Extracellular Release as the Major Degradative Pathway of the Insulin-like Growth Factor II/ Mannose 6-Phosphate Receptor*

(Received for publication, March 12, 1991)

Kevin B. Clairmont‡ and Michael P. Czech§
From the Department of Biochemistry and Molecular Biology and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

The presence of a soluble, truncated form of the IGF-II/Man-6-P receptor in serum has suggested that cleavage from the cell surface may be an initial step in the degradation of this protein (MacDonald, R. G., Tepper, M. A., Clairmont, K. B., Perregaux, S. B., and Czech, M. P. (1989) J. Biol. Chem. 264, 3256–3261). In order to test this hypothesis, we pulse-labeled cultured BRL-3A rat liver cells with [35S]methionine and [3H]cysteine and measured the fate of labeled receptor at various times after incubation with unlabeled amino acids. It was found that the appearance of labeled IGF-II/Man-6-P receptor in the medium accounts quantitatively for the loss of labeled receptor from the BRL-3A cells. In similar experiments with Chinese hamster ovary cells, L6 rat myoblasts, and chick embryo fibroblasts, labeled receptor from the cell membranes decreases with a time course corresponding to the appearance of soluble receptor in the medium. The release of labeled receptor into the medium can be blocked by the addition of the protease inhibitors aprotilin, chymostatin, or phenylmethylsulfonyl fluoride, but not antipain, leupeptin, and benzamidine. The results are consistent with the hypothesis that the degradation and loss of cellular IGF-II/Man-6-P receptors occurs by a nonlysosomal mechanism involving their proteolysis and removal into the extracellular fluid.

Many receptors bind to their ligands at the cell surface prior to their endocytosis through coated vesicles, dissociation from ligand, and recycling back to the plasma membrane (Geuze et al., 1983; Hopkins, 1983; Klausner et al., 1983; Geuze et al., 1984; Goldstein et al., 1985; Brown et al., 1986). The IGF-II/Man-6-P receptor exemplifies this characteristic by remaining on the cell surface for a significant length of time to reach maturity (4–8 h), which requires its conversion by glycosylation from the initially detected 245-kDa form to the mature 260-kDa form. During this maturation process the receptor also gains the ability to bind to its ligands. The receptor has a long half-life (24–48 h), which is not altered by agents which disrupt lysosomes. These reports suggest that the degradation of the receptor is not lysosomal (Creek and Sly, 1983; Sahagian, 1984). The mechanism of receptor degradation has remained an important open question.

A possible mechanism for cellular degradation of the IGF-II/Man-6-P receptor was suggested by the initial report of a form of this receptor in serum (Kriess et al., 1987). Subsequent work demonstrated that this serum receptor could bind IGF-II and Man-6-P containing ligands simultaneously, that it was cytoplasmically altered or truncated as compared with the cellular receptor (MacDonald et al., 1989), and that the circulating receptor was proteolyzed into smaller fragments (MacDonald et al., 1989; Causin et al., 1988). It has also been shown that the IGF-II/Man-6-P receptor can be found in urine (Causin et al., 1988). These data suggested to us that the serum IGF-II/Man-6-P receptor might be a major intermediate form in the degradation pathway of this receptor protein. In the present experiments, this hypothesis was tested by following the fate of IGF-II/Man-6-P receptor in cultured cells pulse-labeled with [35S]methionine and [3H]cysteine. We demonstrate here that the loss of labeled IGF-II/Man-6-P receptor in BRL-3A cells upon incubation of cells with unlabeled amino acids can quantitatively account for the appearance of labeled receptor in the medium. This process is blocked by inhibitors of serine proteases including chymostatin. The data suggest that cellular receptor is degraded by proteolysis at the cell surface followed by release into the medium.

EXPERIMENTAL PROCEDURES

Materials—IGF-II/Man-6-P receptors for antibody generation were purified from rat placental plasma membranes by IGF-II-Sepharose chromatography as previously described (Oppenheimer and Czech, 1983). The anti-IGF-II/Man-6-P receptor antisera used were those previously described (MacDonald et al., 1989). Cell lines were purchased from the American Type Culture Collection. Cell cultured reagents are from Gibco. Sodium iodacetate (Fisher Biotech) was recrystallized twice from methanol. All other chemicals were at least reagent grade.

Radioactive Labeling of Cells—Prior to labeling, cells were grown to 90% confluence. Medium was then removed and replaced by serum-free minimum essential medium buffered with 15 mM Hepes and lacking the labeled amino acid. To this was added 0.15–1.0 μCi of a mixture of [35S]cysteine and [3H]methionine, or Tr-[35S]label (ICN, Costa Mesa, CA) or EXPRE[35S]S-S Label (Du Pont-New England Nuclear). Cells were incubated for 30–60 min at 37°C, then the medium was removed and replaced by serum-free Dulbecco’s modified Eagle’s medium (DME). Incubation was continued for the indicated

* This work was supported by National Institutes of Health Grant DK30648. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Anatomy and Cellular Biology, Harvard Medical School, 220 Longwood Ave., Boston, MA 02115-6092.
§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology and Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation St., Worcester, MA 01605.

1 The abbreviations used are: IGF, insulin-like growth factor; Man-6-P, mannos-6-phosphate; DME, Dulbecco’s modified Eagle’s medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ig, immunoglobulin; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
times in serum-free DME or DME supplemented as indicated prior to homogenization.

Preparation of Membranes from Cultured Cell Lines—Cells were scraped into a buffer consisting of 20 mM Hepes, 0.25 mM sucrose, and 1 mM EDTA, pH 7.4, plus protease inhibitors at 0 °C. The protease inhibitors were leupeptin, antipain, and benzamidine at concentrations of 1 μg/ml each, 20 μg/ml aprotinin, 12.5 μg/ml chymostatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Following homogenization, total membranes were obtained by a single centrifugation at 200,000 × g.

To obtain a membrane extract the membranes obtained as described above were solubilized as follows. A volume of the membrane suspension obtained above equivalent to 0.5 mg of protein or the total membrane protein from one well was pelleted by centrifugation for 5 min at 15,000 × g in a microcentrifuge. The supernatant was removed and the pellet resuspended in 50 μl of 15 mM Tris, 0.15 M NaCl, pH 7.4, containing 1% Triton X-100, 1% deoxycholate, and 0.1% sodium dodecyl sulfate with the protease inhibitors described above. Following a 1-h incubation at 4 °C on an end-over-end mixer, the mixture was centrifuged for 10 min at 15,000 × g in a microcentrifuge, and the supernatant fraction was used as the extract. The cellular form of the IGF-II/Man-6-P receptor was isolated from this preparation.

Preparation of Medium for Immunoadsorption—Prior to homogenization of the cells, medium was removed into centrifuge tubes and the inhibitors added as described above. The medium was then centrifuged at 35,000 × g or 200,000 × g to remove cellular or membrane contaminants. Both techniques produced identical results (data not shown). Medium was frozen at -80 °C until needed. Prior to immunoadsorption, a fraction of the medium corresponding to 0.2-mg membranes or the total medium from one well was concentrated using a Centricon 30 (Amicon, Danvers, MA) or a Centrifugal UltraFree with a 30,000 nominal molecular weight limit (Millipore, Bedford, MA) to a volume of 0.1-0.5 ml.

Immunoadsorption of the IGF-II/Man-6-P Receptor—IGF-II/Man-6-P receptors were immunoadsorbed essentially as described (MacDonald et al., 1989). For immunoadsorption from a membrane extract the 50 μl of extract was diluted to 0.9 ml with 50 mM Hepes, pH 7.4, and to a final concentration of 0.15 M NaCl and 5 mM Man-6-P. For medium, 50 μl of extraction buffer was added to the medium, and it was diluted in the same manner as the membrane extracts. Finally, to each was added 0.1 ml of a 50% slurry of anti-Man-6-P receptor antibody Affi-Gel (Bio-Rad) in 50 mM Hepes, pH 7.4, containing 0.1% Triton X-100. These mixtures were incubated overnight at 4 °C on an end-over-end mixer. Unbound material was removed by withdrawing the supernatant following a 1-min centrifugation in a microcentrifuge at 15,000 × g. This material was then washed in 15 mM Tris, 0.15 M NaCl, pH 7.4, containing 0.1% Triton X-100, then once in each 50 mM Hepes, 0.15 M NaCl, pH 7.4, with 0.05% Triton X-100 and 50 mM Hepes, pH 7.4.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—Samples labeled with 35S were reduced by incubation in electrophoresis sample buffer containing 100 mM dithiothreitol. Electrophoresis was performed as described (Laemmli, 1970) on 6% polyacrylamide gels.

 Autoradiography of [35S]-Labeled Gels and Quantitation of Results—Gels containing 35S-labeled material were stained with Coomassie Brilliant Blue and destained. They were then treated with EN'HANCE (Du Pont-New England Nuclear) according to the manufacturer's instructions. The gels were then dried and subjected to autoradiography. Quantitation was performed using an LKB Ultrascan XL densitometer (LKB, Rockville, MD) to scan the autoradiogram using a densitometer slit or by cutting out the bands and counting them in a scintillation counter. Quantitated results shown are means ± S.D. for three repeats of each experiment.

RESULTS AND DISCUSSION

In order to determine the relationship between cellular and soluble forms of the IGF-II/Man-6-P receptor, BRL-3A cells were labeled for 1 h with [35S]methionine and [35S]cysteine, then incubated with unlabeled medium for the times indicated (Fig. 1). Immediately following the pulse, there is no labeled receptor found in the medium, and only the 245-kDa precursor is seen in cells. The observed increase in cellular receptor during the early points of the chase period is accounted for by the specificity of the antibody used; the primary translation product (232 kDa) is not recognized by the antibody until it has been glycosylated to a 245-kDa form (MacDonald and Czech, 1985). Over the next several hours of the chase period the 245-kDa precursor is converted into the 250-kDa mature receptor, and a truncated receptor is first seen in the medium. At later times, cellular receptor is gradually converted into the truncated form of the receptor found in the medium. Thus, the initial processing of cellular receptor to a form recognized by the antibody prevents quantitation of receptor degradation from the cells over the first few hours (<6 h), and degradation of the medium form of receptor at the later time points prevents quantitation of medium receptor for these times (>22 h). However, quantitation of the results from 6 to 22 h demonstrates that 109 ± 39% of the receptor lost from the cells appears in the medium. These results demonstrate that, for this cell line, release of labeled receptor from the cell can account for the loss of labeled receptor from the cells.

In order to determine if release of soluble IGF-II/Man-6-P receptor is cell type-specific or a more general mechanism, a number of cell lines were studied: L6 (rat myoblast), CHO-K1 (Chinese hamster ovary), and SL-29 (chick embryo fibroblast) cells. Each cell type was labeled with [35S]methionine and [35S]cysteine for 1 h, then incubated in unlabeled medium.
for the times indicated (Fig. 2). In each case, labeled IGF-II/Man-6-P receptor accumulated in the cells at 0 and about 3.5 h following addition of unlabelled amino acids, then decreased to lower levels at about 20 h. Labeled serum receptor was absent from the medium at the start of the chase, then accumulated slightly by about 3.5 h, with similar levels seen at about 20 h. These results are similar to the results discussed above for the BRL-3A cells, in that post-translational modification presumably accounts for the additional receptor appearing between 0 and about 3.5 h following the pulse in the cells. During this time, receptor appears in the medium and, as was seen with the BRL-3A cells, serum receptor in the medium is gradually lost, possibly by further degradation and/or reuptake. Taking these factors into account, it appears that a significant portion of the receptor lost from these cell lines appears in the medium.

The hypothesis that proteolytic release of IGF-II/Man-6-P receptors from the cell surface leads to the appearance of truncated receptor in the medium was tested. The sensitivity of this process to a spectrum of protease inhibitors was evaluated in BRL-3A cells. When added to the chase medium following a 1-h pulse period with \(^{[35]S}\)methionine and \(^{[35]S}\)cysteine, the protease inhibitors aprotinin, chymostatin, or leupeptin has no effect on the production of serum receptor in the medium as compared with cells incubated in the absence of protease inhibitors for the chase period.

While proteolysis from the cell surface would seem to be the most straightforward way to produce a serum form of a receptor when a cellular form already exists, this mechanism has not previously been identified as a significant route whereby serum receptors are produced (Herington et al., 1986; Leung et al., 1987; Gower et al., 1988; Gussow and Pleogh, 1987). The results described above strongly suggest that proteolysis of the cell surface receptor is important for synthesis of the serum form of the IGF-II/Man-6-P receptor. Furthermore, the proteolytic release of the IGF-II/Man-6-P receptor from the cell appears to account for the loss of cellular receptor. This suggests that the serum IGF-II/Man-6-P receptor is an important degradative intermediate in the removal pathway of the cellular receptor.

While proteolytic release from the cell surface has not been shown to play a significant role in the production of other serum receptor forms or in the degradation of any other known protein, a similar proteolytic process occurs in the final stage of transcytosis by the polymeric Ig receptor. In this process IgA or IgM is bound to the receptor at the basolateral surface, transported across the cell, and released from the apical surface (Geuze et al., 1984; Hoppe et al., 1985). In the final stage, release of polymeric Ig from the apical surface, receptor is cleaved to release a portion of the receptor, referred to as secretory component, along with the immunoglobulin (Mostov and Blobel, 1982; Solari and Kraehenbuhl, 1984; Sztul et al., 1985). However, while the process of the
release of the secretory component from the polymeric Ig receptor is similar to the release of the serum receptor form of the IGF-II/Man-6-P receptor, differences do exist. First, the cleavage of the polymeric Ig receptor serves to allow for the release of the Ig, while cleavage of the Man-6-P receptor is not known to serve such a purpose. Second, the two processes are different in respect to protease sensitivity since leupeptin can inhibit the cleavage of the polymeric Ig receptor (Mostov and Blobel, 1982) but not the IGF-II/Man-6-P receptor (Fig. 3).

The findings reported here provide strong evidence for the concept that IGF-II/Man-6-P receptors are degraded by an extracellular pathway in the cells studied here. It is possible that some other receptor proteins share this mechanism of degradation. The cation-dependent Man-6-P receptor, which is also excluded from lysosomes, has been demonstrated to appear at the cell surface (Stein et al., 1987), although it is unable to bind to ligand in that compartment (Kyle et al., 1988). It is possible that such a localization is necessary for the degradation of this protein. In addition, a number of nutrient receptors, such as the low density lipoprotein and transferrin receptor, cycle in a manner similar to the IGF-II/Man-6-P receptor. The transferrin receptor has also been demonstrated to have a serum form (Kong et al., 1986) whose levels are thought to correlate with levels of cellular receptor under various conditions (Flowers et al., 1989; Trowbridge, 1989). Furthermore, the low density lipoprotein receptor has been demonstrated to be degraded independently of the lysosome (Grant et al., 1989). An important question for future studies is whether these and other receptor proteins participate in a life cycle involving cellular extrusion prior to their eventual degradation.

Acknowledgments—We thank Caroline Clairmont for assistance in performing some of the experiments and Drs. Carlos Hirschberg, Roger Davis, Silvia Corvera, Gary Sahagian, and Gregorio Gil for helpful discussions.

REFERENCES

Brown, W. J., Goodhouse, J., and Farquhar, M. G. (1986) J. Cell Biol. 103, 1235–1247

Cousin, C., Waheed, A., Braulke, T., Junghans, U., Maly, P., Humbel, R. E., and Von Figura, K. (1988) Biochem. J. 252, 795–799

Creek, K. E., and Sly, W. S. (1983) Biochem. J. 214, 353–360

Flowers, C. H., Skikne, B. S., Covell, A. M., and Cook, J. D. (1989) J. Lab. Clin. Med. 114, 308–377

Geuze, H. J., Slot, J. W., Strous, G. J., Lodish, H. F., and Schwartz, A. L. (1983) Cell 32, 277–287

Geuze, H. J., Slot, J. W., Strous, G. J., Peppard, J., von Figura, K., Hasilik, A., and Schwartz, A. L. (1984) Cell 37, 193–204

Geuze, H. J., Stoorvogel, W., Strous, G. J., Slot, J. W., Bleekemolen, J. E., and Mellman, I. (1988) J. Cell Biol. 107, 2491–2501

Goldberg, D. E., Goei, C. A., and Kornfeld, S. (1983) J. Cell Biol. 97, 1700–1706

Goldstein, J. L., Brown, M. S., Anderson, R. G. W., Russell, D. W., and Schneider, W. J. (1985) Annu. Rev. Cell Biol. 1, 1–39

Gower, H. J., Barton, C. H., Elsom, V. L., Thompson, J., Moore, S. E., Dickson, J., and Walsh, F. S. (1988) Cell 55, 955–964

Grant, K. I., Cacciola, L. A., Coetzee, G. A., Sanan, D. A., Gevers, W., van der Westhuizen, D. R. (1989) J. Biol. Chem. 264, 4041–4047

Gussow, D., and Ploegh, H. (1987) Immunol. Today 8, 220–222

Herinton, A. C., Ymer, S., and Stevenson, J. (1986) J. Clin. Invest. 77, 1817–1823

Hopkins, C. R. (1983) Cell 35, 321–330

Hoppe, C. A., Connolly, T. P., and Hubbard, A. L. (1985) J. Cell Biol. 101, 2113–2123

Kies, W., Greenstein, L. A., White, R. M., Lee, L., Rechler, M. M., and Nisley, S. P. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7720–7724

Klausner, R. D., Ashwell, G., Van Renenwoude, J., Harford, J. B., and Bridges, K. R. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2263–2266

Kong, Y., Nishisato, T., Kondo, H., Tsushima, N., Nitsu, Y., and Urushizaki, I. (1986) Br. J. Haematol. 64, 277–281

Kyle, J. W., Nolan, C. M., Oshima, A., and Sly, W. S. (1988) J. Biol. Chem. 263, 16230–16235

Laemmli, U. K. (1970) Nature 227, 680–685

Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., and Wood, W. I. (1987) Nature 329, 537–543

MacDonald, R. G., and Czech, M. P. (1985) J. Biol. Chem. 260, 11357–11365

MacDonald, R. G., Tepper, M. A., Clairmont, K. B., Perregaux, S. B., and Czech, M. P. (1989) J. Biol. Chem. 264, 3256–3261

Mostov, K. E., and Blobel, G. (1982) J. Biol. Chem. 257, 11816–11821

Oppenheimer, C. L., and Czech, M. P. (1983) J. Biol. Chem. 258, 8539–8542

Sahagian, G. G. (1984) Biol. Cell. 51, 207–214

Sahagian, G. G., and Neufeld, E. F. (1983) J. Biol. Chem. 258, 7121–7128

Solari, R., and Kraehenbuhl, J. P. (1984) Cell 36, 61–71

Stein, M., Braulke, T., Krentler, C., Hasilik, A., and von Figura, K. (1987) Biol. Chem. Hoppe-Seyler 368, 957–947

Sztul, E. S., Howell, K. E., and Palade, G. E. (1985) J. Cell Biol. 100, 1255–1261

Trowbridge, I. S. (1989) J. Lab. Clin. Med. 114, 336–337