Journal of Cellular Biochemistry 37:347–357 (1988)

Insulin and IGF-1 Receptors Contain Covalently Bound Palmitic Acid

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We have studied the biosynthesis of the insulin receptor in a human hepatoma cell line, HepG2. As previously reported, these cells synthesize a disulphide-bonded α_2 β_2 tetrameric insulin receptor. Labelling of HepG2 cells with [^{3}H]palmitate or [^{3}H]myristate followed by immunoprecipitation with a polyclonal antireceptor antibody revealed the incorporation of palmitate, but not myristate, into the β-subunit and αβ-precursor of the receptor in a hydroxylamine-sensitive linkage. The extracellular α-subunit was not labelled, demonstrating the specificity of incorporation. Acylation of the insulin receptor was an early event as judged by fatty acid incorporation into the αβ-precursor and prevention by protein synthesis inhibitors. Pulse-chase studies demonstrated the expected processing of the αβ-precursor to mature α- and β-subunits, but no evidence for preferential turnover of the fatty acid moiety was found. The site of acylation appears to be in the transmembrane or cytoplasmic domain since proteolytic treatment of intact cells produced a truncated β-subunit still containing label. Binding studies showed that HepG2 cells contain approximately half as many insulin-like growth factor-1 receptors as insulin receptors, raising the possibility that this receptor may also be acylated. Indeed, immunoprecipitation with the antiinsulin receptor serum of MDCK cells expressing IGF-1 receptors, but not insulin receptors, revealed bands corresponding to the αβ-precursor, α- and β-subunits, of which the αβ-precursor and β-subunits incorporated [^{3}H]palmitate but the α-subunit did not.

Key words: palmitate, acylation, thioester, transmembrane signalling

The mechanism of transmembrane signalling by receptors for extracellular stimuli is an area of intense current investigation. Many receptor molecules, after receiving the stimulus, are known to interact with membrane-bound transducing molecules known as G-proteins. These G-proteins transfer the signal to effector molecules such as adenylate cyclase [1,2] or phosphatidyl-inositol bisphosphate phosphodiesterase [3–5].

The insulin receptor (IR) provides an example of an integral transmembrane protein which is capable of mediating multiple diverse biological effects of insulin in different

Abbreviations used: IR, insulin receptor; IGFR, IGF-1 receptor.

Received July 28, 1987; accepted November 12, 1987.

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cell types [6,7]. This tetrameric disulphide-bonded α_2 β_2 molecule, whose α-subunits bind insulin, possesses intrinsic tyrosine kinase activity in its transmembrane β-subunits [8–10]. The physiologically important substrates on which this kinase acts remain to be identified, but it is possible that the activation of the IR upon ligand binding directly initiates a phosphorylation cascade, as is presumed to occur with other receptors which possess intrinsic kinase activity [11]. However, evidence also exists suggesting that the IR interacts with G-proteins to regulate the activity of adenylate cyclase and cyclic nucleotide phosphodiesterase [12,13] and possibly other signalling systems. Thus, insulin has also been shown to trigger the hydrolysis of a novel glycolipid, which may lead to the release of both a water-soluble head group [14,15] and diacylglycerol [16,17]. It is thus possible that the IR is involved in regulating the concentration of a variety of second messengers which directly or indirectly control the activity of intracellular kinases and phosphatases [18].

The molecular basis of these signal transduction events is poorly understood. In studying these processes at the molecular level, we have been focussing on the role of covalent modification of signal-transducing molecules with fatty acids, which has been shown to be vital for the function of G-proteins such as $p21^{ras}$ [19,20] and effector molecules such as $pp60^{src}$ [21,22]. In addition, a number of cell surface receptors for extracellular ligands—e.g., transferrin receptor, histocompatibility antigens, and IgE receptor [23–26]—have been shown to be acylated. This type of modification is not universal, however, since epidermal growth factor receptor and low-density lipoprotein receptor are not acylated [27–29]. During the course of our studies we have observed the covalent attachment of palmitate in thioester linkage to the β-subunits of human IR and canine insulin-like growth factor-1 receptor (IGFR). This appears to be a stable modification which is added soon after synthesis and persists throughout the lifetime of the protein. A preliminary account of this work has been presented elsewhere [30], and we present here a more detailed characterization of the acylation of IR and IGFR.

MATERIALS AND METHODS

Cells and Antibodies

An antiserum to the purified human IR [31] was raised in rabbits by monthly injection of approximately 10 μg subcutaneously in Freund's adjuvant. The human hepatoma cell line HepG2 was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, U.K.) and type II Madin-Darby Canine Kidney (MDCK) cells [32] were from Gibco Ltd., Middx., U.K. Cells were cultured in Dulbecco's Modified Eagle's medium with 10% (v/v) foetal calf serum (Sera Lab Ltd., W. Sussex, U.K.), streptomycin (100 μg/ml), and penicillin (60 μg/ml) in a 95% air-5% CO_2 atmosphere.

Metabolic Labelling and Immunoprecipitation

For labelling with fatty acids, medium was supplemented with 5 mM Na pyruvate. Cells were labelled for the indicated times with 100–150 μCi/ml [9,10-^{3}H]palmitic acid (55 Ci/mmol, Amersham International, Bucks, U.K.) or 100 μCi/ml [9,10-^{3}H]myristic acid (22.5 Ci/mmol, NEN, Darmstadt, F.R.G.). Cells were labelled with 50–100 μCi/ml [^{35}S]methionine (>800 Ci/mmol, Amersham) in medium lacking methionine for a short label or with 10% of the normal amount of methionine for a long label, and with

dialysed foetal calf serum. Chase medium consisted of normal medium supplemented with ten times the normal amount of methionine.

After labelling, cells were briefly washed thrice with ice-cold 50 mM Na phosphate-buffered saline (PBS) and lysed by the addition of cold RIPA buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) NP40, 0.5% (w/v) Na deoxycholate, 0.1% (w/v) SDS, 0.2 mM phenylmethanesulphonyl fluoride, 1% (v/v) Trasylol, pH 7.5) for 10 min on ice. Dishes were scraped and the insoluble residue was pelleted for 10 min at 4°C in an MSE Microcentaur microfuge at 12,000g. The supernatant was precipitated overnight at 4°C with a saturating amount of antiserum (5–10 μl) followed by protein A-Sepharose CL-4B beads (10 μl packed volume, Pharmacia) for 2 hr. The beads were washed thrice with 1 ml RIPA buffer, once with 100 mM Tris-Cl, pH 6.8 and eluted with 50 μl gel loading buffer containing 100 mM dithiothreitol [33].

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli [33] on 0.9 mm-thick 7.5% gels, followed by fluorography [34]. Dried gels were exposed to preflashed Kodak XAR5 film at −70°C. Hydroxylamine treatment was performed on strips of gel before fluorography as previously described [28,35]. The molecular weight markers were myosin heavy chain (200 kD), β-galactosidase (116 kD), phosphorylase (92 kD), albumin (68 kD), and ovalbumin (43 kD).

Binding Studies

Highly purified bovine insulin was a gift from Dr. D. Brandenburg, University of Aachen, and recombinant human IGF-1 was a gift from Drs. H. Peter and K. Scheibli, Ciba-Geigy, Basel. Insulin was radioiodinated with carrier-free Na ^{125}I (Amersham) to a final specific activity of 150–200 μCi/μg as described by Linde et al. [36] and ^{125}I-IGF-1 (specific activity 80–100 μCi/ug) was prepared by the method of Roth [37].

Binding assays were carried out with intact cells grown almost to confluence on 24-well tissue culture plates. Cells were washed with 3 × 1 ml of PBS before addition of labelled insulin or IGF-1 (~15,000 dpm) and unlabelled hormones (10^{-10}–10^{-5} M) in a total of 0.3 ml incubation buffer [38]. After 4 hr at 4°C, medium was aspirated and cells were washed with 3 × 1 ml of cold PBS before solubilisation in 0.2 ml 1 M NaOH. Samples of the solubilised cells were taken for radioactive counting on an NE1600 counter and for protein assay [39] with bovine gamma globulin as standard.

RESULTS

Acylation of the IR β-Subunit

The HepG2 cell line was chosen for these studies because it has been used extensively to study the biosynthesis of liver-specific proteins and has been shown to express between 7×10^4 and 3×10^5 IR per cell, of varying affinity [40,41]. Several polyclonal and monoclonal antibodies [42] were tested for the ability to immunoprecipitate the IR from [^{35}S]methionine-labelled lysates of these cells.The polyclonal serum which has been used throughout these studies was found to be the best for this purpose. Figure 1 shows the results of immunoprecipitation with this antiserum on lysates of metabolically labelled HepG2 cells. Three bands were specifically precipitated from [^{35}S]methionine-labelled cells, which had the expected mobilities of the 190–210-kD αβ-precursor molecule and the mature 130-kD α- and 95-kD β-subunits (lanes a and b). The 190-kD

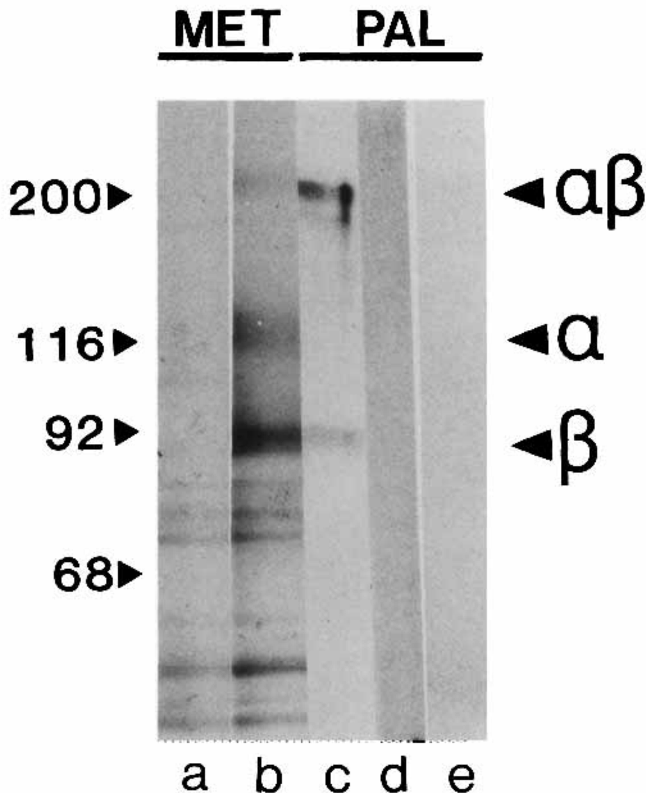

Fig. 1. Acylation of the human insulin receptor (IR). HepG2 cells in 35-mm dishes were labelled in 1 ml medium for 18 hr with 80 μCi/ml [^{35}S]methionine (**lanes a** and **b**) or for 4 hr with 100 μCi/ml [^{3}H]palmitate (**lanes c–e**) as described in Materials and Methods. Lane d was treated with hydroxylamine before fluorography. Lane e shows the abolition of acylation by pretreatment for 1 hr with 10 μm emetine before addition of [^{3}H]palmitate. Lanes b–e are immunoprecipitates using the polyclonal anti-IR serum; lane a was precipitated with nonimmune rabbit serum. The positions of the αβ-precursor and the mature α- and β-subunits are shown. Molecular weight markers are as described in Materials and Methods. Exposure times: tracks a,b = 2 days; tracks c–e = 25 days.

αβ-component appears to be a true intracellular metabolic precursor of the mature α- and β-subunits, whereas the 210-kD component is believed to be a fully carbohydrate-processed cell surface dead-end product which has escaped proteolytic processing [43]. We shall refer to both these bands collectively as the αβ-precursor. Under nonreducing conditions all these bands were absent, and instead a band was seen near the top of the separating gel, as expected for the disulphide-bonded $\alpha_2\beta_2$ tetramer (not shown). After biosynthetic labelling for 4 hr with [^{3}H]palmitic (Fig. 1, lane c) or [^{3}H]myristic acids (not shown), incorporation of the former but not the latter into both components of the αβ-precursor and the transmembrane β-subunit was observed. No label was incorporated into the extracellular α-subunit, testifying to the specificity of the labelling and the lack of reincorporation of label into amino acids. Presumably the label in the αβ-precursor is localised to the β-region. Treatment of gel slices with hydroxylamine (lane d) resulted in almost complete removal of fatty acid label from the αβ and β bands. This is consistent with the label being present in an ester linkage, probably a thioester, and again indicates

the specificity of labelling. In several experiments a relatively high incorporation of [^{3}H]palmitate into the 210-kD component of the αβ-precursor compared to the β-subunits has been observed. Unfortunately, too few counts have been incorporated into the subunits up to this point to allow direct unequivocal identification of the bound label as still being palmitic acid. However, after complete acid hydrolysis >80% of the released label was extractable into hexane, consistent with a fatty acid. Emetine completely blocked acylation of the IR (lane 3), which suggests that it is an early posttranslational event, which is also suggested by labelling of the 190-kD component of the αβ-precursor.

To localise the site of acylation in the β-subunit we performed tryptic digestions on intact cells prelabelled overnight with [^{35}S]methionine or [^{3}H]palmitate. A truncated β-subunit derived from proteolytic removal of the amino-terminal region of the surface-exposed β-subunit was observed with both labels (data not shown), which suggests that the fatty acid is localised in the transmembrane or cytoplasmic domains of the IR. Approximately half of the β-subunit was undigested, consistent with the large internal pool of receptors in serum containing medium [44] (A. I. Magee, unpublished data). In a long label the 190-kD component of the αβ-precursor is not detectable. The 210-kD αβ-precursor was substantially degraded, indicating its predominantly surface localisation, while the α-subunit was little affected under these conditions. In a number of experiments an extra band of M_r of ~60 kD was observed, which labelled with both [^{35}S]methionine and [^{3}H]palmitate and which was precipitated by the anti-IR serum (see Fig. 2). This band, which we have called β′, may be derived from the β-subunit by truncation of the C-terminal half of the molecule; similar β-subunit-derived bands have been observed in other systems [45–47]. However, the possibility that this represents a component unrelated to IR which is recognised by a contaminating activity in the antireceptor serum cannot be ruled out.

Turnover of Fatty Acid on the IR

Omary and Trowbridge [23] have previously shown that the palmitate attached to the transferrin receptor turns over more rapidly than the polypeptide backbone. In order to study the role of the fatty acyl moiety of the IR we have performed pulse-chase studies (Fig. 2). Panel A shows the result of immune precipitation with anti-IR of cells labelled with [^{35}S]methionine or [^{3}H]palmitate. In panel B these data have been quantitated by scanning of the resulting fluorograph. Although there was some indication of a precursor-product relationship in the initial decrease in [^{35}S]methionine labelling of the αβ-component and increase in labelling of mature α- and β-subunits, the αβ-subunits was relatively stable (t½~1.5 days) compared to that described in other cell types [43,48]. This observation, together with the trypsin sensitivity of this component in intact cells, suggests that under the labelling conditions used in HepG2 cells, the high molecular weight αβ-component represents proreceptor which has escaped early proteolytic cleavage and appeared on the cell surface rather than the pool of true receptor precursor. Such a component has been described in other cell types and particularly in transformed cells [49,50].

The kinetics of fatty acid turnover were also studied. No major differences in the stability of the palmitate moiety compared to the protein backbone could be detected, although minor differences may have been missed due to the poor signal-to-noise ratio in quantitating fatty acid labelling (compare the ordinates in the lower panel of Fig. 2). It is notable, for instance, that the labelling with palmitate relative to methionine is greater for the 210-kD αβ-precursor than for the mature β-subunit. Thus it appears that

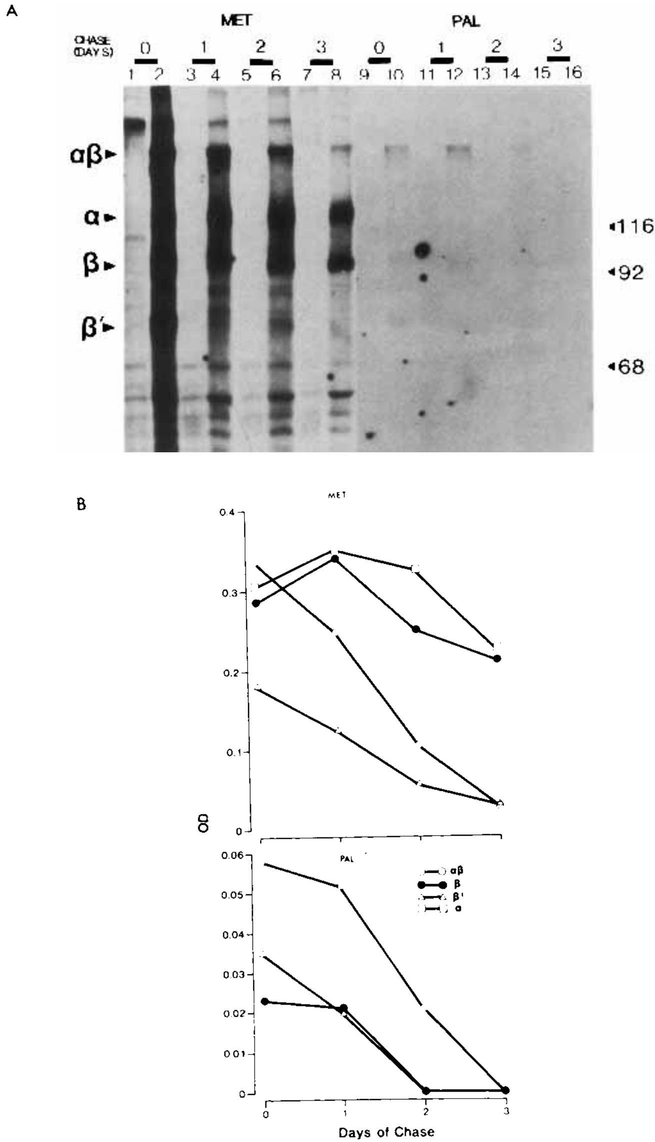

Fig. 2. Turnover of IR and fatty acid. **A**: Subconfluent HepG2 cells in 35-mm dishes were labelled for 2 hr with 50 μCi/ml [^{35}S]methionine (MET) or 100 μCi/ml [^{3}H]palmitate (PAL), followed by chases of 0, 1, 2, 3 days. At each time point cells were lysed and immunoprecipitated with control serum (odd numbered lanes) or anti-IR serum (even numbered lanes). **B**: The resulting fluorograph was quantitated by scanning by using a Zeineh softlaser densitometer. Exposure time = 21 days.

acylation of the IR is a stable modification not turning over significantly during the lifetime of the protein under normal culture conditions.

Acylation of the IGFR

Since HepG2 cells have been reported to express both the IR and IGFR [40,51], and given the considerable similarity in these receptors [52,53], it was possible that the

labelled bands we have observed were actually derived from the IGFR or from a mixture of both receptors. The anti-IR serum used was known to cross-react with IGFR, at least in other human tissues (K. Siddle and M. A. Soos, unpublished observations). Binding studies confirmed that our HepG2 line contained a low level of IGFR [54]. MDCK cells, on the other hand, have been reported to possess only IGFR [54]. Table I shows that while IR was almost undetectable in the strain II MDCK cells used here, the IGFR was present at about three times the level found in HepG2 cells. Thus we performed immunoprecipitation with the anti-IR serum on [^{35}S]methionine- or [^{3}H]palmitate-labelled MDCK cell lysates (Fig. 3). Bands were specifically immunoprecipitated which corresponded in molecular weight to the αβ-precursor and mature α- and β-subunits of the IGFR; thus our antiserum does appear to cross-react with this receptor. The backgrounds were high in these experiments, probably due to reduced cross-species and cross-receptor reactivity of the serum and to the lower amount of IGFR. A similar result was obtained in a separate experiment in which the $\alpha_2\beta_2$ tetramer was observed under nonreducing conditions (not shown). [^{3}H]palmitate was incorporated into the αβ- and β-bands, but not the α-band in a hydroxylamine-sensitive linkage (Fig. 3). The relative intensities of the [^{3}H]palmitate-labelled bands from HepG2 and MDCK cells indicated that the label incorporated in HepG2 cells could not be accounted for by acylation of the IGFR alone. We conclude that both the IGFR and the IR are acylated on their β-subunits with palmitic acid.

DISCUSSION

We have shown that the IR of HepG2 cells and the IGFR of MDCK cells are acylated on their β-subunits. The IR could be biosynthetically labelled with palmitic but not myristic acid, and the sensitivity of the linkage to hydroxylamine suggested that the acylgroup was attached by a thioester bond. We have localised the site of acylation of the IR to the transmembrane or cytoplasmic domains, consistent with the acylation sites of other transmembrane proteins [24,55,56]. There are three cysteine residues in this region of the IR β-subunit which are conserved in IGFR [46,53] and which appear to be the most likely candidates for sites of acylation. It should be possible to test this directly due to the availability of cloned receptor genes. It was not possible in the present experiments to make any estimate of the stoichiometry of acylation; however, the αβ-precursor of the IR consistently had a higher level of acylation relative to [^{35}S]methionine

TABLE I. Binding of Insulin and IGF-1 to HepG2 and MDCK Cells*

	HepG2		MDCK	
	^{125}I-insulin	^{125}I-IGF-1	^{125}I-insulin	^{125}I-IGF-1
Specific binding of tracer (%)	4.7 ± 0.3	0.8 ± 0.1	0.05 + 0.02	2.4 ± 0.3
Insulin giving 50% displacement (M)	3×10^{-10}	2×10^{-6}	—	5×10^{-6}
IGF-1 giving 50% displacement (M)	1×10^{-7}	2×10^{-9}	—	3×10^{-9}

*Binding of labelled hormones to HepG2 and MDCK cells was performed as described in Materials and Methods. Protein content per well was estimated to be 110 ± 28 μg for HepG2 cells and 146 ± 26 μg for MDCK cells.

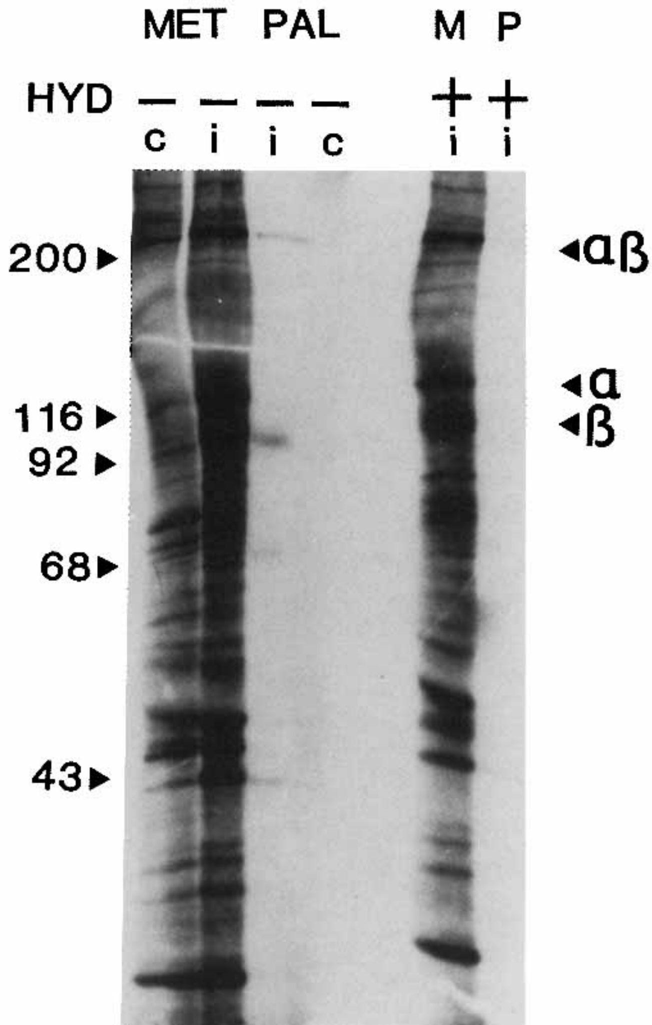

Fig. 3. Acylation of the canine insulin-like growth factor-1 receptor (IGFR). Subconfluent MDCK cells in 90-mm dishes were labelled for 18 hr with 5 ml medium containing 12 μCi/ml [^{35}S]methionine (**MET**) or 190 μCi/ml [^{3}H]palmitate (**PAL**), followed by lysis and immunoprecipitation with control serum (**c**) or anti-IR serum (**i**). Duplicate lanes were treated with hydroxylamine (+) as described in Materials and Methods before fluorography (**M**, methionine; **P**, palmitate). Exposure time = 27 days.

than the β-subunit. This may imply that some fatty acid is lost during maturation of the IR or that the αβ-component observed in the present studies is a dead-end product which is incapable of processing to mature subunits and is hyperacylated.

The kinetics of labelling were consistent with incorporation of palmitate predominantly, if not entirely, as an early posttranslational modification of newly synthesised receptor. Thus the presumed fully glycosylated (M_r ~210 kD) αβ-precursor and the high mannose (M_r ~190 kD) form were labelled with [^{3}H]palmitate, consistent with acylation occurring in the early Golgi, as is believed to be the case for other proteins [57–59]. Because of the low levels of radioactive incorporation it was not possible to

identify directly the esterified label and to quantify the relative rates of turnover of acyl group and receptor protein. HepG2 cells appear to possess a relatively high fraction of "precursor" located on the cell surface in an unprocessed form, and it is not certain that the turnover of acyl groups in this component would properly reflect that in the intracellular pool of precursor proper or in mature β-subunits. Pulse-chase studies suggest, however, that the β-subunit acyl group also turned over only rather slowly, at least in the absence of insulin.

Rather different results relating to acylation of IR were recently described by Hedo et al. [60]. Working with human IM-9 lymphocytes, they reported that myristic acid was incorporated into both α- and β-subunits of IR and palmitic acid only into the β-subunits, and that some of this incorporation was hydroxylamine resistant, most particularly in the α-subunit. This was interpreted as indicating the existence of both ester-linked and amide-linked fatty acyl groups, although the possibility of incorporation of label from fatty acids which had been metabolised to amino acids was not ruled out. Thus, although it was shown that labelled material from hydrolysates co-chromatographed on HPLC with palmitic and myristic acids, this analysis was performed on pooled α- and β-subunits, with extraction solvents which would probably not have extracted amino acids for the chromatographic analysis, and without monitoring recoveries of labelled material, which appear to have been low. It appears to us, therefore, that the existence of acylation other than as the thioester linkage of palmitate to the IR β-subunit which we describe was not conclusively established by Hedo et al. [60]. However, it may be that there are cell-type differences in the extent and sites of acylation or that other forms of acylation were below the level of detection in our experiments.

The role of protein acylation is poorly understood, except in the case of $pp60^{src}$ [21,61] and $p21^{ras}$ [19], where acylation seems to be required for association with the inner face of the plasma membrane and transformation. A number of cell-surface receptors have been found to be acylated (see beginning of this paper), but there are also nonacylated cell-surface receptors such as epidermal growth factor and low-density lipoprotein receptors [27–29]. It seems unlikely that in these cases acylation is required for membrane association. Indeed, the surface glycoprotein of vesicular stomatitis virus has been shown to function normally when its acylation site is removed by site-directed mutagenesis [55]. Acylation must thus perform a more subtle function, and it is intriguing that the fatty acid moiety of a number of proteins has recently been found to turn over faster than the polypeptide backbone [20,23,62]. We have found no evidence for accelerated turnover of the fatty acyl moiety of the IR, but this must be investigated rigorously in the context of the intracellular trafficking which the receptor undergoes. Indeed, the palmitate moiety of the influenza virus haemagglutinin has been implicated in the membrane-fusion activity of this protein [63].

ACKNOWLEDGMENTS

We thank Maria Soos for assistance with binding assays and Marilyn Brennan for typing. This work was supported by the Medical Research Council. Ken Siddle is a Wellcome Trust Senior Lecturer.

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NOTE ADDED IN PROOF

We have now examined two independently-derived cell lines both overexpressing cloned human IR cDNA [Ellis et al.: Cell 45:721–732, 1986; Whittaker et al.: Proc Natl Acad Sci 84:5237–5241, 1987] and find little or no incorporation of fatty acids into the receptor. This may mean that the acylated bands we have detected in HepG2 cells are derived only from IGFR. We cannot at present exclude the alternative possibility that these results are due to differences in processing of IR between cell lines.