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N-Myristoylation of p60^{src}

Identification of a myristoyl-CoA:glycylpeptide *N*-myristoyltransferase in rat tissues

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A 16-residue synthetic peptide corresponding to the *N*-terminal sequence of p60^{src} was used as the acyl acceptor in an assay for myristoyl-CoA:glycylpeptide *N*-myristoyltransferase in rat tissues. An additional *C*-terminal tyrosine amide was added to this peptide to facilitate radioiodination and enhance detectability. Reverse-phase h.p.l.c. enabled the simultaneous detection and quantification of the peptide substrate and its *N*-myristoylated product. *N*-Myristoyltransferase activity was highest in the brain with decreasing activities in lung, small intestine, kidney, heart, skeletal muscle and liver. Brain activity was distributed approximately equally between the 100000 *g* pellet and supernatant fractions. The soluble enzyme exhibited a $K_m^{app.}$ of 20 μM for the *src* peptide and an optimum between pH 7.0 and 7.5. Maximum *N*-acylating activity was seen with myristoyl ($C_{14:0}$)-CoA with lower activities found with the $C_{10:0}$-CoA and $C_{12:0}$-CoA homologues. No activity was obtained with palmitoyl ($C_{18:0}$)-CoA but this derivative inhibited *N*-myristoyltransferase activity > 50% at equimolar concentrations with myristoyl-CoA. With a decapeptide corresponding to the *N*-terminal sequence of the cyclic AMP-dependent protein kinase catalytic subunit as the acyl acceptor, the brain enzyme displayed a $K_m^{app.}$ of 117 μM and was about 14-fold less catalytically effective than with the p60^{src} acyl acceptor. Transferase activity was also seen with a 16-residue peptide corresponding to the *N*-terminal sequence of the HIV p17^{gag} structural protein. Inhibition studies with shorter *src* peptide analogues indicated an enzyme specificity for the p60^{src} acyl acceptor beyond 9 residues.

INTRODUCTION

The transforming product of the Rous sarcoma virus is a tyrosine kinase with an M_r of 60000 termed p60$^{v\text{-}src}$. Both it and its cellular homologue, p60$^{c\text{-}src}$, are localized to the inner surface of the plasma membrane [1–5]. The transforming activity of the viral enzyme appears to depend upon this membrane localization [1–7]. It has been shown that this membrane association requires the addition of myristic acid to the *N*-terminal glycine via an amide linkage [7–16]. Mutant viruses in which the v-*src* product contained *N*-terminal amino acid deletions [7,8] or in which the *N*-terminal glycine was replaced with alanine or glutamic acid [12] all failed to incorporate myristic acid. These mutant proteins were still functional kinases *in vitro* and *in vivo*, but instead of being localized to the membrane, they were found predominantly in the cytoplasm. Most significantly, these mutant kinases no longer transformed cells. These as well as other studies indicate that *N*-myristoylation is an essential step in p60$^{v\text{-}src}$-dependent transformation.

It appears that the sequence information within the first 14 *N*-terminal amino acids of the c-*src* or v-*src* kinases is sufficient for their *N*-myristoylation [7,8]. It has been shown that when a heterologous gene is constructed by fusing the *N*-terminal 14 amino acid sequence of p60^{src} to that of a nonmyristoylated cytoplasmic protein, the resulting hybrid gene product becomes *N*-myristoylated and membrane-associated [11]. In addition, since *N*-myristoylation may occur by a co-translational process [14], it is possible that nascent polypeptide chains are the actual substrates of the *N*-myristoyltransferase(s) [14]. In this paper we have used a 16-amino-acid synthetic peptide homologous to the *N*-terminal amino acid sequence of p60^{src} as the acyl acceptor in an assay for the *N*-myristoyltransferase(s) responsible for p60^{src} *N*-myristoylation in mammalian tissues. We have used this assay to establish a wide distribution of *N*-myristoyltransferase activities in rat tissues. In addition, the preliminary characterization of the *N*-myristoyltransferase activity from rat brain allowed a comparison with a recently reported yeast enzyme which was assayed using a synthetic octapeptide corresponding to the *N*-terminal amino acid sequence of cyclic AMP-dependent protein kinase catalytic subunit [17–19]. *N*-Myristoylation is an essential step in p60$^{v\text{-}src}$ transforming activity. In addition, it may be important for the transformation activities of a variety of viral myristoylated *gag–onc* fusion proteins [20–22]. Consequently, *N*-myristoyltransferase(s) could represent an important new target for the inhibition or reversal of these *N*-myristoylation-dependent transformations.

EXPERIMENTAL

Preparation of rat tissue high speed supernatant fractions

Adult male Sprague–Dawley rats (150–200 g) were killed by cervical dislocation. Tissues were removed,

Abbreviation used: cAMPdPK, cyclic AMP-dependent protein kinase.

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minced with scissors and homogenized in 20 mM-Tris/HCl, pH 7.4, containing 1 mM-EDTA, 1 mM-dithiothreitol and 8 μg of pepstatin, 20 μg of leupeptin and 0.5 mg of aprotinin/ml (1 g of tissue/6 ml) for 15 s at 50% maximum power with a Polytron homogenizer (model PCU) equipped with a PT-10-ST probe (Brinkman Instruments, Westbury, NY, U.S.A.) at 0–4 °C. The homogenates were centrifuged at 100000 *g* for 60 min in an ultracentrifuge (model L5-50, Beckman Instruments). The high speed supernatant fractions were removed and the pellets washed twice by resuspension in the original volume of homogenization buffer using a Dounce homogenizer. The pellets were then recentrifuged and finally resuspended as described above. Samples of the original homogenates and the washed high speed pellet and supernatant fractions from brain were stored at −70 °C and thawed immediately prior to use.

Synthetic peptides

Synthesis and purification of peptides was performed by Peptide Technologies Inc. (Washington, DC, U.S.A.). Peptide amides were synthesized by standard solid phase methodologies on a *p*-methylbenzhydrylamine resin [23]. The synthetic *N*-myristoylated peptides were prepared by acylating the free terminal amino group with myristate by carbodi-imide activation before the resin was deblocked and cleaved. The crude peptides were purified by counter-current preparative reversed phase h.p.l.c. or ion exchange chromatography. The purity and identity of the peptides were established by reversed phase h.p.l.c., t.l.c. and amino acid analysis.

Iodination and purification of radiolabelled peptides

An additional noncoded *C*-terminal tyrosine was included in some peptides to facilitate radioiodination by the method of Hunter & Greenwood [24]. A peptide (2 nmol in 10 μl of deionized water) and 10 μl of 0.33 M-potassium phosphate buffer, pH 7.0, were added to the original vial containing 1 mCi of carrier free $Na^{125}I$ (New England Nuclear). The iodination was initiated by the addition of 10 μl of 0.5 mM-chloramine T (2.5 nmol) and allowed to proceed at room temperature for 1 min. The reaction was terminated by the addition of 40 μl of 0.31 mM-sodium metabisulphite (12.4 nmol), allowed to stand for 3 min prior to the addition of 100 μl of 1 mM-NaI. The iodinated peptides were purified using a C_{18} Sep-Pak cartridge (Waters, Bedford, MA, U.S.A.). The reaction mix was applied to the cartridge, followed by a 0.5 ml 0.1% trifluoroacetic acid wash of the reaction vessel. The separation of labelled peptide from contaminating ^{125}I was achieved by eluting the Sep-Pak with increasing concentrations of acetonitrile in a water/0.1% trifluoroacetic acid/acetonitrile mix (3 ml with 0% acetonitrile, 2 ml 10%, 4 × 0.5 ml 25%, 3 × 1 ml 50% and 3 × 1 ml 75%). Eluted fractions were collected and the radioactivity determined by gamma counting. The $p60^{src}$ and cAMPdPK *N*-glycylpeptides were eluted with 25% acetonitrile, the $p60^{src}$ and cAMPdPK *N*-myristoylglycylpeptides and the *gag* *N*-glycylpeptide were eluted with 50% acetonitrile, and the *gag* *N*-myristoylglycylpeptide was eluted with 75% acetonitrile. Appropriate fractions were combined, dried with a stream of N_2 at 50 °C, dissolved in 100% dimethyl sulphoxide and stored at −20 °C.

Radioiodination of the *src* *N*-glycylpeptide produced one major (80–90%) and one minor radioactive peak which eluted between 5 and 6 min on reversed phase h.p.l.c. (see Fig. 1). When these two peaks were purified by reversed phase h.p.l.c. (see below) and individually tested as *N*-myristoylation substrates after mixing with 100 μM-nonradioactive *src* peptide, then the minor substrate peak exhibited < 1% of the activity of the major substrate peak.

N-Myristoyltransferase assay

Except where otherwise stated, enzyme reaction mixtures included 100 μM-$p60^{src}$, 400 μM-cAMPdPK or 750 μM-$p17^{gag}$ *N*-glycyl[^{125}I]peptides, 100 μM-myristoyl-CoA, 1 mM-dithiothreitol and 20 mM-Tris/HCl (pH 7.4) in a final volume of 0.1 ml. Reactions were carried out in 13 mm × 100 mm test tubes (previously soaked for 1 h in 0.1% polyethyleneimine, drained, and air dried) at 37 °C for the indicated times. Reactions were terminated by the addition of 100 μl of ice-cold 10% trichloroacetic acid in methanol. The tubes were placed on ice for 10 min, and then centrifuged for 10 min at 750 *g* in a table-top centrifuge. The enzymic reaction product was quantified by analysing aliquots of the supernatant fraction by reversed phase h.p.l.c. as described below. All acyl-CoA derivatives were obtained from Sigma Chemical Co.

N-Myristoylglycylpeptide stability was established by incubating 6.9 μM-*src* or 7.5 μM-cAMPdPK *N*-myristoylglycyl[^{125}I]peptides with the same preparation and protein concentration of rat brain 100000 *g* supernatant fraction and under the same conditions as utilized in the K_m experiments (see legend to Fig. 2). After a 15 min incubation, > 98% of both starting *N*-myristoylated peptides were recovered by reversed phase h.p.l.c.

Stoichiometry of *N*-glycylpeptide acylation

The stoichiometry of peptide acylation was determined by incubating 100 μM of either radioiodinated (2.63×10^5 c.p.m./nmol) or nonradioactive $p60^{src}$ *N*-glycylpeptides and 40 μM-[1-^{14}C]myristoyl-CoA (1.01×10^4 d.p.m./μmol) (New England Nuclear) with 125 μg of rat brain 100000 *g* supernatant protein for 30 min as described above. The radioactive *N*-myristoylglycylpeptide products were collected from reversed phase h.p.l.c. (see below) and the radioactivities were determined by liquid-scintillation or gamma counting.

Identification of *N*-myristoylglycine linkage

The enzymic formation of the *N*-myristoylglycine linkage was established by incubating 100 μM-nonradioactive *src* *N*-glycylpeptide and 107 μM-[1-^{14}C]myristoyl-CoA (45.5 mCi/mmol) with 125 μg of rat brain 100000 *g* supernatant protein for 60 min as described above except for the inclusion of 0.25% bovine albumin. The *N*-[1-^{14}C]myristoylglycylpeptide product was isolated by reversed phase h.p.l.c. and proteolytically digested with alkaline Pronase (Sigma) and Pronase E (Sigma) as previously described [25]. The *N*-[1-^{14}C]myristoylglycine was extracted from the digestion mixture with chloroform/methanol (2:1, v/v) and identified by comparison with an authentic *N*-[9,10-^{3}H]myristoylglycine standard by reversed phase h.p.l.c. [25].

Analytical methods

Reversed phase h.p.l.c. was performed using a Vydac C_{18} protein/peptide 5 μm column (4.6 mm × 250 mm) (Separations Group, Hesperia, CA, U.S.A.) with a

Brownlee Aquapore C_8 RP-300 pre-column (Applied Biosystems Inc., Santa Clara, CA, U.S.A.). In the normal enzyme assay, peptides were separated using a linear gradient from 5% acetonitrile/0.1% trifluoroacetic acid to 65% acetonitrile/0.1% trifluoroacetic acid over 10 min followed by 5 min with 65% acetonitrile/0.1% trifluoroacetic acid (flow rate 2 ml/min). The major and minor *N*-glycyl[^{125}I]peptide substrate peaks were completely resolved using a gradient from 0% acetonitrile/0.1% trifluoroacetic acid to 20% acetonitrile/0.1% trifluoroacetic acid over 15 min.

T.l.c. was carried out on plastic backed cellulose thin layer sheets (Eastman Kodak, Rochester, NY, U.S.A.) in a solvent of butanol/pyridine/acetic acid/water (15:10:3:12, by vol.). Non-radioactive peptides were detected with 1% ninhydrin. Radioactive peptides were detected by autoradiography with Kodak XAR-2 X-ray film with Lightening Plus intensifying screens (DuPont Co., Wilmington, DE, U.S.A.) at −70 °C.

For protein determinations, tissue fractions were treated with 1 M-NaOH for 30 min at room temperature and the soluble proteins were determined by comparison with a bovine albumin standard curve by the method of Lowry [26].

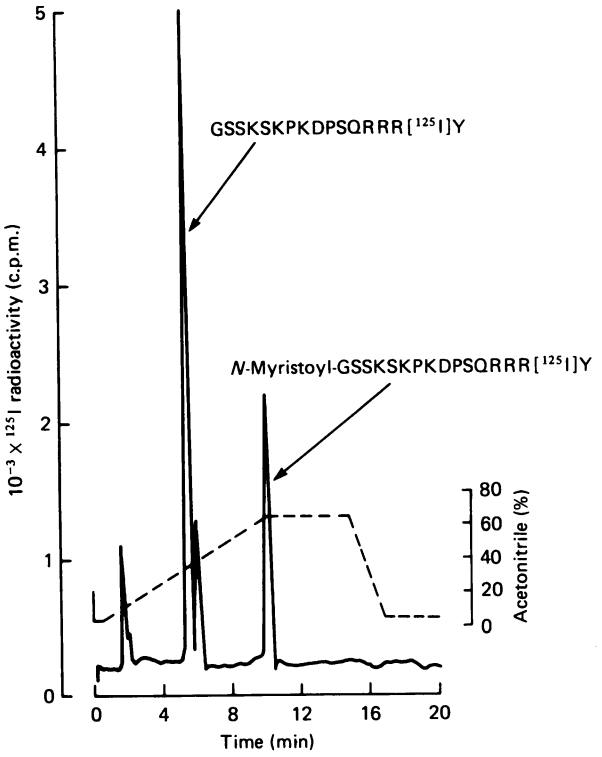

Fig. 1. Separation of the p60^{src} *N*-glycyl[^{125}I]peptide (GSSKSKPKDPSQRRR[^{125}I]Y) acyl acceptor from the *N*-myristoylglycyl[^{125}I]peptide (*N*-myristoyl-GSSKSKPKDPSQRRR[^{125}I]Y) product by reversed phase h.p.l.c.

The elution pattern shown in this Figure was from the analysis of an enzyme incubation of 100 μM-*N*-glycyl[^{125}I]-peptide and 400 μM-myristoyl-CoA in 20 mM-potassium phosphate buffer (pH 7.0) with 190 μg of rat brain 100000 *g* supernatant protein for 60 min as described in the Experimental section. The peptides (total of 1 μmol) were eluted with a linear gradient from 5 to 65% acetonitrile in 0.1% trifluoroacetic acid. The broken line traces the percentage of acetonitrile in the elution program. Radioactivity (c.p.m.) was detected by in-line analysis of ^{125}I.

RESULTS

Myristoyl-CoA:glycylpeptide *N*-myristoyltransferase

The presence of a p60^{src} *N*-myristoyltransferase activity in rat tissues was studied by an enzyme assay which utilized a synthetic peptide homologous to the *N*-terminal 15 amino acid residues of p60^{src} as an acyl acceptor (GSSKSKPKDPSQRRR[^{125}I]Y). The addition of a ^{125}I-tyrosine amide to the *C*-terminal end of this peptide allowed high detection sensitivity. This *N*-glycyl[^{125}I]-peptide and its corresponding *N*-myristoylglycyl[^{125}I]-peptide enzymic product were resolved by reversed phase h.p.l.c. (Fig. 1). The identity of the product was confirmed by comparing its retention time with that of an authentic synthetic *N*-myristoylglycyl[^{125}I]peptide and by co-chromatography with the synthetic *N*-myristoylglycyl[^{125}I]peptide on thin layer cellulose (*N*-glycyl-[^{125}I]peptide, R_f 0.13; *N*-myristoylglycyl[^{125}I]peptide, R_F 0.62).

The stoichiometry for rat brain *N*-myristoylation was determined by incubating non-radioactive or radioiodinated p60^{src} *N*-glycylpeptides with [^{14}C]myristoyl-CoA and comparing the relative proportion of radioactive labelled peptide and myristate in the enzymic product. Under otherwise identical assay conditions, the *N*-myristoylglycylpeptide product isolated by reversed phase h.p.l.c. contained 98.5±2.8 (n = 3) pmol of *N*-glycyl[^{125}I]peptide and 98.4±4.3 (n = 3) pmol of [^{14}C]-myristate. This confirms the 1:1 stoichiometry expected for the *N*-myristoylation of the *N*-glycylpeptide substrate.

The *N*-myristoylglycine linkage expected for the *N*-myristoylated *src* peptide was confirmed by incubating the rat brain 100000 *g* supernatant fraction with [^{14}C]myristoyl-CoA and nonradioactive p60^{src} *N*-glycyl-peptide and analysing for *N*-[^{14}C]myristoylglycine after total proteolytic digestion of the enzymic product. All recovered radioactivity was found as the *N*-[^{14}C]-myristoylglycine with no trace of N^{ϵ}-[^{14}C]myristoyl-lysine, thus confirming the *N*-myristoylation of the *N*-terminal glycine.

N-Myristoyltransferase activity was identified in rat brain using the p60^{src} *N*-glycyl[^{125}I]peptide acyl acceptor in the assay described in the Experimental section. Using this assay, the enzyme activity found in the crude brain homogenate was distributed approximately equally between the 100000 *g* supernatant fraction (55%) and the twice-washed crude membrane pellet (45%). All subsequent characterizations described were carried out on the enzyme activity found in the soluble fraction.

The *N*-myristoyltransferase activity in rat brain was heat-labile with maximum activity dependent upon the presence of myristoyl-CoA as the acyl donor (Tables 1 and 2). *N*-Myristoyltransferase activity was saturated with respect to myristoyl-CoA above 40 μM. At a saturating co-substrate concentration the enzyme displayed a $K_m^{app.}$ of 20 μM for the p60^{src} *N*-glycyl[^{125}I]-peptide acyl acceptor (Fig. 2). With saturating concentrations of substrates, the assay was linear for about 15 min with 120 μg of the brain supernatant fraction protein. The assay was also linear up to approx. 200 μg of brain supernatant fraction protein after a 10 min

Table 1. Characterization of the *N*-myristoyltransferase assay

The 100000 *g* rat brain supernatant fraction (140 μg of protein) was determined in the complete assay described in the Experimental section using the p60^{src} *N*-glycyl[^{125}I]-peptide as the acyl acceptor. In this experiment, the 100% activity in the complete assay corresponds to an activity of 683 pmol of *N*-myristoylglycyl[^{125}I]peptide produced/10 min incubation.

Reaction	*N*-Myristoyl-transferase activity (% of control)
Complete assay	100
−Dithiothreitol	107
−Myristoyl-CoA	0
−Brain supernatant fraction	0
+Heated supernatant fraction (100 °C, 5 min)	2.6

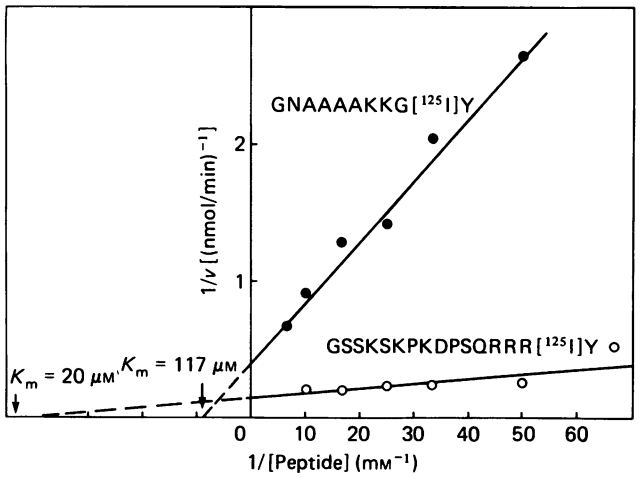

Fig. 2. Double-reciprocal analysis of rat brain *N*-myristoyl-transferase(s)

Assays as described in the Experimental section plus the inclusion of 0.25% bovine albumin were incubated in duplicate with 59 μg of rat brain 100000 *g* supernatant protein for 6 and 12 min. Peptide analogues of p60^{src} (GSSKSKPKDPSQRRR[^{125}I]Y) (○) and of cAMPdPK catalytic subunit (GNAAAAKKG[^{125}I]Y) (●) were used as acyl acceptors.

reaction. The enzyme displayed a pH optimum between pH 7.0 and 7.5 (Fig. 3).

N-Myristoyltransferase acyl-CoA specificity

The relative *N*-myristoyltransferase acyl-CoA donor specificity was examined with the p60^{src} *N*-glycyl[^{125}I]-peptide acceptor using various acyl-CoA substrates in place of myristoyl-CoA (Table 2). Retention times of the single product peaks were dependent upon the acyl chain lengths of the various acyl peptides. The highest *N*-acyltransferase activity was seen with myristoyl-CoA as cosubstrate and was expressed as 100% (Table 2). Lower acyltransferase activities also were seen for n-decanoyl ($C_{10:0}$)-CoA, lauroyl ($C_{12:0}$)-CoA and myristoleoyl ($C_{14:1}$)-CoA. In contrast, little or no significant activity was seen with palmitoyl ($C_{16:0}$)-CoA or stearoyl ($C_{18:0}$)-CoA as cosubstrates under identical conditions. These results demonstrate a clear catalytic preference of the rat brain *N*-acyltransferase for myristoyl-CoA. Furthermore, the inhibition of *N*-myristoyltransferase by the $C_{10:0}$ and $C_{12:0}$ acyl-CoA homologues suggests that a single enzyme accounts for these transferase activities by utilizing alternative cosubstrates. By similar reasoning, the > 50% inhibition by the catalytically inactive $C_{16:0}$ acyl-CoA cosubstrate homologue suggested that the palmitoyl-CoA may inhibit *N*-myristoyltransferase by acting as a dead-end inhibitor.

N-Myristoyltransferase *N*-glycylpeptide acyl acceptor specificity

The kinetic characteristics of rat brain *N*-myristoyl-transferase for the p60^{src} *N*-glycyl[^{125}I]peptide acyl acceptor was compared with *N*-glycyl[^{125}I]peptides corresponding to the *N*-terminal 9 amino acid residues of the cAMPdPK catalytic subunit (GNAAAAKKG[^{125}I]Y) and with the encoded *N*-terminal 2–16 amino acid residues of the human immunodeficiency viral p17^{gag} structural protein (GARASVLSGGELDRW[^{125}I]Y). Except for the *N*-terminal glycine there is no primary sequence homology between these three peptides. At fixed saturating myristoyl-CoA concentrations, the p60^{src} and cAMPdPK peptide acyl acceptors displayed K_m values of 20 and 117 μM and relative $V_{max.}$ values of 11.8 and 5.0 pmol·min^{-1}, respectively (Fig. 2). A comparison of $V_{max.}/K_m$ values (i.e. 5.9×10^5 and 0.42×10^5 pmol·min^{-1}·M^{-1}, respectively), which should be proportional to pseudo-second-order rate constants, suggested a 14-fold greater catalytic efficiency of the enzyme for the p60^{src} acyl acceptor compared with the cAMPdPK acyl acceptor. The stability of the p60^{src} and cAMPdPK *N*-myristoylglycyl[^{125}I]peptides under the conditions of the kinetic experiments was confirmed, substantiating that the differences in transferase activities were not due to a differential stability of the enzymic product. The brain enzyme exhibited negligible activity toward a p17^{gag} peptide acceptor at 100 μM but low activity was apparent using 750 μM-acyl peptide acceptor. These results demonstrate a rather marked preference for the p60^{src} acyl acceptor compared with these two other peptide acceptors. It remains to be determined if this catalytic preference reflects the activity of multiple enzymes or results from the catalytic preference of a single enzyme for different peptides under these conditions. However, the results do suggest an *N*-myristoyltransferase recognition of these synthetic peptides that extends beyond the common *N*-terminal glycine residue.

Peptide inhibitors of *N*-myristoyltransferase

Various sized synthetic *N*-glycylpeptides and their *N*-myristoylglycylpeptide counterparts corresponding to the *N*-terminal amino acid sequence of p60^{src} were examined as inhibitors of *N*-myristoyltransferase (Table 3). Non-radioactive *N*-myristoylglycylpeptides of 3, 5, 7, 9 or 15 amino acid residues at a 50-fold molar excess over the *src* *N*-glycyl[^{125}I]peptide acyl acceptor showed an increasing inhibition of *N*-myristoyltransferase with increasing peptide length. The data in Table 3 indicate that an *N*-acyl peptide of more than three residues is required for inhibition; however the magnitude of this

Table 2. *N*-Myristoyltransferase acyl-CoA substrate specificity

Enzyme assays were carried out with 100 μM of the p60^{src} *N*-glycyl[^{125}I]peptide acyl acceptor and 100 μM of the indicated acyl-CoA either in place of or in addition to 100 μM-myristoyl-CoA as described in the Experimental section, except for the inclusion of 0.25% bovine albumin with 118 μg of rat brain supernatant protein. The retention times of radiolabelled peptides are *N*-decanoylglycyl[^{125}I]peptide (8.9 min), *N*-lauroylglycyl[^{125}I]peptide (9.9 min), *N*-myristoleoylglycyl[^{125}I]peptide (10.2 min), *N*-myristoylglycyl[^{125}I]peptide (10.8 min) and *N*-palmitoylglycyl[^{125}I]peptide (11.6 min). No radioactive peak was detected corresponding to *N*-stearoylglycyl[^{125}I]peptides; however, the radioactivity above background between the expected elution times (12.4–13.8 min) is reported. The 100% reaction corresponds to 90 pmol of *N*-myristoylglycyl[^{125}I]peptide formed in a 15 min incubation. All other radiolabelled acyl peptide products are expressed as a percentage of the *N*-myristoylglycyl-[^{125}I]peptide.

		N-Acylglycyl[^{125}I]peptides (%)					
Acyl-CoA co-substrates	Retention times (min) ...	8.9	9.9	10.2	10.8	11.6	12.4–13.8
Myristoyl ($C_{14:0}$)-CoA		–	–	–	100	–	–
n-Decanoyl ($C_{10:0}$)-CoA		33.2	–	–	–	–	–
Lauroyl ($C_{12:0}$)-CoA		–	81.7	–	–	–	–
Myristoleoyl ($C_{14:0}$)-CoA		–	–	38.2	–	–	–
Palmitoyl ($C_{16:0}$)-CoA		–	–	–	–	4.9	–
Stearoyl ($C_{18:0}$)-CoA		–	–	–	–	–	6.9
Myristoyl-CoA + n-decanoyl-CoA		–	–	–	77.6	–	–
Myristoyl-CoA + lauroyl-CoA		–	21.6	–	71.6	–	–
Myristoyl-CoA + palmitoyl-CoA		–	–	–	43.2	–	–

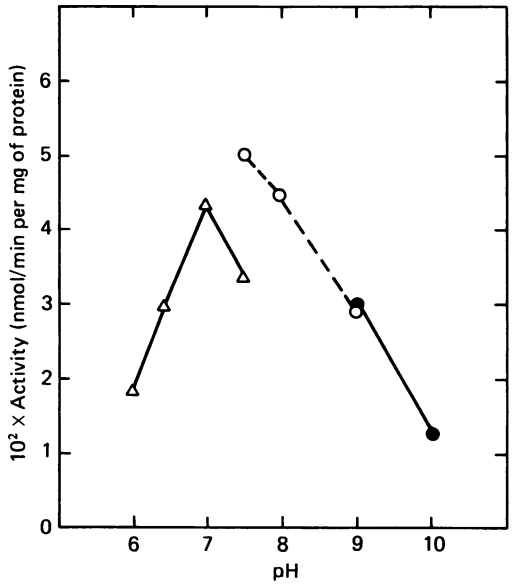

Fig. 3. pH optimum for rat brain *N*-myristoyltransferase(s)

Enzyme was incubated for 15 min with 120 μg of rat brain 100000 *g* supernatant protein in 20 mM-potassium phosphate (△), 20 mM-Tris/HCl (○) or 20 mM-glycine (●) buffers.

size-dependent inhibition is uncertain due to the increasing insolubility of the shorter peptides under our assay conditions. In contrast with the marked inhibition by *N*-myristoylglycyl peptides, comparable inhibition by a series of nonmyristoylated peptides was only evident for the p60^{src} homologues with more than seven residues. Both the 16-residue p60^{src} and p17^{gag} peptides inhibited > 85%. However, a 10-residue cAMPdPK peptide inhibited about 30% compared with a similar sized 9-residue *src* peptide that inhibited by about 60%.

Table 3. Peptide inhibitors of *N*-myristoyltransferase

Enzyme assays were carried out with 118–156 μg of the rat brain 100000 *g* supernatant protein according to the assay described in the Experimental section, except that 100 μM-myristoyl-CoA and 15 μM of the p60^{src} *N*-glycyl[^{125}I]-peptide were used as substrates and 0.25% bovine serum albumin was included in each assay. The peptide inhibitors were present at a concentration of 750 μM and the reaction was carried out for 15 min. The 100% value corresponded to an enzyme activity of 281 pmol of *N*-myristoylglycyl-[^{125}I]peptide formed. The results represent the mean of duplicates except where indicated otherwise.

Inhibitor	*N*-Myristoyltransferase activity (% of control)	
None	100	
Myristoyl-GSS	104*	
Myristoyl-GSSKS	25.9*	
Myristoyl-GSSKSKP	20.1	
Myristoyl-GSSKSKPKD	8.93±2.97	(n = 4)
GS	101.8	(n = 1)
GSS	92.7	
GSSKS	90.6	
GSSKSKP	76.0±6.37	(n = 4)
GSSKSKPKD	41.5±5.52	(n = 4)
GSSKSKPKDPSQRRRY	6.4	
GARASVLSGGELDRWY	16.1	
GNAAAAKKGY	71.5±8.44	(n = 4)

* Turbidity during assay indicated incomplete substrate solubility.

N-Myristoyltransferase tissue distribution

The tissue distribution of *N*-myristoyltransferase(s) was examined by assaying the 100000 *g* supernatant fractions from a number of different rat tissues. Using

Table 4. Rat tissue distribution of *N*-myristoyltransferase activities

Rat tissue enzyme activities were determined with 50–100 μg of protein of the indicated 100000 *g* supernatant fractions by a 10 min incubation in the assay described in the Experimental section using the peptide acyl acceptor corresponding to p60^{src} (GSSKSKPKDPSQRRR[^{125}I]Y).

Tissue	*N*-Myristoyltransferase activity (pmol·min^{-1}·mg of protein^{-1})
Brain	165
Lung	124
Small intestine	83
Kidney	31
Heart	19
Skeletal muscle	11
Liver	9

the p60^{src} *N*-glycyl[^{125}I]peptide acyl acceptor, the highest specific enzyme activity was found in the brain with relatively decreasing activities in lung, small intestine, kidney, heart, skeletal muscle and liver (Table 4). A similar tissue distribution was observed when the cAMPdPK *N*-glycyl[^{125}I]peptide was used as the acyl acceptor (results not shown).

DISCUSSION

No viral genes are known to encode for enzymes catalysing *N*-myristoylation. Consequently, the transferase(s) necessary for p60$^{v\text{-}src}$ *N*-myristoylation must be of cellular origin and they must play a role in normal cell function. Besides the cellular p60$^{c\text{-}src}$ kinase encoded by the *src* proto-oncogene which is also *N*-myristoylated [16], at least two other important cellular enzymes have been shown to be *N*-myristoylated: the catalytic subunit of cAMPdPK [27] and the calmodulin binding phosphatase, calcineurin B [28]. Both of these latter proteins can also be membrane associated and are thought to be regulators of normal cell growth and differentiation [29–31].

The enzyme assay described in this study was designed to identify the *N*-myristoyl transferase(s) responsible for p60^{src} *N*-myristoylation in mammalian tissues. The peptide acyl acceptor we used included the *N*-terminal 1–14 p60^{src} amino acid sequence thought to be necessary for *N*-myristoylation *in vivo* [12]. Using this p60^{src} *N*-glycyl[^{125}I]peptide as the acyl acceptor, we have identified *N*-myristoyl transferase activity in every rat tissue examined. It may be significant that brain, which has the highest specific activity of any tissue examined, is also known to be a rich source of p60$^{c\text{-}src}$ in developing neural tissues [32,33]. The lower levels of transferase in other rat tissues correspond to recent evidence indicating that normal human liver, kidney, and skeletal muscle contain 15–30-fold lower levels of p60$^{c\text{-}src}$ compared with normal human brain [34]. Brain is also known to be a rich source of two other *N*-myristoylated proteins, calcineurin B and cAMPdPK [35,36]. The level of bovine calcineurin B is at least 10-fold higher in brain compared with most other tissues [35]. The rat tissue distribution of cAMPdPK also is similar to that of *N*-myristoyltransferase except for the presence of kinase activity in heart at levels nearly equal to that found in brain and lung [36]. We examined the possibility that heart contains *N*-myristoyltransferase activity with specificity for cAMPdPK by utilizing a decapeptide corresponding to the *N*-terminal sequence of cAMPdPK catalytic subunit as the acyl acceptor. The *N*-myristoyltransferase tissue distribution using the cAMPdPK acceptor was identical with that found with the p60^{src} acceptor, suggesting that a single enzyme is responsible for the *N*-myristoylation of both peptides.

A similar *N*-myristoyltransferase activity has been recently described in yeast and murine tissue culture cells by the transfer of radiolabelled myristoyl-CoA to a non-radioactive octapeptide homologous to the *N*-terminal sequence of cAMPdPK catalytic subunit [17–19]. The rat brain enzyme described in our studies exhibits a number of properties similar to that previously described yeast enzyme. (1) In both rat brain and yeast, *N*-myristoyltransferase activity was found in crude membrane as well as in soluble fractions, indicating the possible association of the enzyme as a loosely bound peripheral membrane enzyme. (2) Both enzymes displayed a high specificity for myristoyl-CoA as the acyl donor. Although the $C_{10:0}$ and $C_{12:0}$ acyl-CoA derivatives also functioned as acyl donors *in vitro*, the myristoyl-CoA was the preferred substrate. In contrast, no activity was detected with the $C_{16:0}$ or $C_{18:0}$ acyl-CoA derivatives. Considering the low natural abundance of short chain saturated fatty acids ($< C_{14}$) compared with myristate in rat tissues [37] and in yeast [38] it is probable that the enzymes described in both studies function as strict *N*-myristoyltransferases *in vivo*. A similar specificity of p60^{src} *N*-acylation for myristic acid has been determined by metabolic labelling [7,15]. In addition, whereas the $C_{16:0}$ acyl-CoA exhibited negligible activity for either the rat or the yeast enzymes, it did inhibit *N*-myristoyltransferase activity in both. However, in contrast with the yeast enzyme in which the $< C_{14:0}$ acyl-CoA derivatives were better inhibitors than the $C_{16:0}$ acyl-CoA, for the rat enzyme the $C_{16:0}$ acyl-CoA was about twice as effective an inhibitor as was the $C_{10:0}$ or $C_{12:0}$ acyl-CoAs. (3) Both the rat and the yeast enzymes catalyse the *N*-myristoylation of *N*-glycylpeptides homologous to the *N*-terminal sequences of p60^{src}, p17^{gag} and cAMPdPK catalytic subunit. Although there is general agreement in the relative kinetic constants for the two enzymes, a strict comparison is not possible because of the use of slightly different *N*-glycylpeptide acyl acceptors and different assay conditions. Keeping this in mind, we found about a 3-fold greater activity with the p60^{src} compared with the cAMPdPK *N*-glycylpeptides in rat brain. This compares to a greater than 2-fold higher activity exhibited by the yeast enzyme for the cAMPdPK acyl acceptor compared with the p60^{src} acyl acceptor but apparently the reverse specificity in a murine cell line [18].

N-Myristoyltransferase(s) display an absolute requirement for *N*-terminal glycine [9–16]. Besides this *N*-terminal glycine there are no other sequence homologies among the five known *N*-myristoylated proteins except possibly a frequently occurring hydroxyamino acid in position five. Thus, *N*-myristoylation may be catalysed either by a family of transferases each with a unique sequence specificity for a different protein acyl acceptor or by a single enzyme with a broad peptide sequence

recognition specificity. It is also possible that there is no consensus *N*-myristoylation sequence beyond *N*-terminal glycine and that acylation is dependent upon an as yet unrecognized conformation-dependent recognition site [10]. An analogous conformation-dependent specificity within the first 40 *N*-terminal residues has been postulated for *N*-acetylation [39].

Although our analysis does not allow a final assessment of these possibilities, the different catalytic rates observed with the $p60^{src}$, $p17^{gag}$ and cAMPdPK acyl acceptors and the results of the inhibition studies indicate that the brain enzyme(s) exhibit amino acid sequence requirements beyond the *N*-terminal glycine. More specific information on acyl acceptor amino acid sequence preference is suggested by the inhibition of *N*-myristoyltransferase by synthetic *N*-glycylpeptides corresponding to the *N*-terminal sequence of *src*. Assuming that these peptides inhibit by functioning as alternative competitive substrates, then the significant inhibition which was evident only with peptides with more than five residues indicates an enzyme recognition of the peptide acyl acceptor beyond position 5. A similar dependence of the yeast enzyme on the presence of a lysine at position 7 is consistent with this conclusion [18]. In addition, the increasing inhibition by 7-, 9-, and 16-residue peptides may indicate a $p60^{src}$ *N*-myristoyltransferase preference either for specific amino acid residues beyond position 9 or for a peptide substrate of larger than nine residues as well.

N-Myristoylation is thought to be an important cellular event. The fact that such a rare fatty acid is incorporated with such strict specificity into cellular enzymes with broad regulatory effects suggests that this process may have a central role in the control of normal cellular growth and differentiation. In addition, the association of *N*-myristoylation with a number of transforming tyrosine kinases as well as its obligatory role in $p60^{v\text{-}src}$ transformations suggest that it may be a potential new target for blocking or reversing certain types of malignancies.

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