experiments corroborated the immunofluorescence labelling distribution of ERα and ERβ. These findings support the concept of a direct action of steroid/thyroid hormones on mitochondrial functions by way of their cognate receptors and also suggest a direct involvement of ERα in nucleolar-related processes.

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1. Introduction

Estrogens play a central role in regulating processes related to female reproduction [1], but also act on male reproductive [2] and on non-reproductive tissues [3] by way of their cognate receptors (ERα and ERβ). In the absence of estrogen, ER is sequestered within nuclei of target cells and maintained in an inactive state by association with heat-shock proteins. The binding of estrogen induces a conformational change in ER, which promotes ER homodimerization and subsequent high-affinity binding of the receptor to estrogen response elements of DNA. Once bound to DNA, the ER-complex initiates the sequence of events leading to the expression of estrogen-responsive genes (see reviews: [4,5]). Two distinct estrogen receptors, the alpha (ERα) and beta (ERβ) isoforms, have been identified [6]. The two receptors share common structural and functional domains, bind estrogens with similar affinity and also bind to estrogen response elements. However, as suggested by the difference in structure, ERα and ERβ differ with respect to their tissue distribution, transcriptional targets and phenotypes in knockout models [7]. ERα and ERβ regulate unique as well as overlapping sets of genes in U2OS osteosarcoma cells [8–10].

Estrogens have an important effect on skeletal development and linear growth of bones during puberty, maintaining homeostasis of the adult skeleton during its continuous regeneration by the process of bone remodeling. The major cell protagonists in these processes are the osteoblast and
the osteoclast. Estrogens stimulate the expression of genes involved in bone formation and decrease the number of remodeling cycles by attenuating the differentiation of osteoclasts and osteoblasts [11,12]. They also promote osteoclast apoptosis and they prevent apoptosis of osteoblasts and osteocytes [13]. The effects of estrogens on bone osteoclasts and osteoblasts [11,12]. They also promote remodeling cycles by attenuating the differentiation of involved in bone formation and decrease the number of the osteoclast. Estrogens stimulate the expression of genes mitochondrial genome of the hepatocyte [16,17]. Similar respiratory chain, encoded both by the nuclear and the increased levels of several mRNAs for proteins of the respiratory chain, encoded both by the nuclear and the mitochondrial genome of the hepatocyte [16,17]. Similar findings were reported for cultured rat hepatocytes and HepG2 human hepatocarcinoma cells treated either with ethinyl estradiol or with estradiol [17]. These estrogen effects on mitochondria were associated with increased respiratory chain activity, increased distribution of glutathione to mitochondria and nuclei, as well as with decreased apoptosis [18]. Furthermore, they were inhibited by the specific anti-estrogen ICI182780, indicating the involvement of the estrogen receptor [19]. Several studies have reported estrogen binding and the presence of estrogen-binding proteins in mitochondria [20–22] and recent studies have localized both ERα and ERβ in the mitochondria of rabbit uterine and ovarian tissue [23–27], in MCF7 [25] and in HepG2 cells [26]. Other research groups, however, have detected solely the ERβ isoform in the mitochondria of human lens epithelial cells [27], of rat primary neurons, primary cardiomyocytes and a murine hippocampal cell line [28]. Furthermore, estrogen response element-like sequences have been detected in the mitochondrial genome [29] and, recently, Chen et al. [30] demonstrated in electrophoresis mobility shift assays (EMSA) binding of ERβ on mitochondrial EREs. The regulatory anabolic and antiapoptotic role of estrogens on osteoblasts and liver cells, respectively, and the presence of glucocorticoid [31–34], estrogen [24–28] and thyroid hormone [35–37] receptors in the mitochondria of a variety of animal cells and tissues have motivated us to explore whether the hypothesis that mitochondria are sites of a primary hormone action [29–31,37,38] could also be expanded to estrogen action on osteoblasts and hepatocytes. In such a case, ER should be detectable in the mitochondria of these cells. We therefore proceeded to localize ERα and ERβ in the SaOS-2 human osteoblast-like osteosarcoma cell line and in HepG2 hepatocellular carcinoma cells by immunofluorescence labelling and confocal laser scanning microscopy and by subcellular fractionation and immunoblotting with respective antibodies. The results presented in this paper demonstrate a differential subcellular distribution of the two ER-isoforms, with ERβ mainly localized in mitochondria and ERα in nucleoli, pointing to a differing functional role of the two estrogen receptors.

2. Materials and methods

2.1. Cells and culture conditions

Human osteosarcoma SaOS-2 and hepatocarcinoma HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS). Cells were grown at 37 °C in a humidified atmosphere with 5% CO2 and expanded to 75 cm² cell culture flasks. In the experiments involving estradiol treatment, the cells were first transferred for 24 h to phenol red and serum-free DMEM and were subsequently incubated for 30 min in an estradiol-supplemented culture medium at a final concentration of 10 nM.

2.2. Antibodies

Rabbit polyclonal anti-ERα and anti ERβ antibodies were commercially provided by Santa Cruz Biotechnology, California (antibody G20, recognizing an epitope mapping at the C-terminus of human, rat and mouse ERα and antibody H150, recognizing an epitope mapping at the N-terminus of human, mouse and rat ERβ). For double-immunolabeling experiments, mouse monoclonal antibodies against Ki67 (Dako Corp., California), cytochrome oxidase subunit I, lamin 90 and C23-nucleolin (Santa Cruz Biotechnology, California) were used. Secondary FITC- and HRP-conjugated antibodies were also purchased from Santa Cruz.

2.3. Immunostaining

Cells grown on coverslips were incubated for 30 min at 37 °C with 400 nM MitoTracker Red CMXRos (CMX; Molecular Probes Inc, UK), washed 3 × 5 with PBS, fixed for 7 min in ice-cold methanol and transferred to ice-cold acetone for 2 min. Alternatively, cells were incubated for 15 min, at room temperature, with pyronin dye solution in Mellvaine’s buffer. After rinsing in PBS, cells were blocked at room temperature with the control serum (5% in PBS) for an hour, to reduce non-specific binding. For immunofluorescence microscopy, specimens were incubated with primary antibodies (1:20 dilution for ERα and ERβ, 1:100 for Ki67 and 1:50 for C23-nucleolin) for 2 h in a moist chamber at room temperature. Following 3 washing steps with PBS, anti-rabbit FITC-conjugated secondary antibody (Jackson Laboratories) diluted 1:50 was added for 1 h. The specimens were washed 3 × 3 min in PBS and mounted in a 50% glycerol solution. For double immunolabeling experiments, two primary antibodies (against ERα and Ki67 or ERα and C23-nucleolin) were added simultaneously. After rinsing in PBS, ERα was visualized by anti-rabbit FITC-conjugated secondary antibody (1:50 dilution) and Ki67 or C23-nucleolin by anti-mouse IgG coupled to Rhodamine (Santa Cruz; 1:200 dilution). The specificity of ERα and ERβ was tested by pre-incubation of the antibodies with the respec-
tive blocking peptides purchased from Santa Cruz Biotechnology. According to their instructions, the antibodies were incubated with a fivefold (by weight) excess of blocking peptide for 2 h at room temperature and were then added to fixed cell preparations. Each immunostaining experiment was repeated numerous times, using cells from at least four independent cell cultures.

2.4. Confocal laser scanning microscopy

Cell specimens were observed with a Nikon confocal laser scan fluorescence-inverted microscope (EZ2000, Nikon), equipped with two lasers used simultaneously: a helium–neon laser (excitation wavelength at 543 nm) and an argon laser (excitation wavelength at 488 nm). The excitation spectra were separated by a dichroic beam splitter of 475/505 nm, and the emission spectra of the two fluorochromes were separated by a 565-nm dichroic beam splitter. Two detectors were used in parallel and were preceded with a 560- to 620-nm (rhodamine channel), or a 500- to 530-nm (fluorescein channel) narrow-band barrier filter. The partial superposition of the emission spectra of the two fluorochromes was negligible. Specimens were observed through an oil immersion ×60/1.4 objective.

2.5. Subcellular fractionation

Nuclei, mitochondria and cytosol were isolated from SaOS-2 and HepG2 cells by sucrose gradient centrifugation of cell homogenates, as described previously [31,39]. Approximately 18 × 10⁷ cells were allowed to grow exponentially in DMEM supplemented with 10% FBS in 14.5-cm dishes, washed twice with ice-cold PBS, harvested in 5 volumes of isotonic buffer (20 mM Hespes–KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 2.5 mM EGTA, 250 mM sucrose), in which protein inhibitors were added (1 mM diethiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin 5 μg/ml, 2.5 mM pepstatin, 20 μg/ml antipain and 1 mM leupeptin), sonicated twice with an ultrasonicator, at level 40 for 20 s and centrifuged, at 12,000 × g, for 15 min at 4 °C. The resulting supernatants, which represent the soluble mitochondrial (mitosol) and nuclear (nucleosol) fractions, respectively, were aliquoted and kept along with the respective pellets (nucleated and mitosed) at −70 °C.

2.6. Protein (Western blot) analysis

Cytosol, nucleosol and mitosol were electrophoresed on a 10% SDS-polyacrylamide gel. The reducing agent was added in a 1:10 ratio. Proteins were then transferred to nitrocellulose membranes, blocked for 2 h at room temperature in 5% non-fat dry milk with TBS–0.1% Tween 20. The blots were subsequently incubated overnight, at 4 °C, either with anti-ERα or anti-ERβ polyclonal antibodies (1:333 dilution in 3% non-fat dry milk with TBS–0.1% Tween 20). After incubation, the membranes were washed twice in TBS–0.1% Tween 20, for 15 min. Horseradish peroxidase-conjugated secondary antibody (1:1000 dilution in 1% non-fat dry milk with TBS–0.1% Tween 20) was then added, for 90 min, at room temperature, and after washing twice, the detection of protein bands was carried out using an enhanced chemiluminescence system (Amer-sham Biosciences, UK). Each Western Blot experiment was repeated at least twice in subcellular fractions derived from each of four independent fractionation procedures.

3. Results

3.1. Immunofluorescence localization of ERα

We investigated the localization of ERα in methanol–acetone-treated SaOS-2 and HepG2 cells. In both cell types, staining with a specific anti-ERα antibody revealed a faint, diffuse cytoplasmic signal (Fig. 1b, e, h, k). This
staining was not associated with mitochondria, as revealed by double-staining experiments with the mitochondrial marker CMX (Fig. 1a, d, g, j; merged images: Fig. 1c, f, i, l). However, an intense nuclear staining was observed mainly in the form of aggregates, whereas the nucleoplasm was faintly stained. These nuclear aggregates correspond to nucleoli, as shown by double-staining experiments, using an anti-Ki67 antibody (Fig. 2a), pyronin (Fig. 2d) and an anti-C23-nucleolin antibody (Fig. 2g, j). Ki67 is a marker of proliferating cells, localized mainly in perinucleolar chromatin, pyronin stains RNA, including nucleolar RNA [40,41], and C23-nucleolin is a nucleolar protein involved in nucleolar RNA biogenesis [42,43]. All three nucleolar markers produced a staining pattern coincident with the ERα signal (Fig. 2b, e, h, k; merged images: Fig. 2c, f, i, l). The specificity of the anti-ERα antibody was tested by preincubation with the specific blocking peptide. Under such conditions, the fluorescence was reduced to back-
ground levels and the nucleoli were no longer stained (data not shown). These findings indicate that a substantial fraction of ERα in SaOS-2 and in HepG2 cells is concentrated in distinct nuclear structures, identified as nucleoli. Upon 30 min administration of estradiol to SaOS-2 and HepG2 cells grown in phenol red- and serum-free medium, the intense nucleolar-associated staining did not change (Fig. 1d, e and j, k, l). Each experiment was repeated numerous times, using cells from at least four independent cell cultures. No considerable variability of the intensity of ERα staining and no difference of the ERα localization reported above were observed between different cells of the same culture or between different cell cultures.
3.2. Immunofluorescence localization of ERβ

We further studied the subcellular localization of ERβ in methanol–acetone-treated SaOS-2 and HepG2 cells. In these cells, the anti-ERβ antibody stained both the nuclei and the cytoplasm (Fig. 3b, e, h, k). The nuclei of HepG2 cells showed a more intense staining than those of SaOS-2 cells. In addition to a faint, diffuse cytoplasmic labeling, there was a striking concentration of the fluorescent signal in numerous perinuclear dot-like structures in a pattern resembling the distribution of the mitochondria. In fact, the mitochondrial marker CMX produced a nearly coincident staining pattern (Fig. 3a, d, g, j; merged images: Fig. 3c, f, i, l), proving the mitochondrial presence of ERβ. The specificity of the anti-ERβ antibody was tested by preincubation with the specific blocking peptide. Under such conditions, the fluorescence was reduced to background levels and the mitochondria were no longer stained.

Fig. 3. Localization of ERβ in SaOS-2 (a–f) and HepG2 (g–l) cells. Cells were incubated with CMX, fixed in methanol–acetone and treated with antibody against ERβ, followed by incubation with FITC-conjugated secondary antibody. CMX staining: a, d, g, j; ERβ staining: b, c, h, k; ERβ staining, estradiol treated: d, e, j, k, l; merged images: c, f, i, l. Bars indicate 10 μm.
These findings indicate that a substantial fraction of ERβ is concentrated in the mitochondria of SaOS-2 and HepG2 cells. Upon 30 min administration of estradiol to cells grown in phenol red- and serum-free medium, no change in the distribution of ERβ was observed (Fig. 3d, e, f and j, k, l). Each experiment was repeated numerous times, using cells from at least four independent cell cultures. No considerable variability of the intensity of ERβ staining and no difference of the ERβ localization reported above were observed between different cells of the same culture or between different cell cultures.

3.3. Biochemical identification of ERα and ERβ in subcellular fractions

Subcellular fractions (cytosol, nucleosol and mitosol) from HepG2 and SaOS-2 cells were subjected to Western blotting using anti-ERα (Fig. 4) and anti-ERβ (Fig. 5) specific antibodies. As shown in Fig. 4, a band of 66 kDa corresponding to ERα was detected, almost exclusively, in the nuclear fraction of both HepG2 (I) and SaOS-2 (II) cells, a finding consistent with the above reported immunofluorescence data. In HepG2 cells, two other faint bands with molecular weights of 55 and 45 kDa were observed in the nuclear fractions. The 55 kDa protein was also detected in the nucleosol of SaOS-2 cells, additionally to another occasionally observed band of 60 kDa (see also [44]). Cytochrome oxidase subunit I and lamin 90, markers for mitochondria and nuclei, respectively, were also assayed by Western blotting, as proof of the purity of the obtained subcellular fractions. In Western blots using the ERβ antibody, a band of 64 kDa corresponding to ERβ was detected in all subcellular fractions of the two cell populations studied (Fig. 5I, II), particularly in the mitochondrial studied, also in accordance with the above shown immunofluorescence findings. A second protein of 50 kDa was also detected in the nucleosol of both cell types and in the mitosol of HepG2 cells. This protein probably represents degraded ERβ produced either during the subcellular fractionation or as a result of a physiological processing mechanism.

4. Discussion

The presence of steroid and thyroid hormones in the mitochondria of various cells and tissues has been demonstrated by various researchers (see review,[45]). As regards estrogen receptors, Monje and Boland [24] detected ERα and ERβ in mitochondria from ovarian and uterine tissue, and Yang et al. [28] found ERβ almost exclusively localized in mitochondria from rat primary neurons, primary cardiomyocytes and a murine hippocampal cell line. Chen et al. [25] demonstrated both ERα and ERβ in the mitochondria of MCF-7 cells and ERβ in mitochondria of HepG2 cells [26]. Furthermore, these authors showed that estrogens significantly enhanced the amount of these receptors in a time- and concentration-dependent manner, accompanied by a significant increase in the levels of mitochondrial DNA-encoded genes and that ERβ binds to mitochondrial EREs [30].

Due to the central regulatory role of estrogens on bone physiology and in the maintenance of a healthy bone mass through their effect on osteoblasts and osteoclasts [11,12] and due to the role of estrogens in hepatocyte energy production and apoptosis [17–19], we have explored the potential presence and subcellular localization of ERα and

![Western blot detection of ERα in HepG2 (I) and SaOS-2 (II) cells. The subcellular fractions were Western blotted as described in Materials and methods. Mitosol (Msol), nucleosol (Nsol) and cytosol (SC) were probed with antibodies to ERα (A), to cytochrome oxidase I (B) and to lamin (C).](image-url)
ERβ in the osteoblast-like SaOS-2 osteosarcoma cell line and in HepG2 hepatocarcinoma cells.

By immunocytochemical localization and confocal microscopy using specific antibodies to ERα, we detected both a nuclear and cytoplasmic localization of ERα in the two cell types. Interestingly, in the nucleus, ERα was localized in structures, which on the basis of their labeling with antibodies to nucleolar proteins (Ki67 and nucleolin) and with pyronin, which strongly stains nucleolar RNA, were characterized as nucleoli. Using the ERβ antibody, a predominantly mitochondrial localization of ERβ was observed, with minor presence in nuclei and cytoplasm. These results were corroborated by Western blotting, demonstrating in nuclei both estrogen receptor isoforms, whereas in the mitochondria only ERβ could be detected. Although ERβ is localized in the mitochondria, it does not contain the classical amphipathic, alpha helical mitochondrial targeting sequence at the amino-terminal region of most, but not all, mitochondrial proteins ([46], see also Ref. [15]). In HepG2 cells, three nuclear ERα species were resolved by gel electrophoresis, one corresponding to the well-characterized 66 kDa isoform and two with MW of 55 and 45 kDa, the latter one probably representing the species described by Denger et al. [44] in human primary osteoblasts, acting as a strong ERα inhibitor. The 55 kDa species was also detected in SaOS-2 nuclei, in addition to a 60 kDa protein. These smaller molecular weight polypeptides could represent functionally active or inactive degradation products generated during a physiological cleavage process or artefacts due to the mitochondria isolation procedure.

The specificity of the reactions with the antibodies was ascertained, as no cross-reaction of ERα with the ERβ antibody was observed. Furthermore, preincubation of the cells and the respective extracts and antibodies with blocking peptides abolished both the immunofluorescence staining and the Western blotting bands.

The presence of ERβ in the mitochondria of SaOS-2 and HepG2 cells corroborates previous findings with glucocorticoid [31–34] and thyroid hormone receptors [35–37], as well as recent findings with estrogen receptors [24–28]. In contrast to Monje and Boland [24] and Chen et al. [25], but in accordance to Yang et al. [28], we detected ERβ, but not ERα in the mitochondria of animal cells. The differences of our data, compared to the results obtained by the other investigators, could be attributed to the fact that we used different cell lines and antibodies with different sensitivity and affinity to the two estrogen receptor forms. These differences, as well as cell cycle variabilities, could have affected the expression and the localization of ERα and ERβ. Furthermore, some of the apparent differences in the intensity of the immunofluorescence staining in the various cell compartments and of the Western Blot bands could be explained by the fact that antibodies have different reactivities when they are used in an in situ technique than when they react in a solution containing isolated protein from specific cellular compartments. Our data, which are mainly qualitative, show characteristic patterns of ERα and ERβ expression both in immunofluorescence and in Western Blot studies.

Mitochondrial glucocorticoid, thyroid hormone and estrogen receptors have been associated with the observed increased levels of mitochondrial DNA-encoded gene transcripts after treatment with glucocorticoid, thyroid hormones and estrogens (see reviews, [26,37]). In addition, other receptors belonging to the nuclear receptor superfamily, such as RXR [47] and the vitamin D receptors [48], have been found in the mitochondria and have been implicated in mitochondrial gene transcription.

Glucocorticoids activate the transcription of genes encoding enzymes of oxidative phosphorylation. Since the complexes of the respiratory chain are composed of subunits encoded both by nuclear and mitochondrial genes, gene transcription should be coordinately regulated in both cell compartments. The prevailing concept for this coordinate action at the level of transcription is a primary steroid induction of nuclear genes encoding not only subunits of oxidative phosphorylation enzymes, but also regulatory proteins, which subsequently activate transcription of the mitochondrial-encoded subunits [49,50]. Additionally, a primary, direct action of steroid and thyroid hormones on mitochondrial gene transcription has also been proposed [26,29,36–38,47,51–55], ensuring the optimal coordination of nuclear and mitochondrial transcription of respiratory subunits.

The findings of Chen et al. [25,26,30] concerning the localization of ERβ in MCF7 and HepG2 cells, the binding of ERβ on mitochondrial EREs and estradiol enhancement of mitochondrial DNA transcription mediated by ERβ [25]
and the presented findings strongly support the direct action of steroid hormones on mitochondrial transcription.

The localization of ERα in HepG2 and in SaOS-2 nucleoli reported in this paper is a novel finding and raises the question of the physiological role of the receptor in nucleolar function. Previous immunofluorescence localization studies of ERα in female reproduction tissue and cells, and more recently, using green fluorescent labelled receptors transfected into a variety of cell types (COS-1, MCF-7) [32,56,57] have demonstrated a granular-diffuse nuclear localization of the receptor, however, not a nucleolar one. This divergence from our results could be due to the high growth potential of the cancer cell lines used in our experiments—evident from the highly prominent nucleoli of these cells expressing the Ki67 antigen, compared to the relatively quiescent cells of the female reproductive tissues. It is interesting to note that the glucocorticoid receptor β also localizes in the nucleoli of these cells (Psarra, A.-M.G., et al., unpublished data) and that Schulz et al. [58] have shown in pull-down experiments an interaction between glucocorticoid receptor and nucleolin. The exposure of SaOS-2 and HepG2 cells to estradiol does not affect the localization of ERs. This could also be attributed to the activated state of the cells used, which are constantly subjected to growth stimuli, which, by signal transduction mechanisms and phosphorylation, can activate ERs (see review, [59]), in a ligand-independent way. Thus, ERs in these cancer cell lines are in an active state, at the site of action and, therefore, are not redistributed by the hormone. Since Chen et al. [25] have reported nuclear translocation of ERβ in MCF7 cells, these differences could be attributed to the use of different cell lines and cell treatment conditions.

As regards the functional significance of this finding, steroid hormones are known inducers of nuclear gene transcription mediated by the respective steroid receptors [4,5]. Hormonal induction of premessenger RNA (hn RNA) is always accompanied by increased synthesis of preribosomal RNA [60–63]. In studies concerning the action of estrogens on RNA synthesis in the uterus, Whelly [64] and Brantley and Whelly [65] demonstrated an increased transcriptional activity of isolated uterine nucleoli, early after estrogen administration to ovariectomized, immature rats and showed that it was an effect of RNA chain elongation. Whelly [66] also demonstrated that rat uterine nuclei contained two types of estrogen binding sites, one with high affinity and low capacity [I] and the second with low affinity and high capacity [II]. The type II sites were localized in the nucleoli and their number increased after estrogen treatment. The fine mechanism of hormonal induction of rRNA has not yet been elucidated. A direct role of steroid receptors on nucleolar rRNA synthesis by way of interaction with putative GREs within the rRNA gene has been discussed [67]. On the basis of the above findings and the presence of ERα in the nucleoli and its colocalization with the nucleolar phosphoprotein nucleolin, whose role is implicated in the early stage of preribosomal RNP biogenesis, assembly, processing and transport to the cytoplasm [40,41], the possible involvement of this receptor in preribosomal RNA synthesis, processing and trafficking should be explored.

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References


