A Rapid Posttranslational Myristylation of a 68-kD Protein in \textit{D. discoideum}

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Abstract. Cells incubated with $[^3]H$myristate were shown to rapidly and specifically acylate a 68-kD protein, p68, in a developmentally-regulated manner. The fatty acid incorporated into p68 was identified as myristate, and is linked to the protein via an amide bond, apparently to an NH$_2$-terminal glycine. The acylation of p68 in \textit{D. discoideum} displays some unusual properties. Unexpectedly, myristylation of p68 is a posttranslational event and occurs in the presence of inhibitors of protein synthesis. Another unusual finding was that although p68 is a stable protein, the acyl moiety is removed with a half time of $\sim$15 min.

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Palmitylation (20, 21) and GPI anchoring (16, 21) have both been shown to occur in \textit{D. discoideum}, but little information is available concerning the modification of proteins by myristate. In the course of our studies on the GPI anchor of gp80, we examined the incorporation of different fatty acid radiolabels into the general population of proteins synthesized by \textit{D. discoideum}. In so doing, we found a rapid and striking myristylation of one protein, of $\sim$68 kD, which we refer to as p68. Modification of this protein by myristic acid displays properties not previously reported. The data in this manuscript demonstrate that p68 is rapidly myristylated, via an amide bond linkage, and then demyristylated, and that these changes in the modification of the protein occur posttranslationally.

Materials and Methods

Cells and Culture Conditions

Ax-2 amebae (26) were exponentially grown in HL5 medium (22). Starvation was initiated by washing cells free of growth medium and resuspending them at a density of $10^7$ cells/ml in 20 mM phosphate buffer pH 6.4. Cells were starved as spinner suspensions (2) and monitored for morphological changes microscopically (6).

Preparation of Radiolabeled Cell Extracts

Cells were incubated with 1 mCi/ml $[^3]H$myristate or $[^3]H$palmitate in buffer supplemented with 10 mM pyruvate, 10 mM MgCl$_2$ for the times indicated. For analysis of total cellular proteins by SDS-PAGE (7), cells were either boiled in sample buffer or lysed in NP-40 NET (0.5% NP-40 in 25 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA) and extensively delipidated with chloroform/methanol (2:1) as described by Towler and Glaser (23). Samples were acidified with 0.1N HCl in between the extractions. This acidification was necessary for complete removal of noncovalently bound lipids (15, 21). Extractions were performed until no additional counts were released into the organic phase. When the effects of proteases were to be examined, extracts were incubated in the absence or presence of 1 mg/ml pronase or V-8 for 15 min at 37°C. To determine the subcellular localiza-
tion of myristylated proteins, cells were lysed by passage through 5-μm polycarbonate filters in a Tris-HCl buffer containing nine protease inhibitors as described (10). The resulting lysates were centrifuged at 30,000 g for 20 min to obtain crude membrane and cytosolic fractions. Protein synthesis was determined by incubating cells with 0.1 mCi/ml [35S]methionine. When present, protein synthesis inhibitors, 500 μg/ml, were added at various times before the addition of the label. Unless indicated otherwise, autoradiograms were exposed for 2 d at ~70°C using a lightning plus screen by DuPont Co. (Wilmington, DE).

Hydroxylamine Treatment

Cells were incubated with [3H]fatty acid for the indicated times. Extracts were prepared and either treated with hydroxylamine and analyzed by SDS-PAGE, or the gels themselves were treated with hydroxylamine. In either case, treatments consisted of 1 M hydroxylamine, pH 7 or 10, and are described by Olson et al. (13). Duplicate samples were treated with 1 M Tris-HCl pH 7 or 10 as control incubations. The results were identical at both pHs.

Chemical Analysis of the Fatty Acids

After extensive delipidation of cell extracts as described above, the protein pellet was hydrolyzed with 0.5 N HCl in acetonitrile/H2O (9:1) for 90 min at 100°C as described by Aveldano and Horrock (1). Free fatty acids were recovered by chloroform extraction and analyzed by TLC on KC18 reverse phase plates developed in acetonitrile/acetic acid (1:1) as described by Schultz and Oroszlan (17). Delipidated samples were also analyzed by SDS-PAGE, and the band corresponding to p68 was excised and treated with acetonitrile to release the free fatty acid label. In some experiments, fatty acids were also released by strong acid hydrolysis (5). The products were analyzed by TLC as described above. To identify the amino acid linked to myristate in p68, the gel band corresponding to the protein was incubated with pronase E according to Towler and Glaser (23). The digestion product was recovered in chloroform and analyzed on KC18 reverse phase plates developed in either acetonitrile/acetic acid (1:1) or 75% acetonitrile in 0.08% trifluoroacetic acid adjusted to pH 7.5 with triethylamine. Both systems were effective in separating the myristyl-amino acid standards from each other and their respective individual components. Myristyl-amino acid standards were synthesized according to Towler and Glaser (23). When sufficient radioactivity was present (i.e., for the various standards), the plates were sprayed with Enhance and analyzed by autoradiography. Subsequently, all rows of the plates were sectioned, scraped, and counted in scintillation fluid.

Materials

Radiolabeled fatty acids and Enhance were purchased from New England Nuclear (Boston, MA), while [35S]methionine and [14C]glycine was obtained from ICN K&K Laboratories (Plainview, NY). KC18 TLC plates were purchased from Whatman Inc. (Clifton, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Fatty Acylation of p68

The life cycle of D. discoideum has two distinct phases, vegetative growth and development. The latter is initiated by removing cells from nutritional medium and incubating them in buffer. During the first 6–8 h of the starvation period, cells develop aggregation competence and form multicellular aggregates. We compared the proteins synthesized by cells that had been starved for 2 h to those proteins modified by fatty acids. Fig. 1 A shows the proteins labeled when 2-h starved cells were incubated with [35S]methionine for 5, 30, and 60 min (lanes 1–3). The pattern obtained was quite different from that obtained when cells were radiolabeled for similar times with either [3H]myristate (lanes 4–6) or [3H]palmitate (lanes 7–9). This would indicate that, in the latter cases, radiolabeling did not reflect a conversion of the fatty acids to amino acids. Distinctly, myristate preferentially identified a protein of ~68 kD, p68, when cells were incubated for a short time with the radiolabel (lane 4). In this experiment, a lower molecular mass band of ~60 kD was also observed. We have determined that this protein is a degradation product of p68. This degradation did not occur if delipidation of samples was performed in the presence of protease inhibitors. Labeling of p68 was maximal by 5–10 min of cell incubation with [3H]myristate and appeared to be unstable. Longer incubation times resulted in a decrease in the label despite the fact that radiolabeling of numerous other proteins was occurring.

P68 was not efficiently labeled when cells were incubated with [3H]palmitate for 5–10 min. A number of other pro-
The Acyl Moiety of p68 Turns Over

Although p68 was rapidly labeled when cells were incubated with [3H]myristate, that label was removed with longer incubation times, even though other proteins were being radio-labeled. Those results suggested that, with increasing times (1) myristate was being converted to products that could no longer be used to label p68 and (2) either the protein or the incorporated fatty acid moiety was unstable. Several experiments were undertaken to address these possibilities.

We examined the nature of the fatty acids incorporated into proteins after increasing times of cell incubation with [3H]myristate. During the first few minutes of incubation, when p68 was the only detectable protein to be radio-labeled, the radiolabel released from the cell extract comigrated on TLC with myristate (Fig. 2, rows A and B). To verify that the recovered product was truly representative of that incorporated into p68, we also analyzed the radiolabel released by acid or acetonitrile hydrolysis of gel-purified p68 (row C). The product comigrated with myristate on TLC. The label released by pronase E digestion of gel-purified p68 was also analyzed and, as expected of myristylated proteins, the product comigrated with the myristyl-glycine standard (row E). Analysis of the fatty acids released from the population of proteins labeled after a 30-min incubation of cells with [3H]myristate revealed that longer chain fatty acids were now present (row D). Consistent with the observation that labeling of p68 decreases during this time period, the amount of [3H]myristate recovered was also decreased. It would appear that the lack of labeling of p68 during these longer incubation times coincides with the metabolic conversion of myristate to other fatty acids.

The experiment depicted in Fig. 3 was designed to assess if the loss of label from p68 reflects an instability of the protein or of the fatty acid modification. Cells were incubated with [3H]myristate until radiolabel in p68 was decreasing (compare lanes 1 and 2). At that time, additional [3H]myristate was added. P68 was again rapidly labeled (lanes 3-7).
Figure 3. Time course of radiolabel turnover. 2-h starved cells were incubated with [3H]-myristate for increased times from 10 (lane 1) to 30 min (lane 2). After the 30-min period, additional radiolabel was added to part of the population and incubation continued for another 1 min (lane 3); 2 min (lane 4); 3 min (lane 5); 5 min (lane 6); 10 min (lane 7); 15 min (lane 8); 30 min (lane 9); 45 min (lane 10). Proteins labeled by cells that had been incubated for an additional 10, 15, or 30 min without fresh radiolabel are shown in lanes 11, 12, or 13, respectively. The arrow marks the position of p68.

As seen previously, continued incubation times led to a loss of radiolabeled p68 (lanes 8-10). The data would argue that p68 was present throughout the periods monitored but that the fatty acid modification was readily removed. That p68 is indeed a stable protein was demonstrated by performing the labeling experiments in the presence of cycloheximide. As shown in Fig. 4, cycloheximide treatment effectively inhibited protein synthesis as monitored by the incorporation of [35S]methionine into proteins (lane 1). However, it did not inhibit the incorporation of [3H]myristate into p68 (lane 3). In the experiment shown, 2-h-starved cells were preincubated for 5 min with the drug before the addition of the label. Variations of that protocol included increasing the period of cell preincubation with the drug (up to 1½ h), increasing the labeling period, or increasing the hours cells were starved before the addition of cycloheximide. We also treated vegetative cells with cycloheximide for 2 h and then starved those cells in the presence of the drug for additional times. In all cases we observed efficient radiolabeling of p68 with [3H]myristate. To eliminate the possibility that p68 may be a protein whose synthesis is resistant to the effects of cycloheximide, we also examined other inhibitors effective in this system, i.e., anisomycin and emetine, for their effects on p68 radiolabeling with [3H]myristate. No inhibition was observed although efficient arrest of protein synthesis was achieved. The data indicate both that p68 is a stable protein and that its modification with myristate is a posttranslational event.

Features of p68 Acylation

In general, myristate modification of a protein core occurs via an amide bond. That linkage can be diagnosed by its resistance to treatments such as basic methanolysis and hydroxylamine (4, 18, 19, 24). To confirm that the fatty acid label in p68 is in the form of an amide bond, we examined its resistance to those treatments. An example is shown in Fig. 5. 2-h starved cells were incubated with [3H]myristate for either 5, 30, or 60 min. Samples were analyzed by SDS-PAGE and one half of the resulting gel was treated with hydroxylamine (lanes 5–8). Included in this experiment was myristate-labeled variant surface glycoprotein (VSG), a surface protein of Trypanosoma brucei, whose fatty acid is present in the diacylglycerol portion of the GPI anchor (3, 9). This linkage is an ester and can be hydrolyzed by hydroxylamine treatment. It can be seen that, although the radiolabel incorporated into VSG was almost totally eliminated by such treatment (compare lanes 4 and 8), little or no loss from p68 was observed. The same result was obtained if cell extracts were treated with hydroxylamine before analysis by SDS-PAGE. The data indicate that the fatty acid is linked to p68 by an amide bond. It should also be noted that few, if any, of the D. discoideum proteins labeled during the 60-min incubation with [3H]myristate proved to be sensitive to this treatment.

Experiments were performed to gain a better understanding of the nature of p68. Cell fractionation experiments showed that myristylated p68 localized primarily to the membrane fraction. We are unable, at this time, to monitor the protein devoid of fatty acid label. Thus, it is not clear if acylation of the protein determines its membrane localization. P68 did not appear to be O-glycosylated. The molecular weight of the protein produced in the modB mutant DL118 was the same as that produced in the wild-type strain...
used in these experiments. The modB mutant (8) is unable to modify proteins with type 2 carbohydrate, which are probably O-linked oligosaccharides (12). Thus, p68 is not modified by such oligosaccharides nor is that type of modification important for the activities that result in its fatty acylation. We have also determined that extensive incubation of cells with tunicamycin to inhibit N-linked glycosylation did not affect p68 acylation or the molecular weight of the acylated protein. Given the stability of the protein, however, the lack of an effect of tunicamycin on the apparent molecular weight of the protein could reflect the fact that little or no synthesis of the protein was occurring during the incubation.

That p68 is indeed a protein was verified by its proteolytic sensitivity. Fig. 6 shows that p68 (lane 1) was totally digested when cell extracts were incubated with protease (lane 4). In this experiment, cells were incubated with $[^{3}H]$myristate for 10 min and the autoradiogram was exposed for several weeks. V-8 digestion produced one radiolabeled band of $\sim 22$ kD (lane 2), consistent with the fact that myristylation occurs at a unique site, the NH$_2$-terminus of the protein. When cell extracts were incubated in buffer alone, p68 was hydrolyzed by an endogenous protease (lane 3). This activity resulted in the limited cleavage of protein to produce a radiolabeled band of 60 kD. P68 was not degraded if extracts were incubated in buffer with protease inhibitors.

**Discussion**

We have demonstrated that cells rapidly acylate p68 when they are incubated with $[^{3}H]$myristate. P68 is clearly the predominant protein so labeled in the first 5 min of cell incubation and the fatty acid incorporated into the protein was shown to be myristate. Acylation of p68 can be detected within 1 min of cell incubation with the radiolabel but occurs for only a limited time. The lack of further labeling of p68 upon continued cell incubation with $[^{3}H]$myristate does not reflect a turnover of the protein substrate for this reaction since p68 was found to be a stable protein. When cells were supplied additional $[^{3}H]$myristate, rapid acylation of p68 again occurred and again it was restricted to a limited time period. After the time p68 is maximally labeled, other proteins incorporate radiolabel, but the radioactivity is in compounds that are metabolic products of myristate. It would appear that the labeling of p68, for only the first few minutes of cell incubation with fatty acid, reflects a restriction of the pool of myristate available for its modification. The above mentioned observations also suggest that p68 may be one of few proteins in *D. discoideum* (detected by our procedures) that is myristylated during aggregation. Extensive exposure
of autoradiograms (several weeks as opposed to several days) of cellular proteins labeled during a 1- or 2-min incubation did not reveal additional labeled proteins. Extracts from cells labeled for 10 min did show additional bands upon long exposure of the autoradiogram (Fig. 6) but their labeling was insignificant when compared to p68. Most proteins in \textit{D. discoideum} are primarily labeled by the conversion products produced upon continued incubation of the cells with \textsuperscript{3}H\textsuperscript{m}yristate. The same conversion products are obtained from palmitate and account for a similar pattern of protein labeling when cells are incubated with \textsuperscript{3}H\textsuperscript{p}almitate for 30 or 60 min. There were one or two proteins that did appear to be preferentially labeled when cells were incubated with \textsuperscript{3}H\textsuperscript{p}almitate and it may be that those proteins have incorporated palmitate, as opposed to its metabolic products.

As expected for myristylated proteins, the fatty acid in p68 is present in an amide bond, as evidenced by its resistance to hydrolysis with hydroxylamine or basic methanolysis. We also noted that a majority of the radiolabeled proteins, identified by SDS-PAGE when cells were incubated for 30-60 min with either \textsuperscript{3}H\textsuperscript{m}yristate or \textsuperscript{3}H\textsuperscript{p}almitate, were resistant to hydroxylamine treatment. Similar observations have been made in other lower eukaryotes, \textit{Tetrahymena} (15) and \textit{Physarum} (11). In the case of \textit{D. discoideum}, we observed that both fatty acid labels are converted to similar metabolic products during these longer incubation periods which, as discussed above, accounts for the labeling of the same proteins at those times. The data in any of these systems, however, do not rule out the possibility that additional proteins are modified by fatty acids that are linked via ester or thio ester bonds since the proteins examined reflect a selected fraction that are likely to be more abundant proteins and/or those that are not already stably modified by endogenous fatty acid pools. In \textit{D. discoideum}, the ras protein has been shown to be fatty acylated via an ester linkage but the identification of that modification required immunoprecipitation of this minor cellular component (27). Additionally, it is of interest to note the experiments of Towler and Glaser (23) showing that, in higher eukaryotic cells, at least 30% of the palmitate derived from \textsuperscript{3}H\textsuperscript{ac}etate was linked to proteins via amide bonds while less than 10% was present in amide bonds when \textsuperscript{3}H\textsuperscript{p}almitate was supplied as the source of radiolabel. Thus, cells may possess various acylating enzymes which preferentially modify proteins by ester or amide bonds.

As discussed below, the \textit{D. discoideum} enzyme that acylates p68 displays some unusual properties.

Studies in higher eukaryotes have indicated that myristylation occurs on a limited segment of a protein, at its NH\textsubscript{2}-terminus. Consistent with that generality was the localization of the myristate radiolabel in p68 to a 22-kD fragment produced by V-8 digestion. In addition, analysis of the product generated upon complete pronase digestion of p68 suggests that the myristate label is on a glycine residue. However, unlike what has been generally attributed to the process of myristylation, the acylation of p68 occurs posttranslationally, as evidenced by the inability of protein synthesis inhibitors to alter this process. Another exception to the rule that myristylation occurs cotranslationally has been reported recently. Isolated mitochondria incorporate myristate into a 52-kD membrane protein. The fatty acylation is not inhibited by chloramphenicol and is not translation linked (25). Chloramphenicol also did not alter the acylation of p68 in \textit{D. discoideum} (A. Silva, unpublished observations). The rapidity with which p68 is acylated in intact cells could suggest that this is not a mitochondrial event. Although an additional report of the posttranslational myristylation of a specific protein has appeared (14), the identity of the fatty acid incorporated was not chemically confirmed.

Another unexpected finding is the observation that p68 is rapidly deacylated. Although p68 was shown to be a stable protein and readily acylated in cells incubated with cycloheximide for several hours, the myristate radiolabel displayed a half time of \(\sim\)15 min. The rapid cellular metabolism of added myristate limits the radiolabeling of p68 to the first few minutes of cell incubation. Subsequent periods are essentially the equivalent of a "chase" period during which time the turnover of the radiolabel in p68 could be observed. The addition of nonradioactive myristate to 5-min labeled cells did not alter the turnover of the radiolabel in p68 (A. Silva, unpublished observations). The fresh addition of \textsuperscript{3}H\textsuperscript{m}yristate at any time leads to a rapid relabeling of p68. This acylation/deacylation/reaclylation is rather unexpected for protein myristylation since that modification is reportedly stable, as opposed to palmitylation which exhibits such cycles. It has been proposed that such cycles allow for changes in the subcellular distribution of a protein (4, 18, 19, 23).

In the case of p68, we know that the fatty acylated protein is membrane associated but currently we do not have the tools to detect the unlabeled (unacylated) protein and thus assess if it partitions differently.

In contrast to starved cells, acylation of p68 is not predominant in vegetative cells, although it is likely that the protein is there. Incubation of vegetative cells with cycloheximide did not inhibit the acylation of the protein during starvation, even when starvation occurred also in the presence of cycloheximide. This indicates that both p68 and the components involved in its acylation are relatively stable proteins and present during growth. The lack of p68 myristylation during growth also does not reflect an altered uptake of the fatty acid since a number of other proteins are efficiently labeled. It would appear that cells regulate the myristylation of p68, such that it occurs primarily, if not only, upon cell starvation. As the identity of p68 is currently unknown, its role in starvation is not clear. However, it is of interest to note that starved cells possess a protease whose activity results in the limited hydrolysis of the protein. Such an activity could limit or otherwise modulate the function of p68. Continued purification of the protein should answer these and other questions concerning the role of this unusual myristylation in \textit{D. discoideum}, as well as expand our general understanding of fatty acylation and the enzymes that regulate this type of modification in eukaryotes.

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