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BINDING, INTERNALIZATION AND INTRACELLULAR PROCESSING OF ^{125}I-EPIDERMAL GROWTH FACTOR PURIFIED BY ISOELECTRIC FOCUSING

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When epidermal growth factor (EGF) which had been extensively purified by HPLC was subjected to iodination with sodium ^{125}iodide, 5 major species of differing isoelectric points were produced. Some of these species bound to rat fibroblasts with different affinities but were internalized with equal efficiency. Examination of the internalized ^{125}I-labelled molecules revealed processing of all the ^{125}I-EGF species to macromolecules with more acidic isoelectric points. The ^{125}I-EGF species with a pI of 4.5 corresponded in electrofocusing behavior with intact non-iodinated EGF. Other EGF species probably represented molecules which were covalently modified as a result of the iodination procedure.

When cultured cells are exposed to ^{125}I-epidermal growth factor (^{125}I-EGF), the ^{125}I-EGF binds to high affinity receptors situated on the cell surfaces (1). Within 5 min after binding, the bound EGF undergoes clustering, endocytosis, and is ultimately degraded (2). The relationship of the latter events to the mitogenic activity of EGF have not yet been elucidated.

Recently we demonstrated that within 5 min after exposure to cells, ^{125}I-EGF became processed to more acidic species which were associated with cellular organelles, as determined by Percoll density gradient centrifugation (3). The isoelectric points of the ^{125}I-containing EGF species were determined by isoelectric focusing in agarose gels. The processed EGF molecules recovered from the organelle fraction were of the same approximate size as native EGF, as determined by gel filtration chromatography, and were also as immunoreactive as EGF. Apparently the latter forms of processed ^{125}I-EGF were substrates for degradative enzymes; low molecular weight ^{125}I-tyrosine appeared in the culture medium as the amounts of these forms of ^{125}I-EGF within the cell decreased.

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A major experimental limitation which occurs in utilizing ^{125}I-labelled EGF is that, when analyzed by isoelectric focusing, the ^{125}I-EGF consists of at least 2-3 species (4). We reported that following iodination of HPLC-purified EGF, (5) two major species and at least one minor species of ^{125}I-EGF were separable by isoelectric focusing and that these forms of ^{125}I-EGF could be bound to and eluted from the surfaces of cultured cells at 4°C (3). We have now purified 5 distinguishable species of ^{125}I-EGF by preparative isoelectric focusing and determined that each ^{125}I-EGF species was bound to cells, was internalized, and was processed to more acidic forms. One of the ^{125}I-EGF species, of pI 4.5, corresponds in electrofocusing behavior to non-iodinated EGF. These experiments corroborate our earlier findings with heterogeneous ^{125}I-EGF indicating that EGF is processed by cells in a stepwise manner through two acidic high molecular weight forms before it is degraded to low molecular weight compounds. The preparative isoelectric focusing methodology described in this paper can be employed for isolating single species of ^{125}I-EGF in order to study intracellular processing of EGF as well as for kinetic binding studies in which interpretation of data requires the use of a homogeneous ligand.

MATERIALS AND METHODS

Rat-1 cells, a cell line derived from Fischer rat embryo fibroblasts (6), were propagated as previously described (7).

EGF was prepared from mouse submaxillary glands (8) and purified to a single peak by reverse phase high performance liquid chromatography (5). The ultrapure EGF (α-EGF) was iodinated using Na^{125}I (Amersham) and chloramine T (1). Similar results to those reported in this paper were obtained using Iodogen (Pierce Chemical Co.) instead of chloramine T.

Isoelectric focusing was performed as previously described (3) in slab gels, 145 x 205 x 1.5 mm, containing 1% (w/v) Bio-Lyte 3/5 (BioRad Laboratories) or Servalyt AG 3-5 (Accurate Chemical Co.), 0.4% (w/v) BioLyte 5/7, and 0.6% (w/v) BioLyte 3/10. Gels were dried (unfixed) after focusing and exposed to Kodak X-Omat AR film with Radelin T2 x-ray intensifying screens.

RESULTS

When HPLC-purified EGF was iodinated, five major species with isoelectric points in the range of 3.8 to 5.2 were resolved by isoelectric focusing (Figure 1). Each of these species was purified by preparative isoelectric

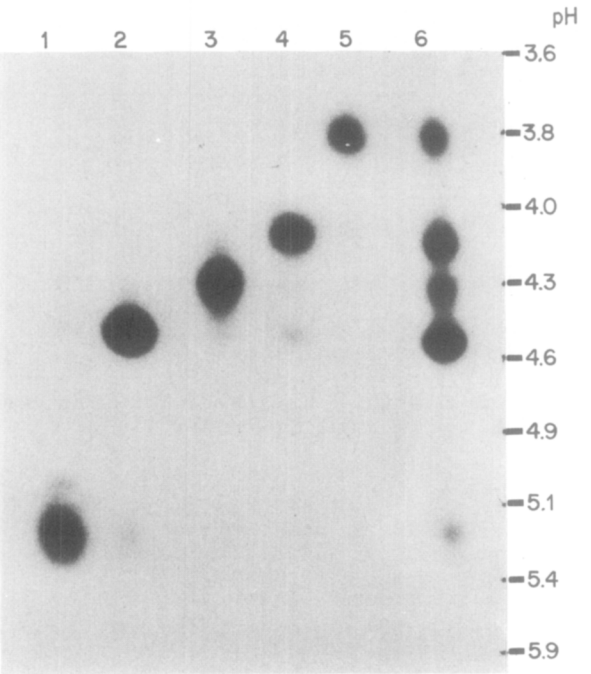

Figure 1. Autoradiographs of isoelectric focusing gels after focusing of ^{125}I-EGF.

As detailed in the legend to Table I, ^{125}I-EGF was applied to isoelectric focusing gels for preparative focusing of ^{125}I-EGF species of differing isoelectric points. Each of these purified species was then applied to an analytical isoelectric focusing gel, whose autoradiogram is shown in this figure (lanes 1-5). The original unfractionated ^{125}I-EGF used to prepare the different ^{125}I-EGF species is shown in lane 6.

focusing (IEF). The most abundant of the iodinated forms (pI=4.5) comprised 32% of the total applied ^{125}I activity, whereas the least abundant species (pI=3.8) represented 8% of the total activity (Table I).

The forms of ^{125}I-EGF purified by IEF were tested for their ability to bind to and be internalized into cells. Rat-1 cells were incubated for 1h at 37^{o}C in the presence of each of the IEF gel-purified species of ^{125}I-EGF. Control plates for each species contained, in addition to the ^{125}I-ECF, an excess of unlabelled EGF to provide a measure of non-specific binding. At the end of the labelling period, culture dishes were rinsed well and extracted first in 0.5N NaCl, pH 2.5, to remove surface-bound ^{125}I-EGF (9,10) and then in 0.05N HCl to remove the remaining (internalized) ^{125}I-compounds. In separate experiments we determined that after binding at 4^{o}C more than 90% of the total ^{125}I activity was removed by the pH 2.5 rinse and that

TABLE I

Isoelectric Point	% Total CPM
5.2	8.2
4.5	32.1
4.3	19.2
4.1	23.8
3.8	16.7

Immediately following iodination and separation on G-25 Sephadex of HPLC-purified EGF, the ^{125}I-EGF was dried in vacuo and applied to an isoelectric focusing gel in 10 µl water. At the completion of focusing, the gel was wrapped in cellophane and exposed to film for several minutes. The regions of the gel corresponding to the presence of ^{125}I-activity were located, excised, and eluted in 1 ml water containing 1 mg/ml bovine serum albumin. The ^{125}I activity present in each of the 5 regions of differing isoelectric points are displayed above as % of the total recovered ^{125}I CPM.

after warming cells to 37°C for 30 min less than 5% of the ^{125}I activity could be removed by the pH 2.5 rinse (data not shown).

All 5 of the ^{125}I-labelled species bound specifically to the cells and were internalized (Table II). However, there were quantitative differences in the binding of the various species. The species with pI's of 3.8 and 5.2 appeared to bind less well than the other species. The similar ratios of internal/external ^{125}I activity for each species demonstrated that they all appeared to be internalized at approximately the same rate.

Previously we showed that cells processed ^{125}I-EGF to more acidic forms (3). In the experiment shown in Figure 2, intracellular ^{125}I-containing macromolecules were obtained after binding and internalization of each of the

TABLE II

pI of ^{125}I-EGF Species	^{125}I CPM External	^{125}I CPM Internal
5.2	2991 ± 97	11,115 ± 147
4.5	3169 ± 102	13,126 ± 132
4.3	3130 ± 7	12,379 ± 211
4.1	3230 ± 68	13,929 ± 42
3.8	2647 ± 29	11,048 ± 190
Unfractionated	2509 ± 29	9,722 ± 84

Rat-1 cells in 35 mm dishes (1 x 10^6 per dish) were incubated for 30 min at 37°C in 0.5 ml serum-free medium (DMEM) containing 1 mg/ml bovine serum albumin plus 1 x 10^5 CPM ^{125}I-EGF. At the end of 1h dishes were rinsed 6x with HBSS containing 1 mg/ml bovine serum albumin. Immediately, 1 ml 0.5M NaCl adjusted to pH 2.5 with acetic acid was added to each dish for 4 min. The solubilized "external" ^{125}I-EGF was then removed and 1 ml 0.05N HCL was added for 30 min. The latter extract constituted the "internal" ^{125}I activity. The ^{125}I-EGF species employed were prepared from unfractionated ^{125}I-EGF as described in Table I. Each value represents the mean ± S.E. of triplicate culture dishes.

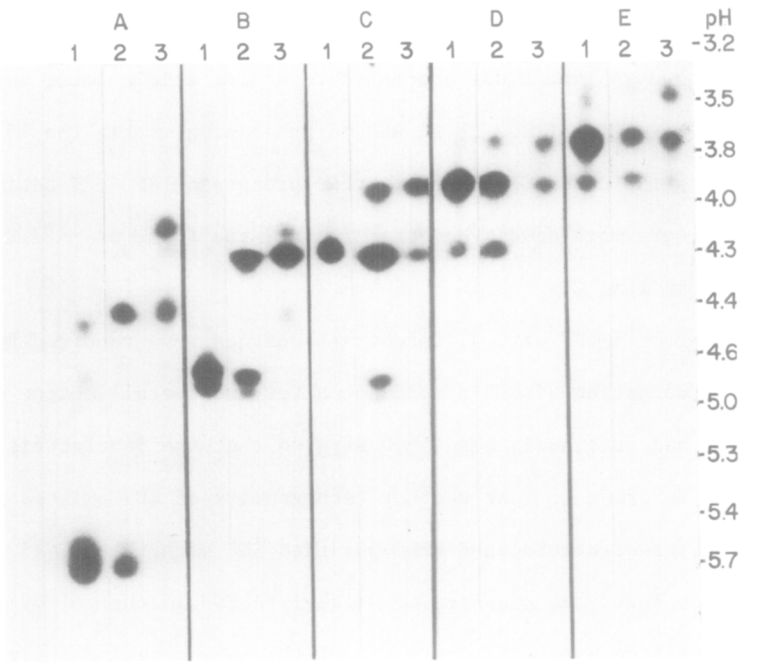

Figure 2. Isoelectric focusing autoradiograph of ^{125}I-EGF species after internalization.

Two groups of culture dishes of Rat-1 cells (1 x 10^6/35mm dish) were incubated at 37°C in 0.5 ml DMEM media containing 1 mg/ml serum albumin and 4 x 10^6 CPM of each of the ^{125}I-EGF species shown in lanes 1-5 of Figure 1. After 15 min one group of cells was removed for extraction of both surface-bound and internalized ^{125}I-EGF (see Table II for extraction procedure). At that time 5 µg non-radioactive EGF was added to the remaining culture dishes for 60 min to serve as a cold chase. These cells were extracted similarly to the first group. Internalized ^{125}I-containing cell extracts were dialyzed against H_2O, concentrated, and applied to isoelectric focusing gels. Dried gels were exposed for autoradiography. ^{125}I-EGF species employed for the experiment had isoelectric points of 5.6 (panel A), 4.5 (panel B), 4.3 (panel C), 4.1 (panel D) and 3.8 (panel E). Lane 1 of each panel represents the ^{125}I-EGF species prior to cell binding. Lane 2 represents intracellular ^{125}I activity 15 min after cell binding, and lane 3 represents intracellular ^{125}I activity after the 60 min cold EGF chase.

purified forms of ^{125}I-EGF and were examined by isoelectric focusing. Cells were incubated at 37°C for 15 min, at which time the surface-bound and internalized ^{125}I-compounds were extracted. An identical set of cells were incubated with unlabelled EGF for 60 min after the 15 min labelling period and then similarly processed by sequential removal of external and internal ^{125}I-compounds. The internalized compounds were examined by isoelectric focusing.

Each of the ^{125}I-EGF forms which was bound was processed to more acidic forms during the course of the experiment (Figure 2). By the end of the 15 min pulse-labelling period, substantial amounts of the ^{125}I-EGF species shown

in lanes A-C had been processed to more acidic forms. Only a trace amount of the species shown in lane D was processed to a more acidic form, and the most acidic ^{125}I-EGF species (lane E) showed no processing within the 15 min period. During the cold EGF chase, further processing of ^{125}I activity to species which were more acidic occurred for all the forms of ^{125}I-EGF except the one shown in lane C.

Species of ^{125}I-EGF with different net charges were reported by Savion et al. (4). Iodination of EGF should not alter the overall charge of the molecule. It had previously been demonstrated that the isoelectric point of EGF was 4.5. In order to test whether hetergeneity of EGF existed prior to iodination, we co-electrofocused HPLC-purified EGF with the pI 4.5 species of ^{125}I-EGF and measured the presence of unlabelled EGF in the gel by a radioreceptor competition assay (Figure 3). All the EGF-competing activity was present in gel fractions which contained the pI 4.5 species of ^{125}I-EGF.

DISCUSSION

Previously we demonstrated that EGF purified by the two-column procedure of Savage and Cohen (7) revealed heterogeneity when resolved by reverse-phase high performance liquid chromatography (HPLC) (8). The HPLC heterogeneity was characterized by a number of 280nm absorbing species, most of which also competed with ^{125}I-EGF for specific binding sites on cultured cells. The most abundant of the HPLC-resolved species (α-EGF) was used for chloramine T-mediated iodination in the experiments described in this paper and constituted 60% of the total 280nm-absorbing material resolved by HPLC (8).

When the α-EGF was iodinated in the presence of chloramine T, five species with differing isoelectric points were produced. We have obtained similar results when iodination was facilitated by Iodogen (Pierce Chemical Co.). The major iodinated species, which had an isoelectric point of 4.5 behaved identically to α-EGF on isoelectrofocusing gels, and therefore probably represented the remaining population of intact iodinated EGF molecules. The remaining ^{125}I-EGF species probably represent EGF molecules which have

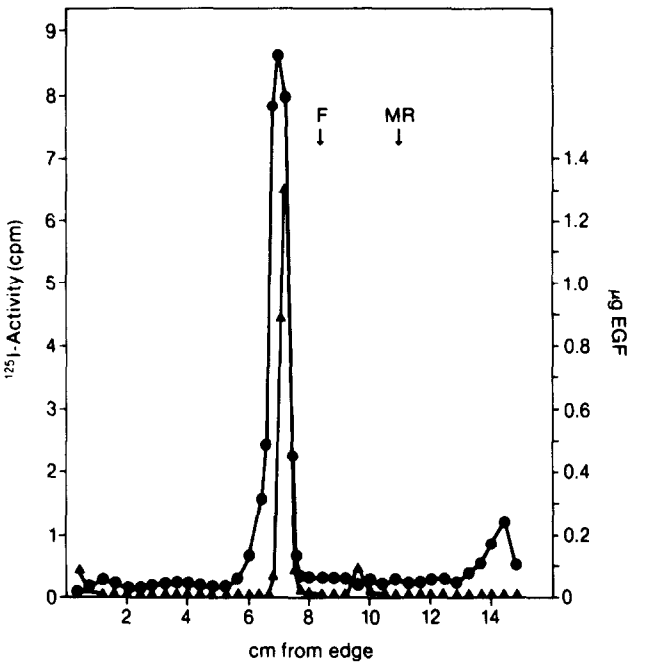

Figure 3. Isoelectric focusing of EGF and the pI 4.5 species of ^{125}I-EGF.

Isoelectric focusing was performed as described in Materials and Methods with 9 x 10^4 cpm of the pI 4.5 species of ^{125}I-EGF and 5 µg of HPLC-purified α-EGF. The wet gel was sliced at 2 mm intervals and the slices numbered starting at the basic edge of the gel. ^{125}I-activity was measured in each gel slice with a gamma counter (●). To elute the EGF, each gel slice was incubated in 1 ml of a 1 mg/ml BSA solution for 18h at 4°C. A 50 µl aliquot was tested for EGF activity using the radioreceptor assay previously described (5). HPLC-purified α-EGF was used for the standard curve and the values were corrected to µg/EGF slice (▲). Ferritin (F), pI 4.3 and methyl red (MR), pI 3.8, were used as markers.

become covalently altered as a result of the iodination procedure, since they were not present prior to iodination.

Despite the altered net charge of the iodinated molecules, they all bound to EGF receptors on Rat-1 cells and were internalized. Although the different species showed different affinities, the ability of all bound ^{125}I-EGF species to be internalized appeared similar.

After binding and internalization, each of the iodinated EGF species became modified to more acidic forms, as distinguished by isoelectric focusing. After formation of acidic species within the 15 min pulse-labelling period, many of the purified ^{125}I-EGF species appeared to be modified again to more acidic species. For most of the purified ^{125}I-EGF species tested, the second acidic species formed during the cold chase appeared at the ex-

pense of the first-formed acidic species. These results are consistent with our previous studies that suggest that EGF undergoes stepwise modification to more acidic forms as it progresses from the cell surface to organelles to the cytosol (3).

Rat-1 cells, which respond to EGF both in induction or ornithine decarboxylase activity (11) and DNA synthesis (12) process the internalized ^{125}I-EGF quantitatively through the two acidic forms prior to degradation to low molecular weight metabolites (3). Whether the intracellular processing of ^{125}I-EGF to more acidic forms is related to its biological activity, however, remains unknown.

Our data suggest that iodination of EGF results in the generation of species of ^{125}I-EGF with charge alterations and with different affinities. Purification of the pI 4.5 species of ^{125}I-EGF, which corresponds in its isoelectric point to non-iodinated EGF, should be performed prior to its use for determining EGF receptor affinity by Scatchard binding analysis.

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