Articles

Photoaffinity Labeling of the Erythropoietin Receptor and Its Identification in a Ligand-Free Form†

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Received July 5, 1990; Revised Manuscript Received September 6, 1990

ABSTRACT: Pure human recombinant erythropoietin (EP) was acylated through a primary amino residue with a cross-linking reagent, *N*-[[3-[[4-[(*p*-azido-*m*-[^{125}I]iodophenyl)azo]benzoyl]amino]propanoyl]oxy]-succinimide (Denny–Jaffe reagent), which is photoreactive and cleavable at the azo residue. The resulting conjugated hormone (DJ–EP) was purified from unmodified EP by reverse-phase high-pressure liquid chromatography and maintained its capacity to bind to receptors for EP on erythroid progenitor cells. The receptor for EP was previously identified as two related proteins of 100 and 85 kDa molecular mass by chemical cross-linking to ^{125}I-EP. Recently, D'Andrea and co-workers [(1989) *Cell 57*, 277–285] cloned a cDNA that codes for a protein of 55–66 kDa, which is thought to be the EP receptor. In this report, cross-linking to the receptor through the monofunctional DJ–EP labeled the same 140- and 125-kDa molecular mass bands (100- and 85-kDa proteins) cross-linked with ^{125}I-EP and disuccinimidyl suberate. Furthermore, cleavage of the azo bond of the DJ–EP receptor complex by sodium dithionite (80 °C, 5 min) demonstrated that proteins of 105 and 90 kDa were labeled in ligand-free form by DJ–EP. This result demonstrates that artifactual cross-linking of multiple proteins or other artifacts of cross-linking do not explain the difference in molecular mass of the EP receptor identified by cross-linking and the receptor identified by expression cloning.

Erythropoietin is the glycoprotein hormone that is essential for the complete maturation of immature erythroid cells into red blood cells (Graber & Krantz, 1989; Sawyer, 1990). Receptors for EP[1] are found on immature erythroid cells in greatest numbers in cells that are most dependent on the hormone and these cells are known as colony-forming units–erythroid. As these cells differentiate into mature erythroblasts, the EP receptor number has been shown to disappear (Sawyer, 1990; Landschulz et al., 1989; Sawada et al., 1987). Receptors for EP were first identified on immature erythroid cells purified from the spleens of mice infected with the anemia strain of Friend virus (FVA cells). These FVA cells have higher and lower affinity receptors for EP, and ^{125}I-EP is rapidly endocytosed and degraded in the lysosomes of these cells (Sawyer et al., 1987a). Cross-linking of ^{125}I-EP bound to membranes from FVA cells identified two bands of radioactivity, which had apparent molecular masses of 100 and 85 kDa when the molecular mass of EP was subtracted from the cross-linked bands (Sawyer et al., 1987b, 1989; Sawyer, 1989). Peptide mapping from the two cross-linked receptor proteins indicated similar if not identical sequences of amino acids, suggesting a single gene and either differential processing of a common precursor or proteolytic generation of the lower molecular mass protein (Sawyer, 1989).

Recently D'Andrea and co-workers (1989a) have cloned a cDNA encoding a 55-kDa protein using expression cloning and screening for ^{125}I-EP binding in transfected COS cells. This receptor protein appeared to be proteins of 105 and 65 kDa when the COS cells expressing the receptor were cross-linked to ^{125}I-EP. The discrepancy in the size of the 55-kDa receptor predicted from the cloned cDNA and the 100-kDa protein observed in cross-linking was proposed to be caused by either the extensive glycosylation of the 55-kDa protein or the simultaneous cross-linking of two 55-kDa peptides to one ^{125}I-EP molecule with the homobifunctional cross-linker disuccinimidyl suberate.

Thus far, experiments in our laboratory have failed to show extensive glycosylation of the 100-kDa protein by enzymatic and chemical deglycosylation of the ^{125}I-EP–100-kDa protein cross-linked complex (Hosoi et al., 1988). Therefore, the possibility of cross-linking artifacts resulting from the use of disuccinimidyl suberate was investigated by using the Denny–Jaffe reagent, *N*-[[3-[[4-[(*p*-azido-*m*-[^{125}I]iodophenyl)-azo]benzoyl]amino]propanoyl]oxy]succinimide (Denny & Blobel, 1984; Jaffe et al., 1980).

Transferrin was first modified with the Denny–Jaffe reagent and was used to identify the transferrin receptor in ligand-free form to establish the appropriate experimental conditions. Then, Denny–Jaffe-modified EP (DJ–EP) was prepared, which maintained the hormone's ability to bind the receptor. EP was acylated through an amino residue to the succinimide moiety

† This work was supported by National Institutes of Health Grants DK-39781 and DK-15555, Veterans Health Sciences and Research Administration Funds, and Joe C. Davis Research Fund.

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[1] Abbreviations: EP, erythropoietin; FVA cells, immature erythroid cells purified from the spleens of mice infected with the anemia strain of Friend virus; DJ, Denny–Jaffe reagent; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

0006-2960/91/0430-329$02.50/0 © 1991 American Chemical Society

of the iodinated, photoactivatable, cleavable cross-linker. The resulting DJ–EP is then capable of cross-linking the receptor for EP through the azido moiety in a photodependent reaction. This is a monofunctional reagent such that only EP is cross-linked to a molecule of protein, thus distinguishing this from the previous experiments using disuccinimidyl suberate in which many proteins may be indiscriminately cross-linked by the bifunctional cross-linker. The DJ reagent is labeled with ^{125}I so that the extent of modification of EP by DJ reagent can be measured. The DJ reagent is iodinated at the photoactive moiety such that the ^{125}I label is transferred from bound DJ–EP to the receptor after the azo bond is cleaved with dithionite.

In this study, DJ–EP cross-linked the same 100- and 85-kDa proteins in FVA cells as were cross-linked with ^{125}I-EP and disuccinimidyl suberate. Furthermore, the identification of the receptor for EP in ligand-free form by DJ–EP delineated similar molecular masses of 105 and 90 kDa. This result shows that cross-linking artifacts have not led to the erroneous migration of a 55–66-kDa receptor protein for EP at 100 kDa on gel electrophoresis.

Materials and Methods

Materials. Recombinant human EP was purchased from AMGEN Biochemicals (Thousand Oaks, CA) and human transferrin was purchased from Calbiochem. *N*-[[3-[[4-[(*p*-Azido-*m*-[^{125}I]iodophenyl)azo]benzoyl]amino]propanoyl]-succinimide (Denny–Jaffe reagent) was purchased from New England Nuclear.

Cells and Plasma Membrane Preparation. Murine immature erythroid cells (FVA cells) were purified from the spleens of CD_2F1 mice infected with the anemic strain Friend virus by velocity gradient sedimentation at unit gravity as described (Koury et al., 1984; Sawyer et al., 1987c). Plasma membranes were also prepared from FVA cells as previously described (Sawyer et al., 1987b) and suspended in a binding/photolysis buffer: 1% bovine serum albumin, 1 mM EGTA, 100 kunits/mL aprotinin, 10 mM phosphate, pH 7.4, and 150 mM NaCl, which was degassed and bubbled with N_2 gas.

Derivatization of EP and Transferrin with the Denny–Jaffe Reagent. All of the procedures were done in the dark with a safety light. An aliquot of Denny–Jaffe reagent shipped in benzene was put into a conical tube and dried under dry N_2 gas. Recombinant human EP (50 units, 0.013 nmol) in 1% dimethyl sulfoxide and 100 mM borate buffer, pH 8.5, was added to the tube coated with the Denny–Jaffe reagent. The solution was kept at room temperature for 120 min to allow the derivatization of a primary amino residue of EP with the *N*-hydroxysuccinimide ester residue of the Denny–Jaffe reagent. Tris-HCl (pH 7.4) was then added to quench the reaction, and the solution was kept at room temperature for 15 min. The reaction mixture was applied to the Bio-Gel P6-DG column equilibrated with 0.1% gelatin, 10 mM phosphate, pH 7.4, and 150 mM NaCl so that the derivatized EP (DJ–EP) was separated from unreacted Denny–Jaffe reagent.

Transferrin was modified by Denny–Jaffe reagent in the same fashion as EP. However, the transferrin was first saturated with iron as described previously (Sawyer & Krantz, 1986) and 4 μg of diferric transferrin was incubated with 100 μCi of Denny–Jaffe reagent.

Reverse-Phase High-Performance Liquid Chromatography Fractionation of DJ–EP. DJ–EP prepared as described above was injected into a Vydac C4 column (The Separation Group, Hesperia, CA), equilibrated with 0.1% TFA in water, and the column was eluted by a gradient made with 0.1% TFA–90% acetonitrile in water. The eluate was collected into glass tubes containing 0.07% bovine serum albumin and 0.1 M ammonium bicarbonate, pH 8.0, in order for immediate neutralization. The radioactivity of each fraction was counted with a γ counter. Each fraction was lyophilized with a Speed-Vac and the dried pellets were reconstituted in deionized, distilled water. The samples were analyzed by $NaDodSO_4$–PAGE as described by Laemmli (1970) and autoradiography. The column was calibrated for the retention time of EP by using radioiodinated EP (^{125}I-EP).

Radioiodination of EP. EP was iodinated with $Na^{125}I$ (Amersham, Arlington Heights, IL) by using 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (IODO-GEN) (Pierce, Rockford, IL) as described before (Sawyer et al., 1987a).

Binding Assay. The binding activity of DJ–EP was examined as described previously for ^{125}I-EP to FVA cells (Sawyer et al., 1987a) with a minor modification. Briefly, (1–2) $\times 10^6$ FVA cells in binding/photolysis buffer were incubated with increasing concentration of DJ–EP at 37 °C for 30 min or at 0 °C for 20 h. After the incubation, the cells were pelleted through a layer of *n*-butyl phthalate. The tubes were frozen and the tips of the tubes were cut off to count the radioactivity associated with cells by using a γ counter. Nonspecific binding was measured by adding 200 units of underivatized EP per milliliter in the binding mixture. This was subtracted from the total binding to obtain the specific binding.

Photoaffinity Labeling of the EP Receptor and Transferrin Receptor. DJ–EP, unfractionated or fractionated with reverse-phase HPLC, or DJ–transferrin was incubated with 0.2–0.4 mg of FVA cell membrane protein at 37 °C for 20 min in the binding/photolysis buffer in 1.5-mL microtubes. Each tube was wrapped with aluminum foil to keep the binding mixture in the dark. The binding mixtures were cooled on ice, transferred into the wells of a 24-well culture plate (Costar), and irradiated with ultraviolet light (365 nm) for 15 min on ice with a UVL-56 lamp (Ultra-Violet Product Inc). The photolyzed samples were washed three times with ice-old 0.1% bovine serum albumin/100 mM NaCl/50 mM glycine/1 mM EGTA, pH 3.5, and once with ice-cold 1 mM EGTA, 10 mM phosphate, pH 7.2, and 150 mM NaCl with centrifugation at 13000*g* for 15 min at 4 °C. After either no treatment or treatment with sodium dithionite to cleave the cross-linker, the membranes were suspended in sample buffer containing β-mercaptoethanol, and the samples were boiled for 5 min, sonicated briefly, and applied to $NaDodSO_4$–PAGE according to the method of Laemmli (1970). The gels were stained with Coomassie Brilliant Blue, destained, dried, and exposed to Kodak-XAR5 X-ray films for autoradiography.

Cleavage of the Azo Linkage. Membranes labeled with either DJ–EP or DJ–transferrin were incubated with 0.2 M or the indicated concentration of sodium dithionite at 80 °C for 5 min. Also, the strips of the dried gel corresponding to the bands of DJ–EP·EP receptor complexes on the autoradiogram were excised out, minced, and rehydrated in 2% $NaDodSO_4$, 2 mM EGTA, and 100 mM borate buffer, pH 8.0, containing 0.2 M sodium dithionite for 15 min at room temperature. An additional volume of 0.2 M sodium dithionite in water was then added and the sample was heated at 80 °C for 5 min. Following the addition of the sample buffer, the gel fragments and the supernatant were analyzed by $NaDodSO_4$–PAGE under reducing conditions and autoradiography.

Results

The transferrin receptor is present on the surface of FVA

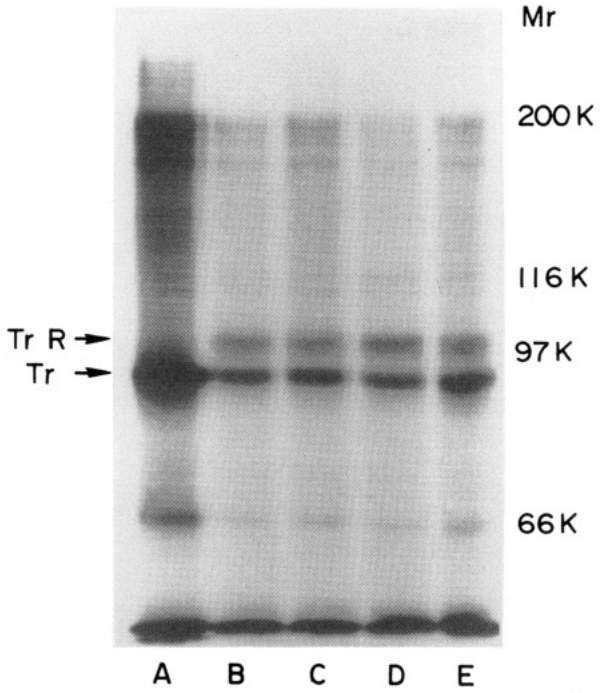

FIGURE 1: Photolabeling of the transferrin receptor by DJ–transferrin. DJ–transferrin was incubated with FVA cell membranes and exposed to long-wavelength ultraviolet light as described under Materials and Methods. $NaDodSO_4$–PAGE and autoradiography showed the high molecular weight complex of DJ–transferrin:transferrin, lane A. Exposure of cross-linked membranes to increasing concentrations of sodium dithionite (lane B, 50 mM; lane C, 100 mM; lane D, 200 mM; lane E, 300 mM) for 5 min at 80 °C before $NaDodSO_4$ resulted in the disappearance of the cross-linked complex and the appearance of the DJ-labeled receptor free of transferrin on the autoradiogram. Positions of transferrin (Tr) and transferrin receptor (TrR) and molecular weight marker are indicated.

cells at a frequency of 200 000 receptors per cell while the EP receptor is only present at 900 receptors per cell (Sawyer & Krantz, 1986; Sawyer et al., 1987). Therefore, DJ–transferrin was prepared to establish the conditions necessary to modify the ligand, cross-link the receptor in a photodependent manner, and cleave the azo bond in the DJ–ligand to identify the receptor in a ligand-free form. DJ–transferrin was prepared as described under Materials and Methods. The resulting DJ–transferrin bound to FVA cells such that 95% of radioactivity was specifically bound (data not shown). Exposure of DJ–transferrin bound to FVA cell membranes to long-wavelength ultraviolet light resulted in the covalent coupling of DJ–transferrin into a high molecular mass band of 180–200 kDa on $NaDodSO_4$–PAGE (Figure 1, lane A). All the bands in Figure 1, lane A, result from specific binding of DJ–transferrin since the addition of an excess of unlabeled transferrin during binding and cross-linking results in virtually no radioactivity on $NaDodSO_4$–PAGE (not shown). Cleavage of the azo bond in the DJ reagent with increasing concentrations of sodium dithionite resulted in the disappearance of the radioactivity in the DJ–transferrin·transferrin receptor complex and greatly reduced the radioactivity in the uncross-linked DJ–transferrin with the appearance of a new band at 100 kDa corresponding to the DJ-labeled transferrin receptor free of transferrin (Figure 1). Immunoprecipitation of the DJ-labeled, 100-kDa band by a monoclonal antibody to the mouse transferrin receptor (RI7-208 from Ian Trowbridge, Salk Institute) verified that this band indeed was the transferrin receptor and the molecular mass agreed exactly with that determined previously in FVA cells (Sawyer & Krantz, 1986).

Table I: Acylation of EP by the Denny–Jaffe Reagent; Effect of the Stoichiometry of the DJ Reagent Coupled to EP on DJ–EP Binding to FVA Cells[a]

mol of DJ reagent/mol of EP	bound DJ–EP		
	total (cpm/10^6 cells)	specific (cpm/10^6 cells)	specific (% of total)
0.05	488 ± 42	267	45
0.06	1041 ± 83	475	46
0.08	1817 ± 54	610	33
0.09	2198 ± 52	460	20
0.12	2066 ± 165	521	25
0.19	715 ± 24	43	6

[a] EP was acylated with increasing concentrations of DJ reagent as described under Materials and Methods. Following separation of DJ–EP from unreacted DJ–reagent by gel filtration, the number of molecules of DJ reagent incorporated per EP molecule was calculated by assuming a complete recovery of EP (possibly underestimating incorporation). DJ–EP binding to FVA cells was then carried out as described under Materials and Methods at 37 °C for 30 min. Results shown are the mean of three determinations ± standard deviation.

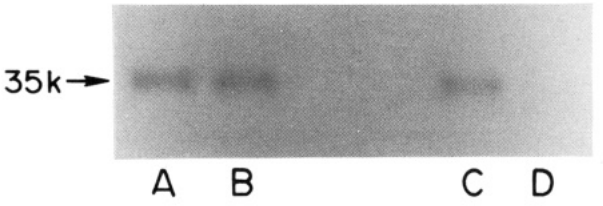

FIGURE 2: Cleavage of the azo bond in DJ–EP with sodium dithionite. DJ–EP and ^{125}I-EP were incubated in the presence and absence of 200 mM sodium dithionite for 5 min at 80 °C and then analyzed by $NaDodSO_4$–PAGE and autoradiography (lane A, ^{125}I-EP minus dithionite; lane B, ^{125}I-EP treated with dithionite; lane C, DJ–EP minus dithionite; lane D, DJ–EP treated with dithionite).

EP was modified with the DJ reagent in the same fashion as transferrin as described under Materials and Methods. The concentration of the DJ reagent was varied in the coupling reaction with a constant amount of EP to optimize the binding ability of the resulting DJ–EP. As illustrated by the data in Table I, EP was modified with the DJ reagent such that the resulting DJ–EP maintained specific binding to FVA cells. However, very little EP could be acylated on a primary amine without inactivation of the EP as measured by the ability to bind the receptor. Only 10% or less of the EP could react with the DJ reagent without near total loss of binding ability.

DJ–EP was analyzed by $NaDodSO_4$–PAGE before and after treatment with sodium dithionite to verify the appropriate labeling of the EP on the cleavable portion of the DJ reagent. As shown in Figure 2, DJ–EP was visualized by autoradiography as a single band (lane C). Treatment with 200 mM sodium dithionite resulted in the almost complete loss of label. No lower molecular weight fragments of DJ–EP were seen on the gel (not shown due to cropping of the autoradiogram). As a control ^{125}I-EP was not affected by the sodium dithionite treatment.

Membranes from FVA cells were cross-linked with DJ–EP as described under Materials and Methods and analyzed by $NaDodSO_4$–PAGE. Bands of 140 and 125 kDa were cross-linked to DJ–EP in a specific and photodependent manner as shown in Figure 3 (lane C). Nonspecific cross-linking (lane B) was very prominent in the area of the gel at or less than 100 kDa such that cleavage by sodium dithionite resulted in lower molecular mass bands that were virtually undetectable above background (data not shown).

DJ–EP was then subjected to reverse-phase high-pressure liquid chromatography on a C-4 column. This was carried out to separate the active DJ–EP from both unmodified EP

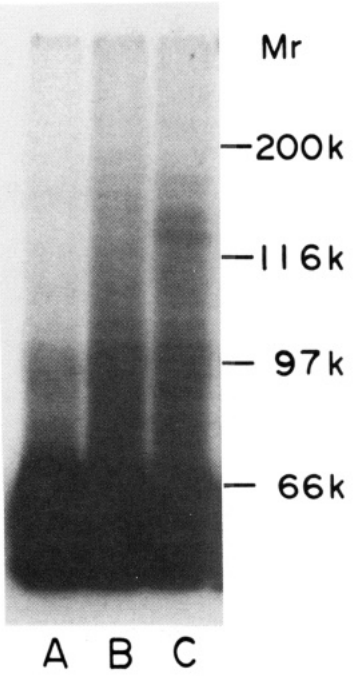

FIGURE 3: Cross-linking of the EP receptors with DJ–EP. Membranes from FVA cells were incubated with DJ–EP, and receptors for EP were photolabeled as described under Materials and Methods and analyzed by $NaDodSO_4$–PAGE and autoradiography (lane C). Nonspecific labeling was determined in the absence of exposure to UV light (lane A) or in the presence of 200 units of unlabeled EP per milliliter (lane B).

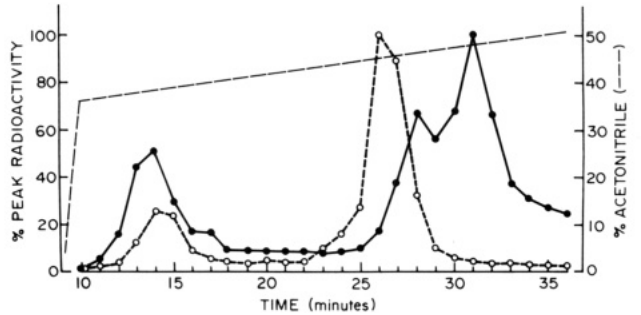

FIGURE 4: Elution profile of DJ–EP and ^{125}I-EP from reverse-phase high-pressure liquid chromatography. ^{125}I-EP (○) and DJ–EP (●) were separately applied to a C_4 column and eluted with the indicated gradient (0.1% trifluoroacetic acid to 0.1% trifluoroacetic containing 50% acetonitrile. Radioactivity in each fraction was counted in a γ counter. No radioactivity eluted before 10 min, and radioactivity eluted before 22 min was not covalently coupled to EP.

and possibly inactive DJ–EP such that more radioactivity could be transferred to the receptor. Figure 4 shows the elution profile of DJ–EP compared to that of ^{125}I-EP. As expected the hydrophobic DJ reagent imparted a longer retention time on DJ–EP compared to ^{125}I-EP. ^{125}I-EP elutes at exactly the same retention time as unmodified EP (not shown). DJ–EP was eluted in two peaks with retention times of 28 and 31 min from the reverse-phase column. Both fractions exhibited equal ability to bind to FVA cells and were pooled for most experiments. Unreacted DJ reagent was eluted at a retention time of 14 min. Therefore, reversed-phase HPLC was routinely used to separate DJ–EP from the unmodified EP and DJ reagent in the initial reaction mixture, eliminating the desalting column step. This resulting DJ–EP bound to FVA cells with more radioactivity than the previous DJ–EP due to less competition from unmodified EP.

Figure 5 illustrates the binding of HPLC-purified DJ–EP to FVA cells. DJ–EP eluted in the second peak was used to

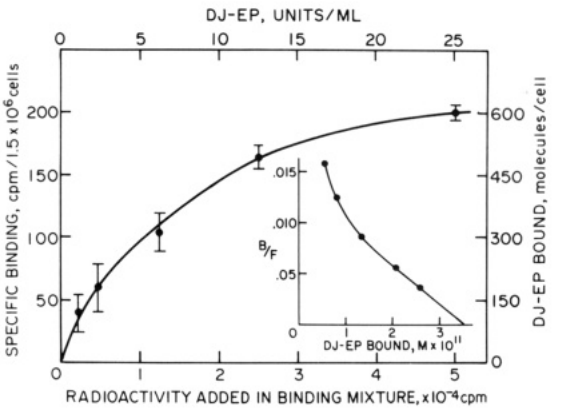

FIGURE 5: Binding of DJ–EP to FVA cells. DJ–EP separated from nonderivatized EP by reverse-phase chromatography was incubated with FVA cells for 20 h at 0 °C as described under Materials and Methods. Nonspecific binding for each concentration of DJ–EP was determined in duplicate in the presence of 200 units of unlabeled EP per milliliter and was subtracted from the total binding resulting in specifically bound DJ–EP. Each point is the mean of three determination and error bars represent standard deviations. Insert shows the data plotted by the method of Scatchard.

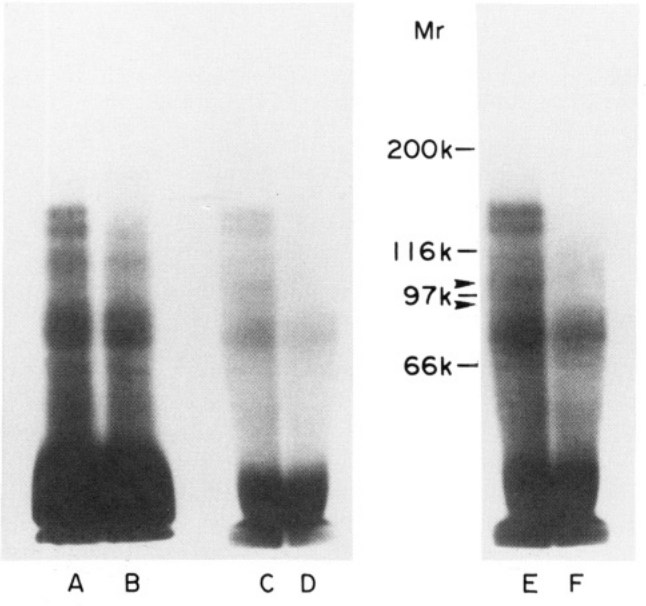

FIGURE 6: Photolabeling of the EP receptor by purified DJ–EP. DJ–EP purified from unmodified EP by reverse-phase chromatography was incubated with membranes from FVA cells and the EP receptors were cross-linked in a photodependent fashion. The material was then analyzed by $NaDodSO_4$–PAGE and autoradiography as described under Materials and Methods. Membranes were incubated in the absence (lanes A, C, and E) or presence of 200 units of unlabeled EP per milliliter (lanes B, D, and F). The azo bond was cleaved by treatment of cross-linked membranes with sodium dithionite (0.2 M) for 5 min at 80 °C (lanes C–F). Lanes E and F are the same as lanes C and D but exposed to film for a longer time. Positions of molecular weight markers are indicated. Arrow heads indicate bands of 105 and 90 kDa.

lessen the possibility of contamination with unmodified EP. The binding is saturable and when plotted by the Scatchard method is biphasic, indicating DJ–EP is recognizing both higher and lower affinity receptors previously identified by ^{125}I-EP (Sawyer et al., 1987a). If the recovery of DJ–EP was 100% (assuming one DJ per EP) and totally free of unmodified EP, the binding would indicate a somewhat higher K_d for DJ–EP binding to FVA cells than that observed with ^{125}I-EP (K_d = 300 and 2000 pM compared to 80 and 500 pM). However, 30% of DJ–EP would be inactive. Alternatively, incomplete recovery of DJ–EP and/or contamination of DJ–

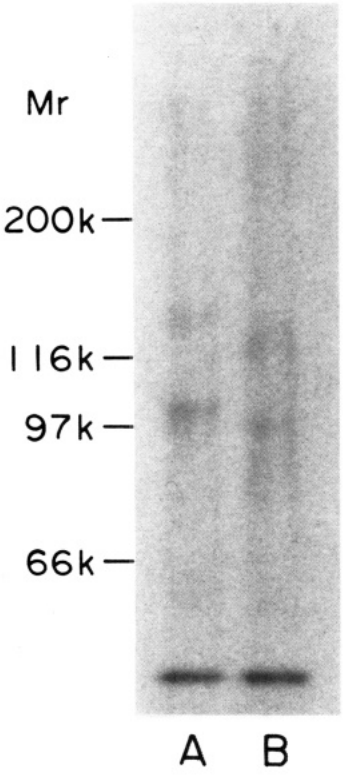

FIGURE 7: Ligand-free labeling of the EP receptor. The azo bond in the DJ–EP·receptor complex was cleaved by treatment with 200 mM sodium dithionite at 80 °C for 5 min. The 140- and 125-kDa bands of DJ–EP·receptor were excised from the dried gel in the experiment shown in Figure 6, lane A, and treated with sodium dithionite as described under Materials and Methods. The material was then analyzed by $NaDodSO_4$–PAGE and autoradiography (lane A, 140-kDa band; lane B, 125-kDa band).

EP with unmodified EP could result in an underestimation of the affinity of DJ–EP binding and the percentage of active DJ–EP by up to 30% (K_d = 200 and 1400 pM).

Figure 6 illustrates the cross-linking of HPLC-purified DJ–EP to plasma membranes from FVA cells as described under Materials and Methods. Bands of 140 and 125 kDa were seen in lane A, Figure 6, which are identical with those seen in lane C, Figure 3, with unpurified DJ–EP and as seen previously with ^{125}I-EP cross-linked to the receptor with disuccinimidyl suberate (Sawyer et al., 1987b; Sawyer, 1989). Nonspecific cross-linking is shown in lane B. In contrast to the experiment shown in Figure 3 (the dried gel was exposed to film for 4 weeks), the autoradiogram in Figure 6 required only 48 h of exposure, indicating the greater radioactivity in the cross-linked EP receptor.

DJ-labeled EP receptor free of EP was observed by cleaving the azo bond in the DJ–EP cross-linked to the receptor by sodium dithionite under conditions established in experiments with DJ–transferrin. In Figure 6, lanes C–F show the resulting bands of replicate samples of FVA membranes cross-linked to DJ–EP followed by treatment with 200 mM sodium dithionite at 80 °C for 5 min. Along with a decrease in the specially labeled bands at 140 and 125 kDa, specific bands appeared at 105 and 90 kDa (lanes C, E). In an alternate experiment, the 140-kDa band and the 125-kDa bands of DJ–EP·EP receptor cross-linked complex were excised from the dried gel whose autoradiogram is shown in Figure 6, lane A. These gel slices were minced and rehydrated in sodium dithionite at 80 °C for 5 min. Following this treatment, the gel fragments and supernatant were analyzed by $NaDodSO_4$–PAGE. As shown in Figure 7, lane A, treatment of the 140-kDa band resulted in a residual 140-kDa band and a new band at 105 kDa, while treatment of the 125-kDa band (lane B) resulted in a residual band at 125 kDa and a new band at 90 kDa. Coomassie Blue staining material was observed at 140 kDa in lane A and 125 kDa in lane B, indicating the lack of effect of sodium dithionite treatment on proteins in general. The corresponding area of the gel in Figure 6 showing nonspecific cross-linking was also excised and treated in parallel, but no radioactivity was detected by $NaDodSO_4$–PAGE and autoradiography (data not shown). Incomplete cleavage of the azo bond in cross-linked DJ–EP resulted from the cleavage conditions of 0.2 M sodium dithionite at 80 °C for 5 min even though almost complete cleavage of free DJ–EP occurred (Figure 2). This observation is likely due to reduced accessibility of sodium dithionite to the azo bond in the cross-linked DJ–EP·receptor complex. Increasing the concentration of sodium dithionite did not increase the cleavage of cross-linked DJ–EP (this was also the case for DJ–transferrin as shown in Figure 1), and increased time of treatment resulted in more cleavage but less recovery of the labeled 105- and 90-kDa bands. Therefore, the experimental conditions were chosen to maximize the production of DJ-labeled receptor free of EP even though some receptor was still in the cross-linked state.

Discussion

We report the use of the Denny–Jaffe reagent to identify the transferrin receptor and EP receptor in ligand-free form following photoaffinity cross-linking with the appropriate ligand. The size of the transferrin receptor is well-known (Trowbridge et al., 1984), and these studies on the transferrin receptor were only undertaken to establish the technical conditions necessary to use the DJ reagent to investigate EP receptors on FVA cells.

While transferrin could be labeled by the DJ reagent in a one to one stoichiomery to yield an active DJ–transferrin, DJ–EP was completely inactive when more than 25% of the EP was modified. This result is consistent with the previous observations that EP was inactivated by modification of primary amino residues (Goldwasser, 1981; Wojchowski & Caslake, 1989). However, DJ–EP labeled with a low stoichiometry (10% labeled) binds with high affinity to the receptor and has biological activity. This paradox may be explained by a change in conformation of EP induced by the modification of the first amino group by the DJ reagent such that another amino group crucial for activity of the hormone becomes many fold more accessible to the DJ reagent than the first amino group. This would lead to the inability to label all of the hormone in an active state. Alternatively, heterogeneity in the conformation of the genetically engineered hormone might also give this result.

The binding of DJ–EP to FVA cells indicated that if DJ–EP is in a conformation different from the native state, then the ability to bind the receptor is not drastically altered. Although the binding affinity of EP to its receptor was decreased 3–4-fold by the addition of the DJ reagent, the DJ–EP still maintains high-affinity binding (0.2–0.3 nM and 1.4–2.0 nM). Just as the extensive carbohydrate on the EP decreases the affinity of the hormone for the receptor when compared to the binding of partially deglycosylated EP (Mufson & Gesner, 1987), it is not surprising that the addition of the bulky and negatively charged DJ–reagent to EP would decrease the affinity of the interaction of DJ–EP for its receptor.

DJ–EP labels two proteins of 100 and 85 kDa apparent molecular mass in the uncleaved cross-linked complex of hormone and receptor (bands of 140 and 125 kDa). These bands are the same molecular weight as previously identified

by chemical cross-linking of ^{125}I-EP to membranes from FVA cells (Sawyer et al., 1987b, 1989; Sawyer, 1989) and other erythroid cells (Sasaki et al., 1987; Fukamachi et al., 1987; Broudy et al., 1988; Tojo et al., 1987; Hitomi et al., 1988; Landschultz et al., 1989). Moreover, cleavage of the azo bond in the DJ–EP receptor complex resulted in the appearance of 105-kDa protein and 90-kDa proteins from the 140- and 125-kDa bands, respectively, which correspond to the 100- and 85-kDa proteins predicted by chemical cross-linking. The radioactivity in the DJ-labeled EP receptor in ligand-free form is faint on the autoradiograms due to (1) the low number of EP receptors, (2) the partial inactivation of DJ–EP, and (3) the poor recovery of receptor radioactivity following cleavage of the azo bond in the cross-linker (this was the case with both DJ–transferrin and DJ–EP receptors). Although expression cloning has identified an EP receptor protein of 55–66 kDa molecular mass (D'Andrea et al., 1989a; Yoshimura et al., 1990), DJ–EP in no case specifically labeled a 55–66-kDa protein in ligand-free form or a 100–110-kDa band in the cross-linked form.

DJ–EP is a monofunctional reagent such that only one EP molecule may be cross-linked to any protein. This is particularly important in interpreting the relationship between the 105- and 90-kDa proteins identified here and the newly cloned receptor for EP, which codes for a smaller protein. D'Andrea and co-workers (1989a) identified a cDNA constructed from murine erythroleukemia cell mRNA, which, when transfected into COS cells, resulted in the appearance of binding sites for ^{125}I-EP. While the cDNA encodes a 55-kDa protein, the cross-linking ^{125}I-EP to receptors on these COS cells labeled proteins of 105 and 66 kDa. Antipeptide antibodies confirm the existence of the 66-kDa protein in erythroid cells (Yoshimura et al., 1990; Li et al., 1990; S. T. Sawyer, unpublished data). It has been proposed that the 105-kDa protein might represent the simultaneous cross-linking of two 55-kDa receptor proteins or the cross-linking of the receptor and two EP molecules or the receptor plus an accessory protein and ^{125}I-EP by the bifunctional cross-linker disuccinimidyl suberate. The work presented here proves that this simultaneous cross-linking is not the case.

Possible origins of the 105-kDa protein include the following: (1) the cloned gene product is somehow additionally modified to become the 105-kDa form of the receptor, or (2) the 105-kDa protein is constitutively expressed in the COS cells and erythroid cells and forms a complex with the 66-kDa receptor protein. Considering the first possibility, posttranslational modification of the 55-kDa peptide by disulfide bridging to another subunit or extensive glycosylation is not likely. The 105-kDa protein was observed on $NaDodSO_4$–PAGE in the presence of high concentrations of reducing agents, which would be expected to break disulfide bonds. Digestion of the cross-linked EP receptor–^{125}I-EP complex with mixtures of N- and O-glycanases and reductive alkaline hydrolysis failed to detect a significant level of carbohydrate (N- or O-linked) on the 105- or 90-kDa proteins (Hosoi et al., 1988). This, together with the presence of *only one* extracellular site for N-linked glycosylation on the cloned receptor protein (D'Andrea et al., 1989a), makes it unlikely that extensive addition of carbohydrate to the 55-kDa peptide results in a 105-kDa protein.

Recently, it has been shown that the genetically engineered 66-kDa receptor protein can interact with EP in extracts of COS cells transfected with either the human or murine cDNA encoding the receptor (Jones et al., 1990; Wognum et al., 1990). Metabolically labeled 66-kDa protein was recovered from detergent extracts of COS cells through an interaction with either EP, anti-EP antibody or biotinylated EP avidin agarose, respectively. These observations are consistent with the identification of both the 66- and 105-kDa proteins by chemical cross-linking of ^{125}I-EP in these COS cells. The simplest interpretation of these experiments is that the 66-kDa protein is a subunit of the receptor containing the binding site for EP, while the 105-kDa protein appears to be another subunit of the receptor. Apparently the 105-kDa subunit does not bind EP or the complex EP–66-kDa protein in detergent extracts. However, this interpretation may not be correct if the 66-kDa protein is greatly overexpressed in relation to the 105-kDa protein in these COS cells. Also, the processing of the 66-kDa protein may be unique in COS cells since the 66-kDa protein is not identified as an EP binding protein by cross-linking in normal erythroid cells although it is detected by antibodies (Sawyer, 1990, S. T. Sawyer, unpublished data). The possibility that the 66-kDa protein interacts with another subunit is supported by the observation that the 66-kDa protein interacts with another subunit is supported by the observation that the 66-kDa protein forms a tight complex with the gp 55 vial protein in cells cotransfected with the cDNA encoding the 66-kDa protein and the Friend spleen focus forming virus (Li et al., 1990).

The cloned EP receptor, 66-kDa protein, belongs to a family of growth factor receptors that includes the interleukin 2 receptor β-chain (p70), the interleukin 4 receptor, and the interleukin 6 receptor, as well as receptors for prolactin and growth hormone (Bazan, 1989; D'Andrea et al., 1989b, Mosley et al., 1989). Recent reports also include the granulocyte-macrophage colony-simulating factor (GM-CSF) receptor (Gearing et al., 1989), interleukin 3 receptor (Itoh et al., 1990), and interleukin 7 receptor (Goodwin et al., 1990) in this family. The high-affinity form of the interleukin 2 receptor is a heterodimer of α- and β-chains that can bind interleukin 2 separately, however, with much less affinity than the heterodimer (Hatekeyma et al., 1989; Sharon et al., 1986; Tsudo et al., 1986; Teshigawora et al., 1987). The receptor for interleukin 6 appears to interact with a 130-kDa signal transduction protein (Taga et al., 1989). These precedents enhance the possibility that the 105- and 90-kDa proteins cross-linked to ^{125}I-EP may be additonal proteins in the active receptor complex not related in amino acid sequence to the 66-kDa receptor that has been cloned. The specificity of the cross-linking reaction would argue that the 100- and 90-kDa proteins are part of the binding site for erythropoietin and are not the result of a random reaction.

In summary, the present study shows that EP interacts with proteins of 105 and 90 kDa on FVA cells. This is in agreement with the previous studies, which have identified receptor proteins of the same or similar molecular weight by cross-linking ^{125}I-EP to the cell surface of a variety of erythroid cells by using bifunctional cross-linkers. Most importantly, cross-linking artifacts do not explain the relationship of the 105- and 90-kDa proteins cross-linked to ^{125}I-EP in erythroid cells with the 66-kDa protein, which is the product of the cDNA identified as the receptor for EP by expression cloning. It is possible that either the 66-kDa receptor protein is posttranslationally modified to become a 105-kDa receptor protein or that the EP receptor is a complex of subunits such that an unrelated 105-kDa protein (and/or 90-kDa proteins) exists in a complex with the 66-kDa receptor protein.

ACKNOWLEDGMENTS

We thank Judith Luna for technical assistance, Mary Jane Rich for typing the manuscript, and Dr. Eugene Goldwasser

for information on the chromatography of EP on reverse-phase HPLC.

Registry No. EP, 11096-26-7; DJ, 109545-43-9.

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