Regulation of carnitine palmitoyltransferase *in vivo* by glucagon and insulin

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Carnitine palmitoyltransferase (CPT total) activity and synthesis increase in states where the insulin/glucagon ratio is low, such as starvation and diabetes [Brady & Brady (1987) *Biochem. J.* **246**, 641–646]. However, the effect of glucagon and insulin on CPT synthesis is unknown. The present experiments were designed to determine the effect of glucagon, cAMP [8-(chlorophenylthio) cyclic AMP], and insulin + cAMP on CPT transcription and mRNA amounts over time after injection. The CPT protein that was purified, used to generate antibody, and cloned in these studies was the 68 kDa mitochondrial protein described previously [Brady & Brady (1987) *Biochem. J.* **246**, 641–646; Brady, Feng & Brady (1988) *J. Nutr.* **118**, 1128–1136; Brady & Brady (1989) *Diabetes* **38**, in the press]. Saline-injected control rats exhibited a 2-fold increase in hepatic CPT transcription rate and CPT mRNA over the 5 h experiment from 09:00 to 14:00 h. The effect was most probably due to the fasting state of the rats during the day. Glucagon injection caused an 8-fold increase in transcription rate by 90 min and a 4-fold increase in CPT mRNA by 90–120 min. The cAMP effect had reached a peak by the first time point taken (15 min). Transcription rate was increased 4-fold and CPT mRNA was increased 3-fold at this time. The combination of cAMP + insulin injection did not produce any significant increase in transcription rate or CPT mRNA over the saline-injected controls. CPT mRNA and transcription rate showed a clear dose-response to glucagon injection from 0 to 150 µg/100 g body wt. Total CPT activity and immunoreactive CPT were not increased during these experiments. The data indicate that glucagon and insulin interact in control of transcription rate and amount of CPT mRNA, but that increases in CPT immunoreactive protein and activity are temporally delayed. This lag probably relates to the half-life of the CPT protein *in vivo*, which has been estimated as 2–7 days.

INTRODUCTION

Carnitine palmitoyltransferase (CPT outer activity) has been described as the rate-limiting enzyme in hepatic mitochondrial β -oxidation of fatty acids (for review, see McGarry & Foster, 1980). However, it is currently not clear whether the mitochondrial 'outer CPT activity' and the 'inner CPT activity' result from the same mitochondrial CPT protein, and this has limited attempts at molecular characterization of CPT regulation. Four different types of regulation of 'outer' or 'inner' CPT activity have been demonstrated: (1) inhibition by malonyl-CoA of the 'outer CPT activity' (reviewed by McGarry & Foster, 1980); (2) phosphorylation of the mitochondrial 68 kDa CPT (the same as described here) (Harano *et al.*, 1985); (3) membrane or micro-environmental lipid alterations (both 'outer' and 'inner' activities) (Pande *et al.*, 1985; Bremer *et al.*, 1985; Woldegiorgis *et al.*, 1985; Declercq *et al.*, 1987; Brady *et al.*, 1987); and (4) alteration of transcription and amount of mRNA (mitochondrial 68 kDa CPT described here) (Brady *et al.*, 1988; Brady & Brady, 1989a,b). Most attention has focused on short-term regulation of 'outer' CPT activity by malonyl-CoA in intact mitochondria. The complexity of the CPT system has prevented a full delineation of the role of synthesis and degradation in regulation of the 'outer' versus the 'inner' CPT activity.

The CPT which we studied in the present work is the

mitochondrial 68 kDa enzyme described in more detail by Brady & Brady (1987). This CPT protein's characteristics correspond to some of those described by several other groups (Clarke & Bieber, 1981; Miyazawa *et al.*, 1983; Woeltje *et al.*, 1987; Ramsay, 1988). We have confirmed that this enzyme does not cross-react with Ramsay's (1988) anti-(peroxisomal CPT_s) antibody, nor does our antibody to the 68 kDa CPT cross-react with her peroxisomal CPT_s (Brady *et al.*, 1989). Thus the 68 kDa CPT isolated from mitochondria is not a peroxisomal protein contaminant. However, we cannot yet assign a definitive location within the mitochondria to the 68 kDa CPT. Although Clarke & Bieber (1981) and we (Brady & Brady, 1987) have published reports suggesting that this enzyme represents the only mitochondrial CPT, there are now several reports suggesting that a separate mitochondrial CPT protein may exist (Declercq *et al.*, 1987; Zammit *et al.*, 1988; Murthy & Pande, 1988). Declercq *et al.* (1987) described a 94 kDa protein which bound malonyl-CoA and tetradecylglycidyl-CoA and tentatively identified it as CPT-I (a separate outer CPT). Whether the inhibitor-binding protein represents the outer CPT or only a protein which binds these CPT inhibitors has yet to be defined, since the CPT catalytic activity of the malonyl-CoA- and tetradecylglycidyl-CoA-binding protein(s) has never been conclusively demonstrated. Zammit *et al.* (1988) estimated sizes of two proteins possessing CPT

Abbreviations used: CPT, carnitine palmitoyltransferase; cAMP, 8-(4-chlorophenylthio) cyclic AMP (this slowly degraded derivative of cAMP is more commonly referred to as CPT cAMP; however, for simplicity, this is abbreviated as 'cAMP'); ELISA, enzyme-linked immunospecific assay.

activity by radiation-inactivation techniques. They estimated that the outer CPT activity corresponded to a 96.7 kDa protein and the inner CPT activity to a 69.7 kDa protein. Murthy & Pande (1987) found one CPT catalytic activity (CPT_o) in the mitochondrial outer membrane and a second CPT catalytic activity in the inner membrane. These CPT activities were differentially inhibited by malonyl-CoA and detergents. On the basis of these studies, those of Clarke & Bieber (1981) and our own (Brady & Brady, 1987; Brady *et al.*, 1988), the evidence for the number and location of CPT activity(ies) is not conclusive, and we have not assigned a specific location within the mitochondria to the 68 kDa CPT protein. However, the present paper reports evidence that the synthesis of the 68 kDa CPT is regulated by hormones.

The conditions under which 'outer', 'inner' or 'total' CPT activity is induced or malonyl-CoA sensitivity of 'outer' CPT activity is decreased correspond to those in which there is a low circulating insulin/glucagon ratio (e.g. starvation, diabetes) (Harano *et al.*, 1972, 1982; Gamble & Cook, 1985; Brady *et al.*, 1985; Brady & Brady 1987; Grantham & Zammit, 1988). However, few studies have directly measured the response of any CPT activity to insulin or glucagon. Nosadini *et al.* (1979) perfused livers with oleate and glucagon and found no increase in hepatic CPT activity in Sprague-Dawley rats as a result of glucagon perfusion. However, they did find increased CPT activity in livers from Zucker rats perfused with glucagon. Grantham & Zammit (1987) found no effect of glucagon injected *in vivo* on kinetics of 'outer' CPT of hepatic mitochondria isolated 20 min after injection. In hepatocyte culture, Harano *et al.* (1982) found a 50–80% increase in total CPT activity when mitochondria were isolated from glucagon-treated hepatocytes. Insulin antagonized this effect. Subsequently Harano *et al.* (1985) suggested that glucagon increased the mitochondrial 68 kDa CPT activity by increasing the affinity of the mitochondrial 68 kDa enzyme for palmitoyl-CoA via phosphorylation. Nothing is known about how glucagon or insulin act on CPT synthesis/degradation at the molecular level. However, we do know that, in spontaneously diabetic BB Wistar-rat liver, total CPT activity and immunoreactive CPT, amounts and transcription rates of CPT mRNA (68 kDa mitochondrial CPT) are elevated if insulin is withdrawn for 24 h and that this elevation is prevented by insulin administration (Brady & Brady, 1989a). We also know that hepatic CPT synthesis (68 kDa mitochondrial CPT) is increased in diabetic animals, as is the translation rate of mRNA to this CPT protein (Brady & Brady, 1987).

The major mechanisms by which hormones act on enzyme synthesis are via transcriptional, post-transcriptional and translational controls (Wynshaw-Boris & Hanson, 1983; Rucker & Tinker, 1986). Thus the purpose of the present studies was to examine the effect of glucagon, cAMP and insulin on hepatic CPT RNA and transcription rate of CPT RNA from DNA (68 kDa mitochondrial CPT). Total CPT activity and immunoreactive CPT were also measured.

METHODS

Animals and treatment

Male weanling Sprague-Dawley rats were maintained on Purina Lab Chow and water *ad libitum* until they

reached 150–200 g body wt. Rats were divided into four groups, which received one of four treatments at 09:00 h on the day of the experiment: (1) saline (0.9% NaCl) injection; (2) injection of glucagon at 150 µg/kg; (3) injection of cAMP (0.2 mg/100 g); (4) injection of insulin (at 07:30 h; 2.5 units/100 g), followed by injection of cAMP (0.2 mg/100 g; at 09:00 h). Rats were killed at the times indicated in Fig. 2(a) (three rats/group) and livers were removed. Total hepatic RNA and nuclei were isolated as described below. In a second set of experiments, rats were injected with glucagon (0, 30, 60, 90, 120, 150 µg/100 g body wt.) and killed 3 h later (three rats per group).

CPT activity, mRNA determination and transcription assay

CPT activity was determined as described at 25 °C (Brady & Brady, 1987). This assay measures total CPT activity after disruption of mitochondria by 5 mM-phosphate buffer/0.05% Tween 20, pH 7.5. The assay contains 100 mM-Tris buffer, pH 8.0, 0.12 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 1.1 mM-EDTA, 80 µM-palmitoyl-CoA and 8 mM-L-carnitine, modified from Bieber *et al.* (1972). We have found that the total CPT activity measured by this method correlates with measures of 'outer CPT activity' using intact mitochondria and putative 'inner CPT activity' using inverted mitochondria (Brady *et al.*, 1985, 1986). Under conditions of detergent solubilization, we have measured malonyl-CoA sensitivity by using various palmitoyl-CoA and carnitine concentrations and analysis by Dixon plots. The plots are abnormal and do not allow accurate estimation of malonyl-CoA sensitivity, although examination of the data suggests little inhibition.

The N-terminal amino acid sequence of purified CPT was determined and has been presented (Brady *et al.*, 1988; Brady & Brady, 1989a). A 17-residue oligonucleotide probe was synthesized on the basis of this sequence and labelled as described by Maniatis *et al.* (1982) with [γ -³²P]ATP by using polynucleotide kinase after hydrolysis of the 5'-terminal phosphate with calf alkaline phosphatase. Use of this probe gave exactly the same results on Northern blotting as use of the RNA probe generated from the full-length cDNA clone. Immunoreactive CPT was determined via both ELISA assay and Western blotting. The ELISA assay has been previously described (Brady *et al.*, 1988). The antibody used in these studies was polyclonal antibody generated in rabbits. This antibody did not cross-react with peroxisomal CPT₂ on Western blots as described by Ramsay (1988), nor did Ramsay's (1988) peroxisomal antibody cross-react with our purified mitochondrial CPT or our isolated mitochondria on Western blots or in the ELISA assay (Ramsay *et al.*, 1987; Ramsay, 1988; Brady *et al.*, 1989). Mitochondria isolated by the technique described by Hoppel *et al.* (1979) have little contamination with microsomal fractions, plasma membrane or lysosomes (Hoppel & Tomec, 1972). Further, we have confirmed that mitochondria isolated by this method do not cross-react with the anti-CPT₂ antibody on ELISA or Western blots (Brady *et al.*, 1989). Thus mitochondria isolated by this method do not contain significant peroxisomal contamination.

Northern and dot-blot hybridizations were done as described previously (Brady *et al.*, 1988; Brady & Brady, 1989a). A Northern blot of control and glucagon-injected

Control Glucagon

Fig. 1. Northern blot of hepatic CPT RNA from control and glucagon-treated rats

Treatment of the animals and total RNA isolation were as described in the Methods section. RNA (5 μ g) was applied to a 1%-agarose gel in a formaldehyde system and electrophoretically separated before capillary transfer to nitrocellulose. Blots were hybridized with either a 32 P-labelled 17-mer (shown) or a 32 P-labelled mRNA probe developed from the pBluescript_{3A15.1.1}. The blots were detected by autoradiography.

rat liver RNA is presented in Fig. 1. Quantification was by measuring peak areas after densitometric scanning of dot-blot.

For the transcription assays, pBluescript_{3A15.1.1} (a clone previously described; Brady *et al.*, 1988; Brady & Brady, 1989) was used after linearization with endonuclease *Bam*HI and alkaline denaturation. pBluescript_{3A15.1.1} appears to represent an almost full-length (>95%) CPT insert. The identity of the clone was confirmed by generating T₃ RNA polymerase transcript with 5'7 meGppp5'G cap from *Hind*III-restricted pBluescript_{3A15.1.1}. The resulting mRNA (2.1–2.3 kb) was translated in the standard reticulocyte lysate system with [35 S]methionine plus RNasin (RNAase inhibitor). CPT activity was detected by using the standard assay modified to include 20% (v/v) dimethyl sulphoxide. No CPT activity was found by using brome-mosaic-virus mRNA as control. The size of the translation product was 70.6 kDa as determined from the SDS/polyacrylamide-gel-electrophoresis autoradiogram. 14 C-labelled protein standards were included for this determination.

For the transcription assays *in vitro* (nuclear run-on assays), the pBluescript_{3A15.1.1} clone was restriction-digested with *Bam*HI and denatured as described by Marzluff & Huang (1984). The pBluescript_{3A15.1.1} was spotted on to nitrocellulose filters (0.5 μ g/dot in a Schleicher & Schuell dot-blot manifold) in 2 $\times$ SSC (0.3 M-NaCl/0.03 M-sodium citrate, pH 7.5) and baked at 80 $^{\circ}$ C for 2 h *in vacuo*. The filters were prehybridized at 52 $^{\circ}$ C before hybridization with the RNA for 48 h. Rat liver nuclei were isolated as described by Morris *et al.* (1987). Nuclei were stored at -70° C after centrifugation through a 2 M-sucrose cushion. Storage buffer contained 50% (v/v) glycerol, 10 mM-magnesium acetate, 0.1 mM-EDTA, 5 mM-dithiothreitol, 100 mM-Hepes, pH 7.5, at a concentration of 2×10^8 nuclei per ml. The transcription assay *in vitro* and RNA extraction were as described by Morris *et al.* (1987).

Materials

Endonucleases *Eco*R1, *Hind*III, *Bam*HI and *Pst*I were purchased from Bethesda Research Laboratories

(Bethesda, MD, U.S.A.). pBluescript and T₇/T₃ mRNA capping kit were obtained from Stratagene (San Diego, CA, U.S.A.). Rabbit reticulocyte-lysate translation and processing kits (endonuclease-treated) and RNasin were obtained from Promega (Madison, WI, U.S.A.). CoA was purchased from Pharmacia-PL Biochemicals (Piscataway, NJ, U.S.A.). Palmitoyl-CoA was synthesized as described by Seubert (1960), by using commercially available palmitoyl chloride (Sigma Chemical Co., St. Louis, MO, U.S.A.). L-Carnitine was kindly given by Sigma Tau (Rome, Italy). Crystalline insulin and glucagon were kindly given by Lilly Research (Indianapolis, IN, U.S.A.). [35 S]Methionine, α -[32 P]ATP, α -[32 P]dUTP and γ -[32 P]ATP were obtained from DuPont-New England Nuclear (Boston, MA, U.S.A.). Nitrocellulose was obtained from Schleicher & Schuell (Keene, NH, U.S.A.). All other chemicals were of the highest available purity.

Statistics

Data were analysed by analysis of variance (ANOVA) for a multifactor design with unequal numbers by using a general linear model with the PC-SAS system (SAS Institute, Cary, NC, U.S.A.). Statistical significant is defined as $P < 0.05$.

RESULTS

Total CPT activity and immunoreactive CPT protein did not change over the time of the experiment (5 h) for any of the four treatments. This is consistent with previous results, where CPT activity was unchanged with short starvation periods and mild diabetes or acute glucagon treatment up to 4 h (Saggerson & Carpenter, 1981; Brady & Brady, 1987; Boon & Zammit, 1988; Grantham & Zammit, 1987, 1988). This is also consistent with data on turnover *in vivo*, which estimated the CPT half-life at 2.0 days (Brady & Brady, 1987). Fig. 2 presents the data on the effect of saline, glucagon, cAMP, and CAMP/insulin injections *in vivo* on hepatic CPT mRNA amounts (a) and transcription rates (b) over time. CPT mRNA amounts and transcription rates for control saline-injected rats increased throughout the day, such that they were elevated about 2-fold by 13:00 h. This diurnal pattern probably reflects the animals' lack of food intake during the day. Glucagon and cAMP injection increased hepatic CPT mRNA amounts and transcription rates over those seen in the controls at several time points, with the glucagon effect occurring more slowly. Injection of cAMP increased mRNA amounts 3-fold and transcription rate 4–5-fold within 15–30 min, with the cAMP effect on mRNA reaching a peak at 90 min and then falling back to control values. The cAMP effect on transcription actually reached a peak by 15 min. Injection of glucagon caused a significant increase in CPT mRNA and transcription rate by 30 min with the effect reaching a peak at 60–90 min for transcription and 90–120 min for mRNA, then falling to control values. When insulin was given to cAMP-injected animals, there was no increase in mRNA amount and transcription rate. The data document that changes in transcription rate and CPT mRNA amounts precede actual increases in CPT immunoreactive protein or activity. The stimulation of transcription rate by glucagon is about 8-fold, whereas that of mRNA amounts is about 3–4-fold. These data further suggest that both

Fig. 2. Time course of induction of CPT mRNA and transcription rate

Rats were treated with saline (control) (■), glucagon (●), cAMP (▼) or cAMP plus insulin (▲) as described in the Methods section. Animals were killed at various intervals (three per time point), and hepatic RNA and nuclei were isolated. CPT mRNA (a) was determined by dot-blot hybridization and quantified, after autoradiography, by scanning densitometry. Transcription rates (b) were determined by nuclear run-on assay. Values were expressed relative to the mean initial value for the control rats. Data were analysed by analysis of variance. The S.E.M. is derived from the mean-square error for this analysis. Data were further analysed for individual differences from the closest control point by Duncan's test. The curves shown represent the product of a polynomial curve-smoothing routine.

Fig. 3. Dose-response curve of induction of CPT mRNA and transcription in response to glucagon

Rats were injected with various doses of glucagon at 09:00 h and killed 90 min later. mRNA (a) and transcription rates (b) were determined as outlined in Fig. 2 legend. CPT transcription rates are expressed here relative to total RNA transcription (p.p.m.). Data were analysed by analysis of variance. The error bars are S.E.M. ($n = 3$). Data were further analysed for individual differences from the zero-glucagon value by Duncan's test. Significant differences are indicated by an asterisk. The curves shown represent the product of a polynomial curve-smoothing routine.

mRNA processing from the RNA initially transcribed and processing of CPT into the mitochondrial membrane may limit expression of CPT activity.

Fig. 3 presents the glucagon dose-response curve for CPT mRNA (a) and transcription rate (b). Increasing the glucagon dose from 0 to 150 $\mu\text{g}/100\text{ g body wt.}$ increased both the CPT mRNA and the transcription rate of RNA and DNA. Transcription rate was significantly stimulated over control by 60 μg of glucagon/100 g. The maximal stimulation of transcription at the 3 h time point was by 120–150 $\mu\text{g}/100\text{ g}$ (2-fold); this contrasts with the 8-fold maximal stimulation by 150 $\mu\text{g}/100\text{ g}$ seen at 60–90 min in Fig. 2, but this is only about 2-fold higher than the response at 3 h seen in Fig. 2. The maximal stimulation of mRNA amounts was also at 120–150 $\mu\text{g}/100\text{ g}$. The stimulation at this 3 h time point was approx. 2-fold, which agrees with the time-course data in Fig. 2. There were no changes in CPT activity or immunoreactive protein under these conditions, again showing that increases in transcription and mRNA precede increased immunoreactive protein or CPT activity.

DISCUSSION

The present work demonstrates that CPT (68 kDa mitochondrial CPT) transcription and mRNA amounts are increased by glucagon, presumably acting through cAMP, and that this increase is prevented by previous administration of insulin. The data further suggest that the lack of change in immunoreactive CPT and CPT activity seen in some situations is due to a processing step beyond simple mRNA synthesis. These data are supported further by results *in vivo* which show that the CPT half-life is 2 days (Brady & Brady, 1987) or 6.5–7.5 days (Ozasa *et al.*, 1983). Thus an effect on enzyme activity or amount might not be obvious by 5 h after hormone treatment. The conclusion that glucagon might regulate CPT synthesis is as expected on the basis of the literature, which shows that CPT activity is increased in situations with low insulin/glucagon ratios, such as 24–48 h starvation and severe diabetes (Harano *et al.*, 1972, 1982, 1985; Bremer, 1981; Brady *et al.*, 1985; Brady & Brady, 1987). However, the present work is the first demonstration that

glucagon and cAMP directly induce transcription rates and mRNA amounts for CPT (68 kDa mitochondrial CPT). In similar studies, Beale *et al.* (1984) found that both glucagon and cAMP induced phosphoenolpyruvate carboxykinase mRNA and that insulin given to diabetic rats depressed this mRNA. We have previously found that insulin given to spontaneously diabetic BB Wistar rats that were deprived of insulin for 24 h depresses both CPT mRNA amount and transcription to the same extents as found in control BB Wistar littermates (Brady & Brady, 1989a). These rats are chronically diabetic and must be maintained on daily insulin to survive. Thus CPT may be regulated by insulin and glucagon in a way similar to phosphoenolpyruvate carboxykinase.

The data reported in the present paper, as well as that previously presented by our group (Brady *et al.*, 1988; Brady & Brady, 1989a), suggest that regulation of CPT is more complex than was originally suspected. Most attention has focused on short-term regulation of the 'outer CPT activity' by malonyl-CoA. Thus far, nothing is known about long-term regulation of the 'outer CPT activity' by synthesis/degradation. The data presented here and in several other papers (Ozasa *et al.*, 1983; Brady & Brady, 1987; Brady *et al.*, 1988, 1989; Brady & Brady, 1989a) suggest that the 68 kDa enzyme is regulated, at least in part, by changes in synthesis. It is apparent from the present studies that total CPT activity measurement and the actual amount of immunoreactive mitochondrial 68 kDa CPT protein lag behind the induction of mRNA and transcription at early times after hormone administration (up to 5 h). Data in H4IIE cells after short-term hormonal treatment confirm that immunoreactive CPT is not increased until 12 h after cAMP addition, whereas transcription rates and mRNA increase by 90 min (Wang *et al.*, 1989). This suggests that the steps after mRNA generation may limit the final CPT activity observed. These steps would include: translational efficiency of mRNA, processing of the nascent CPT (approx. 71.5 kDa) to the mature protein (approx. 68 kDa), and the concomitant insertion of the protein into the mitochondrial membrane. In studies where chronic treatment is involved there is good agreement between total CPT activity, immunoreactive 68 kDa CPT protein, mRNA amounts and transcription rate (Brady *et al.*, 1988; Brady & Brady, 1989a,b). The data presented here also suggest that processing of mRNA from initial nuclear transcripts may be limiting in some cases, as peak transcription rates in response to dose of glucagon were higher than actual amounts of CPT mRNA.

The study of the carnitine palmitoyltransferase(s) has(have) been confounded by the inability to isolate two mitochondrial proteins. The most current interpretations of existing data are: (1) there may be two proteins, but that the outer activity (protein) is quite labile; (2) the outer CPT that has been described by some groups is a protein which binds malonyl-CoA and/or tetradecylglycidyl-CoA, but might be a regulatory unit, since purification of this protein and demonstrated CPT activity have not been conclusively demonstrated; (3) one of the two CPTs may be a contaminant derived from peroxisomes or endoplasmic reticulum. The protein which we isolated may be the inner CPT, but, again, this has not been proved conclusively. Our present data suggest that we need to modify our view of CPT regulation to include a role for this 68 kDa CPT. This

enzyme's activity and its sensitivity to malonyl-CoA have generally been ill-defined in metabolic studies, owing to the inability to separate 'inner activity' completely from the 'outer CPT activity' in intact or broken mitochondria or to purify two enzymes with CPT activity from uncontaminated mitochondria. However, several groups, including ourselves, have purified this CPT and studied its kinetic and inhibition constants and synthesis. Key regulatory questions remain: (1) is the putative outer CPT a regulatory subunit, or does it have CPT activity, and is it regulated by long-term synthesis also, or is it only regulated via malonyl-CoA and mitochondrial membrane changes?, and (2) is the synthesis of both proteins co-ordinated? The answers must await purification and cloning of the binding protein or putative outer CPT.

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